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Assessment of the feasibility of SP-10 as a male fertility marker and validation of a lateral flow immunoassay for indirect sperm concentration analysis

A project dissertation submitted in fulfilment for the degree of MSc by research

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Abstract

Over the past few decades, the emphasis on male gamete has increased as male factor infertility contributes to almost 30% of infertile couples. As a result, the need for development of more accurate assays is now more important than ever. Determination of the concentration of sperm using an indirect assay does not possess the inherent user subjectivity, procedure uncertainty and biological restrictions such as viscosity of manual semen analysis. This thesis aims to assess the feasibility of SP-10 protein to be used as a fertility marker by the assessment of a lateral flow immunoassay.

Preliminary analysis for sample preparation, handling and production outlined a suitable protocol for novel device analysis. Validation of a novel diagnostic sperm counting device as developed by Swiss Precision Diagnostics (SPD) against the laboratory semen analysis was the primary objective of the current study. Suitable concentration of lysis buffer, incubation period and device sensitivity and specificity were then determined by analysing patient samples seeking fertility advice.

A series of analysis results for the preliminary examinations, indicated that stirring the sample is an acceptable compromise to using a high speed vortex for homogenisation of semen. It was also found that the chosen lysis buffer at its current concentration is a suitable agent for acrosomal sperm membrane impairment for releasing SP-10 into the solution from the entire sperm population. Accuracy assessment of the device prototype showed that the accuracy of the device at the lowest threshold ($5x10^6$/ml)

expressed 100% accuracy compared with CASA and haemocytometer readings.

Further analysis is required to definitively accept the thresholds for the percentage absorbance of the high and low sensitivity strips inside the device; particularly at the $15x10^6$/ml threshold where the device expressed 85% accuracy. The validation data shows that indirect measurement of seminal sperm concentration using the novel device can accurately estimate the sperm concentration. However, further optimisation is required before the device is ready for commercial distribution.

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Acronyms and abbreviations

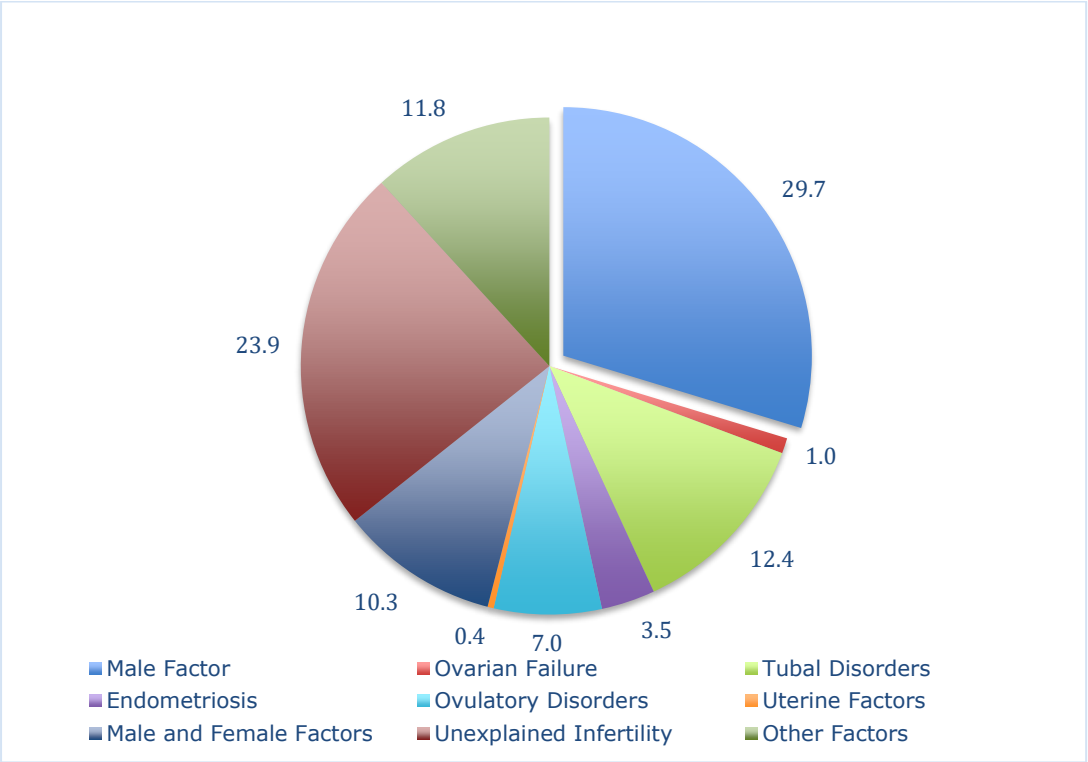
ART: Assisted Reproduction Technology
ASAB: Anti-sperm antibody
CASA: Computer assisted sperm analysis
CBAVD: Congenital bilateral absence of the vas deference
cP: Centipoise
CI: coitus interruptus
DNA: Deoxyribonucleic acid
DPBS: Dulbecco's phosphate-buffered saline
FSH: Follicle stimulating hormone
GnRH: Hypothalamic gonadotrophin releasing hormone
hCG: Human chorionic gonadotrophin
HPG: hypothalamic-pituitary-gonadal
HFEA: Human fertilisation & embryo authority
HOS: Hypo-osmotic swelling (test)
ICSI: Intra-cytoplasmic sperm injection
IUI: Intrauterine insemination
IVF: *in-vitro* fertilisation
LH: Luteinising hormone
MFI: Male Factor Infertility
mPa.s: millipascal per second
NEQAS: National external quality assessment service
PFA: Para-formaldehyde
PGC: Primordial germ cells
PGE_2: Prostaglandins E_2
PR: Progressive motile
PT: Perinuclear theca
ROS: Reactive Oxygen Species
SFA: Seminal fluid analysis
Sg I: Semenogelin I
Sg II: Semenogelin II
SHV: Semen hyperviscocity
SPD: Swiss precision diagnostics
ZP: Zona Pellucida

1.0 Introduction

1.1 Infertility

Fertility is a wide spectrum, ranging from optimal to normal to non-existence. Many couples have the normal male and female gametes that have the potential to conceive naturally. However, they don't manage to achieve pregnancy due to unexplained reasons or simply being unlucky (Comhaire 1996). Infertility has a great psychological and medical impact on women and men alike. Approximately 15% of couples trying to conceive, fail to do so within the first 12 months of unprotected regular intercourse (Thonneau et al, 1991; Sharlip et al, 2002; Iammarronne et al, 2003). Gynaecological complications such as ovarian failure, tubal disorders and endometriosis are often regarded as causes of infertility. However, approximately 30% of couples fail to conceive as a result of male factor infertility (MFI) (Iammarrone et al, 2003; HFEA 2010). Additionally, in 20% of infertile couples, MFI is considered the sole contributor of infertility (Thonneau et al, 1991; Hamada et al 2011).

Figure 1: Approximate proportions of contributing factors to infertility



Male factor contributes to 29.7% of infertility cases. (HFEA 2010).

1.2 Male factor infertility

Following physical examination, infertile men will produce 2 semen samples after 12 months of unprotected intercourse for MFI diagnosis (Hamada *et al* 2011). Seminal fluid analysis (SFA) is the first line laboratory investigation indicating the quality of a semen sample (Iammarrone *et al*, 2003). MFI may occur due to congenital or acquired unexplained conditions. There are several male factors that contribute to decreasing the probability of conception, these include: dysfunctional sex glands, genetic abnormalities, infection, local inflammation, endocrine disturbances,

reactive oxygen species (ROS) and aberrant DNA packaging (Comhaire 1996; Hamada *et al* 2012; Du Plessis *et al*, 2013). In cases where patient history suggests the need or functional disorders such as anejaculation are detected by andrology tests, additional investigations by urologists or other specialists are carried out (Sharlip *et al*, 2002).

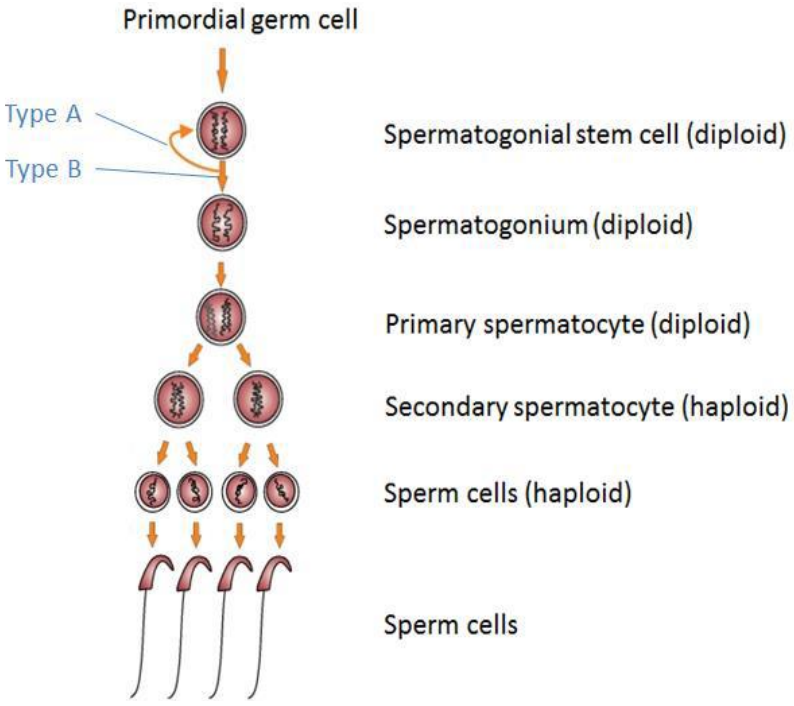
In certain cases, female factor infertility has been ruled out and all sperm parameters appear to be within the reference range. These men are diagnosed with unexplained infertility, which account for 15% of all MFI cases (Hamada *et al* 2011). As the name suggests, the origin of unexplained infertility is unidentified. They could occur spontaneously as a result of an obstruction or other unknown reasons (Hamada *et al* 2011).

1.3 Spermatogenesis

Spermatogenesis is the process by which sperm cells are created from the primordial germ cells (spermatogonia) until they reach maturation in approximately 74 days (de Kretser *et al* 1998). Spermatogenesis occurs in the seminiferous epithelium of the seminiferous tubule which is considered the functional unit of the mammalian testes (Cheng and Mruk 2010). Spermatogenesis can be divided to three major stages: mitotic cell division, meiotic cell division and spermiogenesis (de Kretser et al 1998). Mitotic cell division, involves proliferation of the spermatogonia, which leads to the formation of spermatocytes and subsequent mitotic cell division to maintain their concentration by renewal. The second stage involves two meiotic

divisions of spermatocytes which gives rise to haploid spermatids (containing half the genetic information of diploid cells). Finally during the third phase, the spermatids undergo a series of cytological transformations leading to the production of spermatozoa (Clermont 1972).

Figure 2: Spermatogenesis



Stages of spermatogenesis form primodial germ cells (PGC) to mature spermatozoa (Cheng and Mruk 2010)

Recent advancements in the fields of proteomics and genomics have assisted scientists to understand the process of spermatogenesis in greater depth. It is now possible to study the underlying molecular mechanisms of spermatogenesis using tissue-profiling experiments and cross species comparisons of gene annotations to detect testes specific genes that are essential for testicular function (cheng and Mruk 2010; Calvel *et al* 2010). Despite these advancements, assessment of testicular histology and

cytology remain valuable diagnostic tools for sub-fertile and hypogonadal patients (Comhaire 1996).

The process of spermatogenesis is highly diverse amongst sexual species of the animal kingdom, however successful spermatogenesis always require the mature sperm to have the ability to recognise and successfully fertilise an egg (Calvel *et al* 2010). In addition, spermatogenesis ensures reduction in ploidy from diploid to haploid cells, repackaging of the cell chromatin, physiological cellular changes to streamline the cell organelles for sufficient motility as well as having the ability to produce adequate energy to maintain motility (Calvel *et al* 2010).

To understand the dynamics of spermatogenesis for the purposes of diagnosis and treatment, it is important to focus on factors that can disturb the balance by which this precise process occurs. Factors such as environmental exposure, radiation or chemotherapeutic agents, genetic abnormalities, changes in the hormonal regulation or physiological stress can all have a profound impact on spermatogenesis (Mulhall *et al* 2013).

1.3.1 Regulation of spermatogenesis

Management of spermatogenesis can be incorporated into juxtacrine, paracrine and endocrine factor regulations (Calvel *et al* 2010). The paracrine regulation of spermatogenesis is originated from the secretion of gonadotrophin releasing hormone (GnRH) by the hypothalamus. The hypothalamus-pituitary-gonadal (HPG) axis promotes spermatogenesis and androgen biosynthesis (Mulhall *et al* 2013). GnRH is secreted periodically

into the pituitary blood stream and stimulates the secretions of follicle stimulating hormone (FSH) and luteinising hormone (LH) by the anterior pituitary gland. LH and FSH are peptide hormones; LH is known to be the hormone stimulating the secretion of testosterone in the Leydig cells from the testis whilst FSH supports spermatogenesis by stimulating the seminiferous tubules. Testosterone then stimulates the receptors on the Sertoli cells to regulate spermatogenesis (de Kretser *et al* 1998; De Jonge and Barratt 2006). HPG axis operates a closely regulated negative feedback loop which is maintained by the feedback received form the cascade of steroids and peptides in the system (Mulhall *et al* 2013).

1.3.2 Early preparations for spermatogenesis

Preparations for spermatogenesis begin at the very early stages of life. During the embryonic stage, primordial germ cells (PGC) migrate from epiblast to the testicular anlagen to proliferate and colonise the genital ridge. The migration of PGC is regulated by expression of the surface protein, c-kit which serves as a receptor for stem cell factor (SCF). At this stage PGC are associated with the mesenchymal cells which are the pre-cursers of the Sertoli cells. Following a period of multiplication, PGC begin the formation of a network of tubules that collectively form the seminiferous cords which will eventually give rise to the seminiferous tubules (de Kretser *et al* 1998; De Jonge and Barratt 2006).

At birth, PGC are arrested at mitosis and become non-proliferative type-A spermatogonia until puberty at which time the elevated secretion of GnRH

stimulates an influx of mitotic division of A-type spermatogonia (de Kretser *et al* 1998; De Jonge and Barratt 2006).

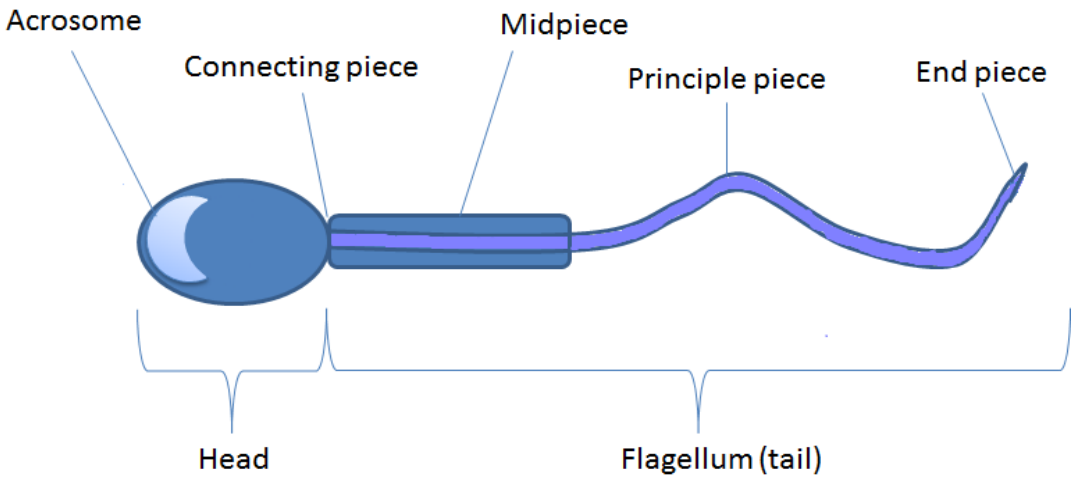
1.4 Spermatozoa

Mature human spermatozoa consist of head, neck region, midpiece and flagellum (WHO 2010). The sperm head consists of the nucleus that contains densely coiled chromatin (haploid DNA) fibres, that at fertilisation result in zygote diploid. During mammalian sperm development, DNA condensing core and linker histones that package DNA in early spermatids, are partially replaced by protamines (positively charged DNA proteins) in the final stages of spermatid maturation. Protamines are essential for hyper-condensation of the nucleus into a hydrodynamic shape, which is essential to motility of spermatozoa (De Jonge and Barratt 2006; Balhorn, 2007).

The sperm nucleus is covered by a nuclear envelop (NE) and is provided protection by the perinuclear theca (PT). PT is a unique cytoskeletal element that forms a shell around the nucleus and provides a rigid cover using stable structural proteins bound by disulphide bridges (Oko and Maravei 1995). PT can be divided into three major segments: subacrosomal layer, equatorial segment and post-acrosomal sheath (PAS), these segments cover the sperm head and play a vital role during fertilisation (Oko and Maravei 1995) (De jonge & Barratt 2006).

Spermatozoa head also contains the acrosome (figure 3). The acrosome is a sac-like secretory granule derived from golgi apparatus, surrounded by inner and outer acrosomal membranes with unique exocytotic properties (Tulsiani *et al* 1998). Interaction between capacitated spermatozoa and zona pellucida is initiated by glycan binding molecules such as glucosyltransferases, binding with the complementary glycans on the surface of zona pellucida. The physiological acrosome reaction occurs following the irreversible molecular bindings on the surfaces of the anterior spermatozoa head plasma and ZP membrane. Spermatozoa acrosome contain several enzymes including: acid glycohydrolases, proteases, esterases, acid phosphatases and aryl sulfatases that are secreted at the site of sperm-ZP binding via acrosomal exocytosis (Abou-Haila and Tulsiani 2000; WHO 2010).

Figure 3: Human sperm cell



Schematic representation of a human sperm: head, midpiece and tail.

Maturation of sperm is a complex biological process which requires the assistance of proteins. The processes of capacitation and acrosome reaction take place outside the male body and particularly rely on the action of proteins. Some of the proteins that are significant in male reproduction are

products of the epididymis and accessory glands and are readily present in the seminal fluid. Other proteins are intracellular and assist in sperm function upon their release from the cell (Ashrafzadeh *et al* 2013). The fertility related proteins can be categorised into the following categories; sperm motility proteins, sperm-ZP interaction proteins, peripheral proteins, acrosome reaction, nuclear and post-translational modification proteins (Ashrafzadeh *et al* 2013).

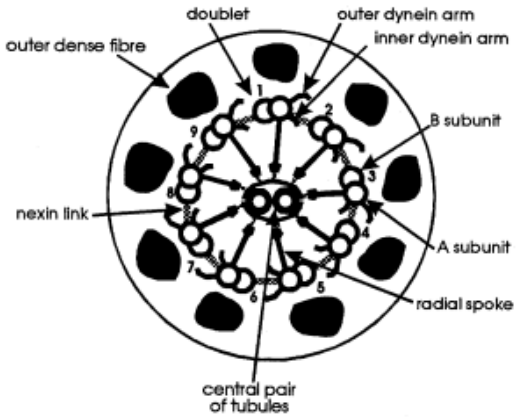
One of the acrosomal proteins is called SP-10. In a global effort to identify sperm surface antigens as potential male contraceptive agents, WHO identified SP-10 as a suitable candidate (Nieschlag and Habenicht 1992). Generally, sperm plasma membrane antigens are regarded as the most fitting class of immunogens for contraceptive vaccine development. SP-10 demonstrates a polymorphic set of immunoreactive polypeptides that range from 18 to 34 kDa (Wright *et al* 1990). The significance of SP-10 in particular as a contraceptive agent is due to its exposure to the plasma surface after acrosome reaction but prior to fertilisation which offers a great target for immunocontraception (Herr *et al* 1990a). Located in the acrosome of the mature sperm head, immunolocalization data points out SP-10 as an antigen present in every stage of the seminiferous cycle and spermiogenesis in humans (Kurth *et al,* 1991). SP-10 is recognised by a monoclonal (MHS-10) antibody and is present in human and a number of other primates (Herr *et al,* 1990b).

The sperm midpiece consists of a central filamentous core wrapped in a mitochondrial sheath (Björndahl *et al* 2010). Mature spermatozoa are streamlined to optimise motility and efficiently reach the oocyte in the female reproductive tract to complete the process of fertilisation. During spermiogenesis, most cytoplasm, some mitochondria and other residual bodies are lost. Late spermatocytes, spermatids and spermatozoa have more condensed and metabolically efficient mitochondria that are rearranged in tubular forms constituting the midpiece of spermatozoa (Amaral *et al* 2013). Sperm mitochondria are essential for the generation of energy in the form of ATP for several key metabolisms as well as maintenance of motility (Piomboni *et al* 2012). The energy production is largely credited to oxidative phosphorylation, although other metabolic pathways such as Krebs cycle and reactions of amino acid metabolism also occur in mitochondria. This organelle also play a key role in generation of ROS, cellular apoptosis, calcium signalling and iron metabolism (Piomboni *et al* 2012).

The structure of the sperm tail consists of the axial filament complex. The core of the spermatozoa tail is made up of a fascicile of 11 parallel longitudinal fibrils. There are 9 symmetrically arranged double microtubules on the outer part of the complex, surrounding 2 central single fibrils named C1 and C2 connected by the central pair bridge, forming a 9 + 2 axoneme. The 9 doublet microtubules consist of a complete A-tubule and an incomplete B-tubule with dynein arms (inner and outer) that are connected to each other by interdoublet links. The 11 fibrils uniformly correspond to the axial filament. The mitochondrial sheath in the mid-piece and the fibrous sheath in the principle piece of the flagellum are wrapped around

the axial filament complex and its associated dense outer fibres. At the end piece of the tail, the axial filament complex exceeds the fibrous sheath and is only surrounded by the cellular plasma membrane (Inaba 2003, Telkka *et al* 1961, Mortimer 1997).

Figure 4: 9 + 2 axoneme of sperm tail



Transverse schematic representation of the flagellum (Mortimer 1997)

1.5 Seminal fluid

Semen is the organic fluid that delivers a concentrated suspension of spermatozoa through ejaculation from the urethra during intercourse (WHO 2010). The vas deferens, seminal vesicles, prostate gland, and the bulbo-urethral glands are all contributing glands in secreting the components of semen (Guyton 1981). The initial 5% portion of the ejaculate originates from the bulbourethral and Littré glands. These glands secrete mucoproteins that assist in lubrication of penis and the penile urethra as well as neutralising any residual acidic urine. The next 15%-30% portion of the ejaculate consists of secretions of the prostate gland, adding a number of biochemically important substances to the seminal fluid. This is followed

by a small fraction contributed by the ampulla and epididymis. Lastly over 60% of the seminal fluid is secreted by the seminal vesicles, ensuring residual spermatozoa are washed out from the ejaculatory ducts and the urethra. The secretion from seminal vesicles contains a large amount of mucus as well as biologically important substances essential for spermatozoa function. The role of the testes in the formation of seminal fluid is the production of millions of sperm which are suspended in a fluid secreted by the epididymis and Sertoli cells that assist the delivery of sperm from the seminiferous tubules to the genital ducts (Owen and Katz 2005; Guyton 1981; Jequier 2011; Du Plessis *et al*, 2013).

Seminal plasma acts as an important physiological medium in male reproduction. A number of highly concentrated organic substances are present in semen that dictate the osmotic state of the seminal fluid and reflect the metabolic pattern of the male accessory glands (Mann and Lutwak-Mann 1981). Some of these substances in chronological order are as follow: citric acid, prostaglandin, fructose, phosphorylcholine, ergothioneine, inositol, glycerylphosphorylcholine, sorbitol, cartinine and acetylcartinine, hypotaurine, scyllitol, glycerylphosphorylinositol, N-acetylglucosamine (Mann and Lutwak-Mann 1981). The seminal plasma also contains many free amino acids such as glutamic acid and molecular peptides. Glutamic acid concentrations have been shown to correlate with sperm concentration and secretions of the prostate gland and epididymis (Keil *et al* 1979).

There are other components of seminal plasma that have important biochemical and functional inputs; Zinc plays an important role in regulation of the 3D structure of SgI and SgII, hence determining the susceptibility of the coagulum to the actions of Prostate specific antigen (PSA) (Elzanaty et al, 2004). PSA is a proteolytic enzyme responsible for gradual degradation of SgI and SgII during semen liquefaction and reduced levels of PSA in semen may postpone seminal liquefaction. PSA levels in semen may be associated with hyper-viscosity (Elzanaty *et al.* 2004). Fructose a secretion of the seminal vesicles and is considered as an indicator of seminal vesicle function. It is readily converted to lactic acid by sperm to provide energy required by sperm in the form of ATP for motility and other cellular mechanisms (Mann & Lutwak-Mann 1948).

Freshly ejaculated semen is not homogenous (Owen and Katz 2005) and the fluids contributed by male sex glands have various compositions. Furthermore, various components of the entire ejaculate only come into contact after they are pushed down the penile urethra, hence these fluids are mixed incompletely during ejaculation (Owen and Katz 2005; Emami *et al* 2009).

Table 1: Constituents of semen and their originating organs

Originating organ	Constituents
The prostate	Acid phosphatase, citric acid, inositol, calcium, zinc, magnesium, prostasomes, prostate-specific antigen, vesiculase, spermine.
Testes and excurrent ducts	Testosterone, inhibin B, transferrin, ceruloplasmin, L-cartinine, Glycerophosphorylcholine, inositol, lactic dehydrogenase C_4.
The seminal vesicles	Fructose, ascorbic acid, prostaglandins (PGE_2), SgI, SgII, nitric oxide synthase
Epididymis	L-carnitine, neutral α-glucosidase, spermatozoa (cauda epididymis)
Ampulla of the ductus deferens	Small amount of fructose

Based on findings of Owen and Katz (2005); jequier (2011)

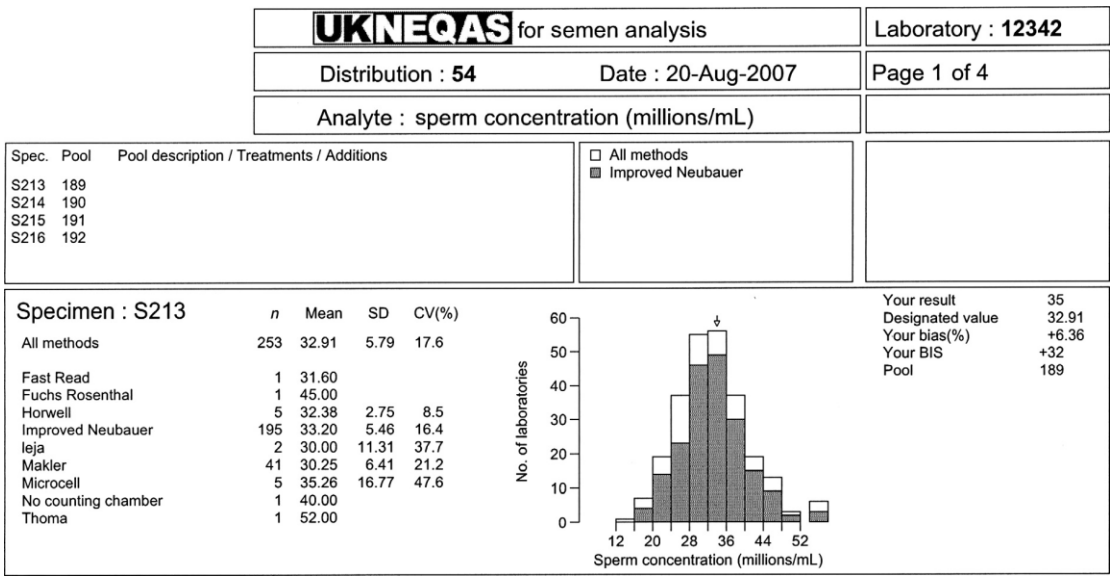
1.6 Seminal fluid analysis

The conventional seminal fluid analysis (SFA) carried out in andrology laboratories across the world provides an insight into the fertility status of the male partner as the primary investigation. The methodology and result interpretation of SFA is done with accordance to the WHO laboratory manual for the examination and processing of human semen (2010). SFA is usually the first investigation for male reproduction that rules out sterility. Additionally, conditions such as globozoospermia are only diagnosed as a result of this test, as they have no other phenotypic manifestation other than round sperm heads that have not developed acrosomes (Perrin *et al* 2013).

One of the main limitations of SFA is its inability to comment on the underlying pathophysiological causes of the microscopic observation

(Guzich *et al* 2001). Additionally, the semen quality parameters may not reliably reflect the true fertilising potential of an individual or the probability of conception (Lipshultz *et al* 2009). Furthermore, evidence of uncertainty, inaccuracy and lack of reproducibility associated with sample handling and measuring errors for SFA are well recorded (Tomlinson 2010; Tomlinson 2016; Sanders *et al* 2017). As per evidence from the UK based external quality assurance scheme shows (Figure 5), lack of standardisation and uncertainty has resulted in transcription errors and varied result interpretations (Tomlinson 2016). 253 participating laboratories took part in the distribution outlined by figure 5 and the reported sperm concentration ranged from 12 mil/ml to 52 mil/ml. The range of reported concentration analysis is unacceptable as concentration of lower than 15mil/ml are diagnosed with having low sperm count (WHO 2010).

Figure 5: Example of the UK NEQAS distribution report



Published results of a sample distribution by NEQAS. reported concentration counts range from 12 x10⁶/ml to 52 x10⁶/ml

SFA is often considered a neglected laboratory test due to lack of standardisation, unreliability and questionable validation methods. The

following summary (table 2) aims to outline reasons for uncertainty over SFA.

Table 2: Factors that cast uncertainty and unreliability over SFA

Limiting factors	Reasons for uncertainty
User subjectivity	Gold-standard SFA is the manual analysis and the diagnosis is reliant upon the andrologist's experience and judgement. Traces of inadequate andrology training in laboratories is evident.
Conflicting opinions on manual analysis	Manual SFA is unchanged and unmodernised for decades. Experts have different opinions on how the test should be performed. Many laboratories use outdated procedures and others perform the test using supplies that are not designed for semen analysis.
Lack of modernisation	There is a lack of technology that is widely accepted, accurate, affordable, and user friendly.
Principles of quality management	Some laboratories overlook the importance of external quality control therefore competency is not validated outside the laboratory. Available external quality controls show many partaking laboratories produce widely inaccurate results over periods of time without the motive to improve the bench techniques or re-evaluate the competency procedures (Figure 5).
Additional Limitations	SFA fails to address the fluctuating nature of sperm production and semen quality and merely provides a snapshot diagnosis based on a few fertility markers. It cannot comment on any underlying pathophysiological complications. SFA must be taken into account in addition to male clinical history and female gynaecological tests, as on its own it may be misdiagnosing.

Limiting factors of SFA and reasons for uncertainty of results (Lipshultz et al 2009)

Semen analysis consists of initial evaluation and microscopic analysis. The initial investigations of semen evaluate the macroscopic characteristics of the sample such as volume, colour, viscosity, pH and consistency. An abnormal measure of any of these characteristics can indicate an underlying problem. The microscopic evaluation of semen quality is by investigating sperm and cellular components of the ejaculate using phase contrast microscopy. Sperm concentration, motility, and morphology will determine

the diagnostic outcome of the analysis. Additional tests may be performed if required depending on the male clinical history and microscopic sperm quality; sperm vitality testing, anti-sperm antibody (ASAB) reaction and sperm DNA integrity analysis (WHO 2010; Agarwal and Said 2011). Table below illustrates the main seminal and sperm parameters as well as their reference values.

Table 3: Latest WHO diagnostic interpretations for semen analysis

Initial evaluation	**Reference range**	**Underlying Causes for abnormal values**
Semen volume	≥1.5ml	Obstruction of the ejaculatory duct. Congenital bilateral (or unilateral) absence of the vas deference (CBAVD). Poorly developed sex glands.
pH	≥7.2	Imbalance between the accessory gland secretions, Obstruction of the ejaculatory duct. Congenital bilateral (or unilateral) absence of the vas deference (CBAVD). Poorly developed sex glands.
Colour	Gray-opalescent	Haematospermia (pinkish-red colour) Medication, infection, jaundice STDs (yellow).
Viscosity	Clear droplets from a pasture pipette	Discussed in table 4
Microscopic analysis	**Out of reference range**	**Diagnosis**
Sperm concentration	<15M/ml <1M/ml	Oligozoospermia Severe oligozoospermia
Sperm motility	<32% PR* <5% PR*	Asthenozoospermia Severe asthenozoospermia
Morphology	<4% normal 0% normal	Teratozoospermia Severe teratozoospermia

The reference range, underlying causes and the associated diagnosis for semen and sperm parameters (WHO 2010). PR*: Progressive motile.

1.6.1 Sample collection and delivery to the laboratory

It is of diagnostic significance for the patients to keep a period of 2-5 days of sexual abstinence prior to semen analysis (WHO 2010). Levitas *et al* (2005) found a correlation between prolonged abstinence period and declined semen parameters in a study analysing 9,489 semen samples. The same study also found that patients with oligozoospermia, tend to have improved sperm motility and morphology results after 2 days of sexual abstinence.

For the purpose of seminal analysis, patients may produce a sample either by masturbation or during intercourse. Ideally, the sample should be produced onsite in an isolated room designated for this purpose by means of masturbation. Onsite sample production limits the exposure of semen to temperature changes and reduces the time between sample production and analysis (WHO 2010). In certain cases where a patient struggles to produce a sample in the clinic or a laboratory lacks adequate facilities for sample production, the sample may be collected at home. Oral and written instructions as well as a pre-weighed non-toxic container should be provided to the patient prior to the appointment date (WHO 2010).

Patients who are unable to produce a sample by masturbation or rather avoid masturbation for religious reasons, may collect the sample in a special non-spermicidal condom during coitus (WHO 2010). There are other means of sample collection in cases where patients require assistance to produce a semen sample. For men who suffer from erectile or ejaculatory

dysfunction, semen can be collected by penile vibratory stimulation or rectal probe electrostimulation (Nehra *et al* 1996). Samples collected from patients who suffer from erectile dysfunction often tend to have high sperm concentrations while the sperm motility is considerably low as a result of infrequent ejaculation (WHO 2010).

Upon sample delivery to the andrology laboratory, samples are incubated at 37°C or room temperature for a minimum of 20 minutes to reach liquefaction. Samples should be analysed within 60 minutes of production as a prolonged period between production and analysis is associated with increased level of seminal pH and decreased motility, thus resulting in an inaccurate report (lipshultz *et al* 2009; WHO 2010).

1.6.2 Sperm vitality testing

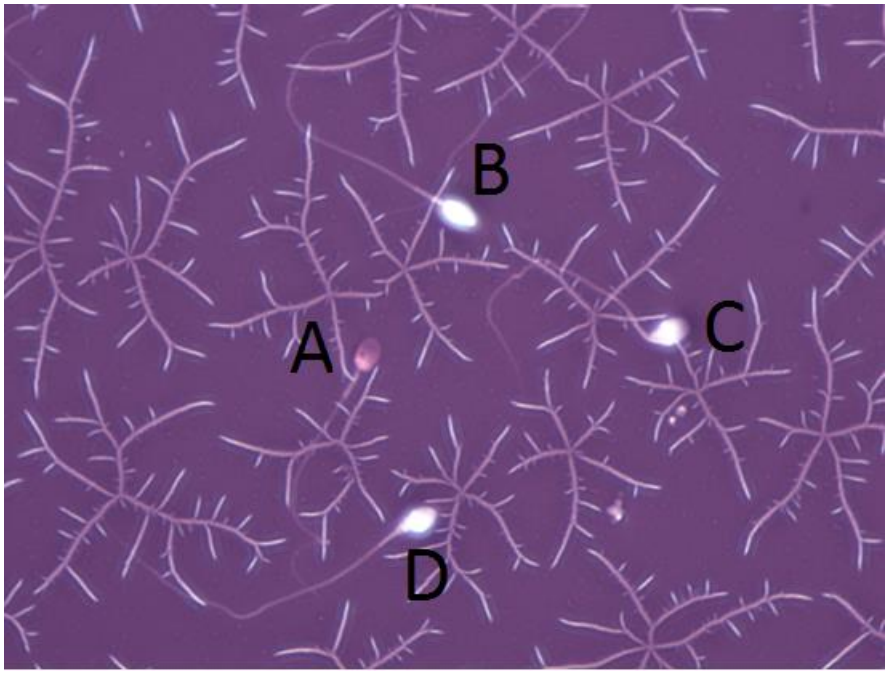
Prior to introduction of sperm vitality testing, motility was the only indicating factor considered as the index of sperm life (Burgos and Di Paola 1951). Development of sperm vitality analysis has enabled us to determine the number of dead cells independently of motility. This test is particularly significant for patients with asthenozoospermia. The standard operating procedure for sperm vitality assessment at the andrology unit at Nottingham university hospital, states that this test must be carried out on samples with lower than 10% motile sperm.

There are two categories of sperm vitality testing; Hypo-osmotic swelling test (HOS) and staining kits. HOS test focuses on the membrane

permeability properties of sperm cell and its ability to withstand exerted hypo-osmotic pressure. This test can distinguish between intact and impaired sperm membrane. A functional membrane will swell and excrete excess fluid out of the cell in equal quantity, while an impaired membrane which is no longer intact will not express swelling after exposure to hypotonic fluid (Talwar and Hayatnagarkar 2015). Vitality analysis by staining uses eosin and nigrosine dyes. Using this method, the dead cells are marked as they take up the colour of the dyes inside the cell indicating that the cell membrane is not intact (Bjorndahl *et al* 2003).

A major limitation associated with vitality testing is that once the cells are exposed to HOS media or the stain solution, they are unsuitable for further analysis and more importantly they cannot be used for any treatment procedures (Talwar and Hayatnagarkar 2015). The findings of Wilton *et al* (1988) suggest that sperm quantity and the percentage of motile sperm can improve for patients who suffer from total or severe asthenozoospermia by frequent ejaculation which results in depletion of residual extragonadal sperm.

Figure 6: Semen sample as stained by sperm VitalStain™ media



A) Sperm head has taken up the dye and appears dark indicating impaired nonviable membrane. B,C and D) appear white therefore have intact cellular membrane, these cells are considered alive.

1.7 Ejaculation

Ejaculation is a complex and highly coordinated physiological process by which semen exits the penis following an orgasm. The process of ejaculation is regulated by the nervous system and is composed to two phases: emission and expulsion (Alwaad *et al* 2015). Emission and expulsion of semen are heavily controlled by the autonomic and somatic nervous systems respectively (Giuliano and Clement 2005).

The emission phase of ejaculation begins with closure of the bladder neck preventing retrograde spillage of seminal fluid. The secretions of the prostate gland are then ejected into the prostatic urethra. Subsequently, contributions of the seminal vesicle, Cowper's gland and periurethral glands

are secreted. The organs involved in the emission phase of the ejaculation receive a high autonomic nerve supply from the pelvic plexus that heavily regulates this process both sympathetically an parasympathetically. The cerebrum also has great control over this phase of ejaculation that is induced through physical or visual stimulation (Alwaad *et al* 2015).

Expulsion follows emission and it represents the ejection of fluid from the urethra. This process is considered to be as a result of spinal cord reflexion. Smooth muscle fibres of the bladder neck remain sealed to ensure the antegrade flow of semen and the external urethral sphincter relaxes. Pelvic, bulbospongiosus and ischicavernosus muscles then have rhythmic contractions propelling semen towards the urethra. The underlying mechanism triggering the rhythmic muscle contractions are not yet fully understood (Giuliano and Clement 2005; Alwaad *et al* 2015).

1.8 The coagulation-liquefaction process

In normal *in vivo* conditions, newly ejaculated semen usually constructs a gel-like coagulum spontaneously to form a depository of sperm in the rear part of the vaginal cavity (Lundwall *et al* 2002; Emami *et al* 2009). Per Baker and Bellis (1995), only the latter part of the ejaculate undergoes coagulation whilst the former part typically doesn't. During intercourse, the initial sperm-rich part of the ejaculate admixes with the cervical mucous which assists the spermatozoa to swim towards the cervix (Sobrero and MacLeod 1962), and the remainder of the seminal fluid gather as a pool inside the vagina (WHO 2010). Laboratory analysis of semen, requires men

to collect the entire ejaculate inside a container or a non-spermicidal condom, where all spermatozoa in the sample are encaptured in a coagulum. It has been found that men who ejaculate frequently tend to produce semen samples that have a longer period of coagulation. The study suggested that samples with at least 48 hours of sexual abstinence, liquefy in a shorter period of time in comparison with samples of shorter abstinence period (Mandel and Bhattacharyya 1986).

The gel matrix formation is due to the function of semenogelin I (SgI), semenogelin II (SgII) as well as fibronectin and lactoferrin. SgI and SgII are coagulating proteins secreted by the seminal vesicles (SgII is also expressed by the cauda epididymis), that form a non-covalent bond via disulphide bridges to form a coagulum upon ejaculation. The coagulum prevents the process of capacitation in freshly ejaculated semen by inhibition of reactive oxygen species (ROS) (Lamirande & Lamothe 2010; Peter *et al* 1998). Fibronectin and lactoferrin are both secreted by the seminal vesicles and the ampulla. Fibronectin is an adhesive glycoprotein that is integrated in the meshwork of the gel-like matrix immediately after ejaculation, whilst lactoferrin, a sperm coating antigen remains within the solution and forms links to SgI and SgII (Lilja *et al* 1987).

Liquefaction is the process in which semen becomes homogeneous by proteolytic degradation of SgI and SgII into soluble proteins, then into smaller peptidic residues during the late stages of liquefaction (Lilja *et al* 1989; Emami *et al* 2009). The degradation is as a result of the proteolytic

activity of prostate specific antigen (PSA), a kallikrein-like, prostatic serine protease (Lilja *et al* 1989; Lee *et al* 1989). Typically, after about 20-30 minutes of ejaculation semen liquefies. The gel-bound SgI, SgII and fibronectin network is fragmented gradually and becomes soluble (Lilja *et al* 1987). Its only after liquefaction of the coagulum that sperm are free from the seminal clot and gain motility in order to swim towards an oocyte for fertilisation (Du Plessis *et al*, 2013).

1.9 Semen hyper-viscosity

Semen usually loses its viscous characteristic during the process of liquefaction. In 12-29% of the ejaculates semen remains viscous post liquefaction, this is known as seminal hyper-viscosity (SHV) (Elzanaty *et al,* 2004; Esfandiari *et al,* 2008). Observations of Ming-Chung *et al.* (1992) concluded that asthenozoospermic samples displayed much higher SHV than normozoospermic samples. In addition, a prospective study carried out by Esfandiari *et al* (2008) found that hyper-viscous samples showed significantly lower fertilisation rates in comparison with the control group post IVF. It has also been reported that SHV correlates with poor fertilisation rates both *in vivo* and *in vitro* (Du Plessis *et al*, 2013).

The exact underlying cause of SHV remains unknown. There have been a number of theories over the past few decades regarding the contributing factors to SHV that have either been dismissed or not yet widely accepted by the scientific community (Du Plessis *et al,* 2013).

Table 4: Potential sources of semen hyper-viscosity

Cause	Theory	References
Neutral α-glucosidase (NAG)	NAG is one of the most significant epididymal markers. A 1998 study, found no correlation between levels of NAG and SHV.	WHO 2010 Carpino & Siciliano (1998)
Prostate specific antigen (PSA)	PSA is a proteolytic enzyme in seminal fluid responsible for gradual degredation of SgI and SgII during liquefaction. A 1989 study concluded that there was no relationship between reduced secretions of PSA and SHV.	Elzanaty *et al*, (2004) Dube *et al,* (1989)
Zinc	A 1998 study found that zinc levels in normal and SHV samples didn't differ significantly.	Carpino & Siciliano (1998)
Fructose	In 1993, a relationship between hypo-function of seminal vesicles and SHV was observed. however in 1998, an experiment produced contradictory results to the 1993 study	Gonzales *et al,* (1993) Carpino & Siciliano's (1998)
+ve bacterial cultures, Leukocytospermia, percentage of ASAB	In 1999 *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Chlamydia trachomatis* were cultured and observed using a direct fluorescence assay. No significant association was found between positive and negative samples. Same study carried out antisperm-antibody analysis and determined the concentration of white blood cells in normal and SHV samples. No significant correlation was report. In 1989 however, a correlation was reported between +ve antibody reaction in semen and SHV.	Munuce *et al,* (1999) Munuce *et al,* (1999) Du Plessis *et al,* 2013 Moulik *et al* (1989)
IL-6, TNF-α, IL-10 and ROS production	In 2014, an association was found between SHV and increased oxidative stress (due to production of ROS) and elevated number of pro-inflammatory interleukins. They concluded that SHV is increased in patients with male accessory gland infections, particularly when the infection extends to the seminal vesicles.	Castiglione *et al*, (2014) Castiglione *et al*, (2014)

Causes of seminal hyper-viscosity and the relevant research.

1.9.1 Laboratory viscosity measurement

Viscosity measurement of a semen sample in the laboratory is carried out by semen aspiration in a disposable pasture pipette. By allowing semen to drip using gravity, the length of the formed threads determine the viscosity of the sample. Samples with normal viscosity usually form clear droplets or short threads, while samples with abnormal viscosity usually form threads of longer than 2 cm (WHO 2010). WHO (2010) suggests using a glass rod as another method for viscosity measurement. This method is carried out by inserting a glass rod into the sample, the rod is then removed the length of any formed threads are estimated. Similarly, samples that form threads of longer than 2 cm are considered to be hyper-viscous.

The methods for viscosity measurements suggested by WHO (2010) are subjective and semi-quantitative and inadequate for quantitative analysis (Rijnders *et al* 2007). Douglas-Hamilton *et al* (2005) suggested another method for viscosity measurement based on the flow velocity of the sample in a capillary. By keeping the diameter of the capillary and the angle of contact constant, the determining factor for the time taken for the semen to fill the capillary would indicate the viscosity of the sample (Rijnders *et al* 2007).

1.9.2 Viscosity measurement using a Viscometer

Following liquefaction, semen behaves as a Newtonian liquid with a stable viscosity, which means the viscosity is not altered depending on the speed at which is it measured (La Vignera *et al* 2011). Human semen viscosity

has been measured in the 1960s and 70s using multiple point capillary and quantitative viscometers (Tjioe and Oentoeng 1968; Dunn and Picologlou 1977). the authors of both mentioned studies found that the viscometer reading for liquefied semen was less than 10 cP at 37°C. In 2011, La Vignera *et al* measured the rheological properties of semen using quantitative viscometry. This study used the viscometer readings to establish a connection between male accessory gland infection and the extension of the inflammatory process to the various glands. The quantitative measure of viscosity in this study was outlined as a valuable diagnostic tool which provides a wide range of readings from normal viscosity to severely viscous without the user subjectivity limitation of conventional viscosity measurement methods.

1.10 Sperm concentration

The concentration of spermatozoa in a semen sample is of clinical significance as it has directly been linked to length of time to achieve pregnancy and ART pregnancy success rates (Slama *et al*, 2002; Hashimoto *et al,* 2010; Arikan *et al,* 2012). Microscopic seminal parameters including sperm concentration have been determined by comparison of mean values of semen characteristics in large groups of fertile men (fathers) against infertile men. In this instance, infertility refers to male partners of couples who haven not conceived following at least 12 months of unprotected intercourse (Freund 1962).

Low sperm count is usually as a result of imperative sperm production or an obstruction in the reproductive tract. Isolated cases of oligozoospermia

are often idiopathic and are accompanied with other abnormal sperm parameters (Mulhall *et al,* 2013). Table below (table 5) aims to summarise the some of the common causes of low sperm count:

Table 5: Primary and secondary testicular failure and low sperm count

Type of failure	Underlying causes
Primary testicular failure	Idiopathic spermatogenesis failure: Partial ejaculatory duct obstruction, valve obstruction, other unknown reason.
	Testicular damage: Infection, vascular, varicocele, surgery, trauma.
	Drugs and toxins: Chemotherapy, radiation, tobacco, alcohol, antimetabolic agents.
	Chromosomal and genetic anomalies: Numerical, structural, translocational, inversions, microdeletions, single gene defects.
Primary and secondary testicular failure	Non-reproductive illnesses: Liver or renal failure, viral infections, thalassemia, obesity.
Secondary testicular failure	Partial GnRH deficiency: Kallman's syndrome, pre-pubertal hupogonadotropic hypogonadism.
	Partial gonadotrophin deficiency: Steroid abuse, Leydig cell hypoplasia, adrenal insufficiency, excess seminal oxidants.

Classifications of testicular failure resulting in reduction of sperm count. Also outlines examples of the underlying causes. Table inspired by Mulhall et al (2013); Mclachlan (2013).

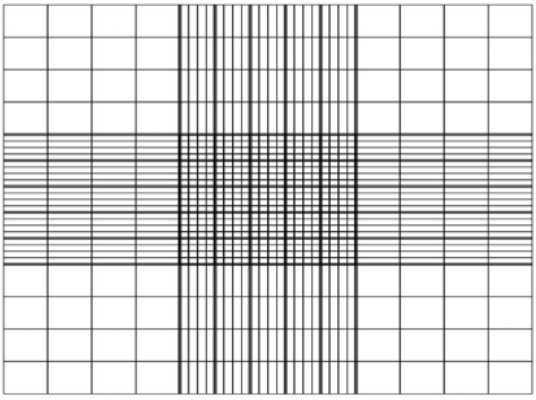
1.10.1 Laboratory analysis of sperm concentration

Semen is usually added to a fixative such as 0.4% PFA in DPBS at a predetermined dilution factor in order to immobilise sperm cells prior to counting. The dilution factor and a rough estimation of concentration is determined during the wet-prep semen analysis (Comhaire 1996). commonly, the number of spermatozoa are counted using the improved

Neubauer haemocytometer (100 µm deep). The concentration of sperm is then calculated per unit volume of semen (mil/ml) (WHO 2010).

Before the semen extracts are removed for dilution, the sample pot is mixed well. To validate the final calculation, two independent semen aliquots are removed and added to the fixative in separate diluent pots. The prepared solutions are vortexed and 10 µl of each solution is injected into the haemocytometer chambers using a positive displacement pipette. Current guideline (WHO 2010) considers samples with lower than 15 mil/ml concentration as oligozoospermic and fewer than 1 mil/ml as severe-oligozoospermic.

Figure 7: Schematic representation of the haemocytometer chamber



The grids on an improved Neubauer haemocytometer as observed under the microscope.

The new improved Neubauer haemocytometer is considered the gold standard method for counting sperm by the WHO (2010). Table 6 aims to compare the haemocytometer with alternative methods of sperm concentration count.

Table 6: Comparison of different methods of sperm concentration count

Chamber	Advantages	Disadvantages
Haemocytometer	Gold standard (WHO 2010), easy to count immobilised sperm	Inaccurate for viscous samples, preparation stages are prone to errors, time consuming. (Tomlinson *et al* 2010)
Makler counting chamber	Designed specifically for sperm concentration and percentage motility count, rapid	Lack of correlation compared to haemocytometer, relatively poor validation. (Seaman *et al* 1996)
Cell-VU sperm counting chamber	Accurate count	Expensive as it is disposed after one use, not as quick as using other methods. (Seaman *et al* 1996)
Micro-cell and Leja slides	Designed specifically for sperm count, simple, rapid	Underestimates sperm count, unreliable using wet prep sample (Bailey *et al* 2007)
CASA	Rapid, assesses count as well as motility and velocity simultaneously, minimised human error	Tends to underestimate concentration (Tomlinson *et al* 2010)

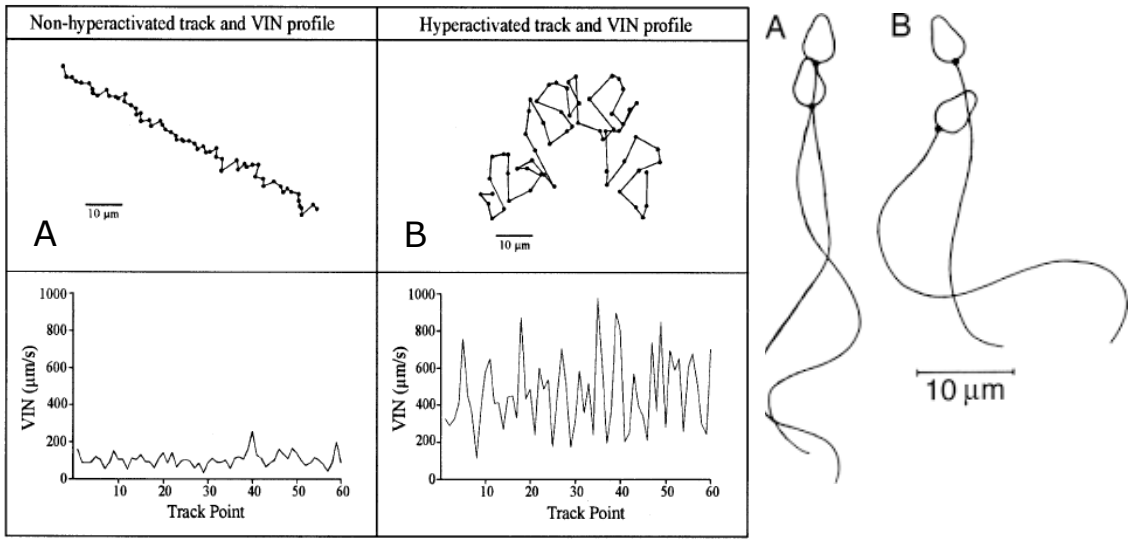
Comparison of haemocytometer with other methods of sperm count.

1.11 Motility

The male gamete is a highly specialised cell and in order to reach the fertilisation site and fertilise an egg it needs to possess the ability to progress (Gaffney *et al* 2011). Motility is an acquired sperm characteristic during the epididymal transit of sperm. Sperm retrieved from the seminiferous tubules are almost entirely immotile and motility is initiated upon dilution in seminal fluid (De Jonge and Barratt 2006). The mechanism of sperm motility is complex; a morphologically normal sperm head moves

from side to side as it progresses as the result of contractions of axonemal fibres in the sperm tail (Jequier 2011).

Figure 8: Cellular motion tracking of hyper-activated sperm



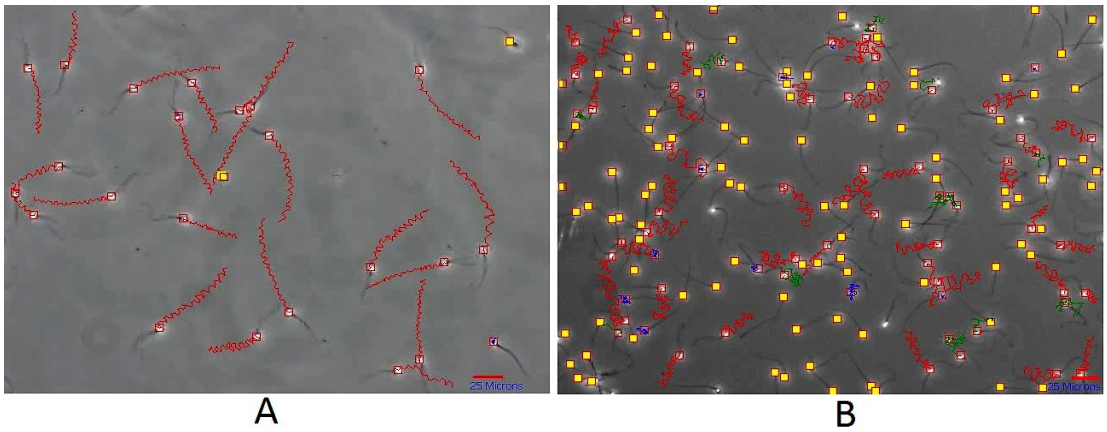
Flagellar contractions to propel the sperm head forward. A) non-hyperactivated B) hyperactivated sperm (Suarez 2008; Mortimer and Swan 1999).

Structural alterations in sperm flagella decrease the motility of sperm in a semen samples (De Jonge and Barratt 2006). Some of these alterations can be observed on a stained morphology slide under X1000 microscopy including coiled, short or bent tails (WHO 2010); other defects such as primary flagellar dyskinesia, missing or excess doublets or microtubules, reduced dynein arms and the absence of nexin links are observed by electron microscopy (Afzelius 2004). Furthermore, the alterations of the flagella can be categorised as phenotypic and genotypic. The phenotypic anomalies may be as a result of varicocele (Villanueva-Diaz *et al* 1999; Alizadeh *et al* 2006), bacterial infections, morphological defects, flagellar impairments or hormonal imbalance (Mundy *et al* 1995; Piomboni *et al* 2012).

The metabolic energy required for sperm motility is generated by mitochondria in the form of ATP. Lowered mitochondrial efficiency results in a reduction of sperm motility (Ferramosca *et al* 2012). In addition, reduced number, altered structure and disorganisation of mitochondria as well as elongation of sperm mid-piece are other mitochondrial complications may result in astheno- or severe asthenozoospermia (Piomboni *et al* 2012).

Prior to fertilisation of an oocyte, sperm will undergo the gradual process of capacitation in the female genital tract which is considered as the penultimate step in sperm maturation (Ferramosca and Zara 2014). During capacitation, sperm goes through a complex series of structural and functional changes including protein phosphorylation and membrane modifications (De Jonge 2005; Ferramosca and Zara 2014). One of the characteristics of capacitation is hyperactivated motility which is distinguished by an increased flagellar bend and asymmetrical whip-like movement in comparison with the initial motility (Torres-Flores *et al* 2011).

Figure 9: CASA tracking of motility before and after capacitation



A. capacitated sperm as tracked by CASA (tail movement is highlighted in red). Increased velocity is observed. B. Initial motility sperm as tracked by CASA (tail movement is highlighted in red).

Recent technological developments have increased the understanding of sperm motility. Using patch-clamping and imaging, the vital role of plasma membrane ion channels in sperm motility have been highlighted (Publicover and Barratt 2011). To reach the site of fertilisation and fertilise an egg, sperm must overcome challenges such as the acidic environment of the vagina, female immune response, the viscous environment of the cervical mucous, transport through the uterus and uterotubal junction and reach the fallopian tube (Suarez and Pacey 2006). To successfully achieve its task, sperm must therefore achieve initial motility, become hyperactive through capacitation, find the location of the egg using chemotaxis and finally fuse with the ZP by acrosomal exocytosis (kirichok and Lishko 2011) It is widely acknowledged that an increase in intracellular Ca^{2+} has a direct impact on increasing sperm motility (Jensen *et al* 2011).

1.11.1 Laboratory analysis of motility

Official guidelines (WHO 2010) recommends that the motility of sperm in a semen sample should be done upon sample liquefaction which is usually after 30 minutes of sample production. The motility analysis should not take place longer than 1 hour following ejaculation to minimise the deteriorating effects of pH, dehydration and temperature changes on motility (WHO 2010). The latest edition of WHO (2010) categorises sperm motility as progressive, non-progressive and immotile.

A minimum of 32% progressive motile sperm is required for the sample to be considered normal. Samples with fewer than 32% motile sperm are diagnosed with asthenozoospermia and fewer than 5% are considered severe asthenozoospermic. Under phase contrast microscopy (X40 magnification), 2X10µl aliquots are extracted independently. This procedure can take place at 37°C or room temperature. A minimum of 200 sperm are analysed from different fields of view under the microscope to eliminate user bias and avoid overcounting motile subjects. WHO (2010) recommends that the samples are mixed well before aliquots are removed from the sample. Given the difference between the two readings is acceptable, the total motility percentage is then determined by calculating the mean value.

Lack of methodological standardisation is still evident within modern andrology laboratories (Tomlinson 2010). Additionally, due to the inherent user subjectivity, homogeneity, sampling errors, quality control methods and level of operating experience, there has not yet been a universal biological standard put in place (Tomlinson 2016).

1.12 Morphology

Sperm morphology was first observed by Anton Van Leeuwenhoek in seventeenth century. He submitted his findings to the Royal society of London in 1677 and described teratozoospermia for the first time (Chemes and Rawe 2003).

Investigations on sperm morphology have found that samples with lower percentage of morphologically normal sperm tend to have lower IVF and IUI success rates. Additionally, morphological defects also have a negative impact on ICSI outcomes (Berkovits *et al* 1999; Liu and Baker 2000; Chemes and Rawe 2003). Morphological abnormalities are often related to spermiogenesis, spermatogenesis, epididymis pathology, genetic defects, exposure to radiation and other environmental factors (Chenoweth 2005).

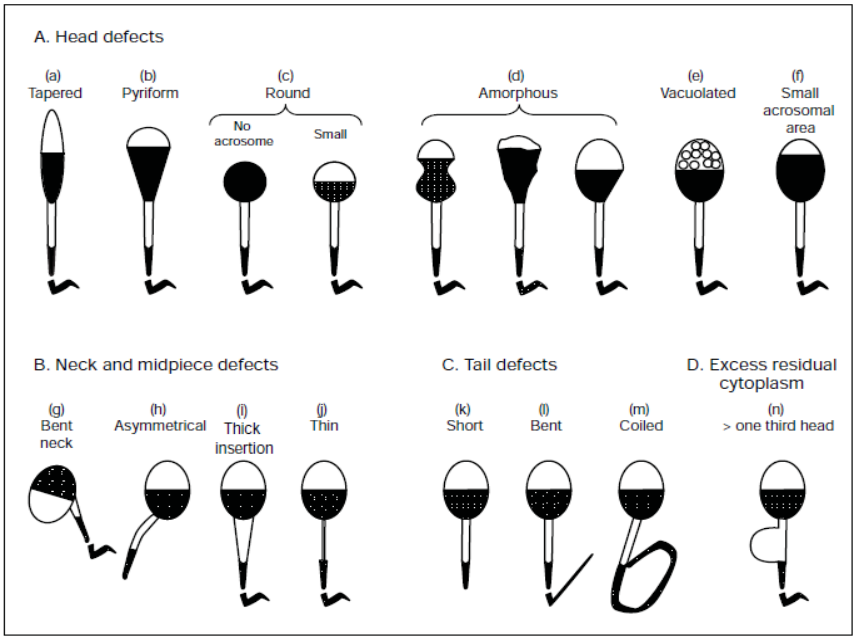
The acceptable forms of sperm have been determined by analysing sperm cells retrieved from postcoital endocervical mucous and the surface of zona pellucida (Fredricsson and Bjork 1977; Menkveld *et al* 1991). Menkveld *et al* (1991) found a significant correlation between the percentage of morphologically normal sperm present in a sample and the number of sperm bound to the surface of zona pellucida.

1.12.1 Laboratory assessment of morphology

As per manual concentration and motility analysis, morphological assessment of semen has limitations and express uncertainty due to the subjective nature of human perception (Tomlinson 2016). Morphology assessment of semen aims to comment on the shape and size of sperm which may reflect its effect on sperm function; such as the ability to possess motility and contain intact DNA.

For conventional morphology assessment of sperm, a 10µl extract of fresh semen is smeared on a glass slide and allowed to airdry. The slide then goes through a dehydration process in ethanol (70%) to preserve the smear before staining. Papanicolaou stain is the recommended stain for this purpose. Shorr and Diff-Quik stains are also deemed acceptable by the WHO (2010) and are used by some laboratories. Once the staining process is complete, a drop of oil immersion is added on the coverslip and the slide is analysed using a light microscope under X1000 magnification. 200 sperm are counted twice for this assessment. Given the difference between the two counts is acceptable, the mean value will determine the percentage of normal sperm in the sample. Samples containing less than 4% normal forms are diagnosed with teratozoospermia and 0% normal forms indicate severe-teratozoospermia (WHO 2010).

Figure 10: Morphological abnormalities of sperm



Head, midpiece and tail defects (A, B and C), excess residual cytoplasm (D). (WHO 2010)

1.13 Automated seminal fluid analysis

Manual analysis of seminal fluid is prone to human errors. The process of sperm concentration count using a haemocytometer involves pipetting, dilution and mathematical calculations that can all be sources of inaccuracy. It is worth mentioning that many laboratories in the UK prefer alternative conventional methods of sperm count such as the makler counting chamber in order to save time. Despite measures taken in the recent years by the WHO (2010) in order to standardise semen analysis across the board, manual seminal fluid analysis remains a poorly reproducible report with varied clinical value in different clinics (Alvarez *et al* 2005; Tomlinson *et al* 2010).

Factors such as failure to evaluate a sufficient number of sperm per analysis, inconsistencies in using a thermo-validated heated stage and a standardised time of analysis are all contributing factors to uncertainty of manual motility measurement (Tomlinson *et al* 2010). Furthermore, the subjectivity element of manual motility and morphology count, only adds to the uncertainty associated with the low reproducibility of the report (Tomlinson 2016). Automated semen analysers also possess the ability to measure the velocity of moving sperm (Table 7).

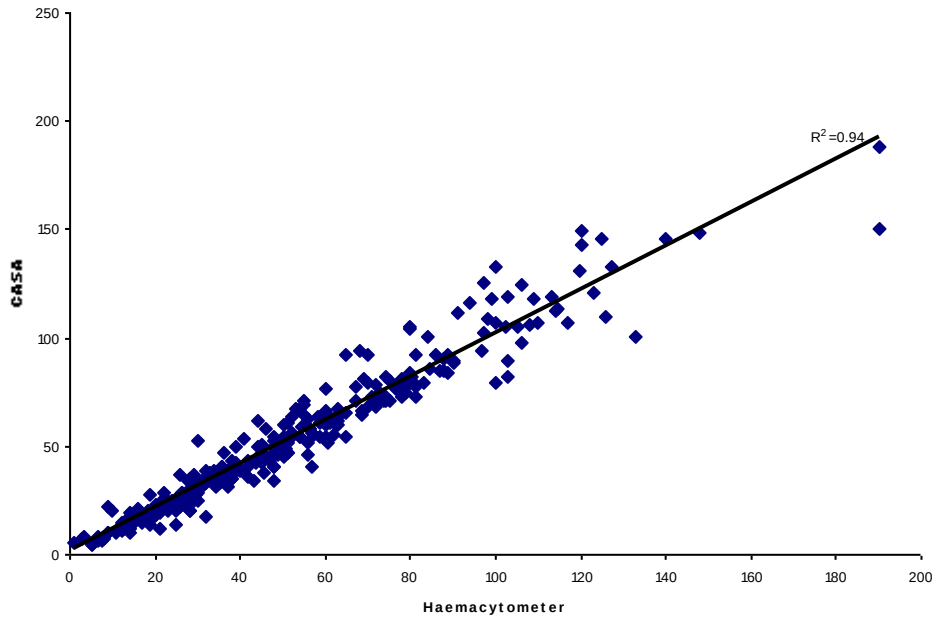
Table 7: CASA classification of sperm motility

Category	Velocity per Sec	Classification
A	>25 µm	Fast progressive
B	5-25 µm	Slow progressive
C	<5 µm	Non-progressive
D	Static	Immotile

Categories of sperm motility based on velocity in the period of 1s.

Tomlinson and colleagues (2010) validated the CASA by analysing 287 samples as measured by improved Neubauer haemocytometer and CASA software. The following scatter plot demonstrates the correlation observed.

Figure 11: CASA sperm count validation data against haemocytometer



CASA concentration count (mil/ml) versus the haemocytometer count (mil/ml) (n=287) Tomlinson et al (2010).

1.14 SP-10 assays and sperm analysis kits

SP-10 possesses characteristics that make it a suitable marker for sperm detection without using a microscope and therefore has the potential to be developed into an over counter test. As previously identified by WHO, Herr *et al* (1999) chose SP-10 as a sperm marker analyte due to its conservation in the human population. Solubility, abundance, immunogenicity and tissue specificity are among other reasons SP-10 is a suitable marker of sperm presence in semen (Herr *et al* 1990a; Herr *et al* 1999).

Currently, there are a number of commercially available home semen analysis kits on the market. Herr *et al* (1999) aimed to develop a cost effective kit for rapid semen analysis which makes a semi-quantitative analysis of sperm concentration above or below the diagnostic threshold. The application of the said device is for the following category of patients: infertile couples, vasectomised men, vasovasectomised men, men who have experiences traumas or undergone surgery and men who are curious about their fertility status. Previous to Herr *et al* (1999), similar immunological devices operated on a basic level by producing a simple qualitative result indicating presence of sperm. The table 8 summarises the commercially available tests for the purpose of sperm count at home.

Table 8: Commercially available sperm counting kits

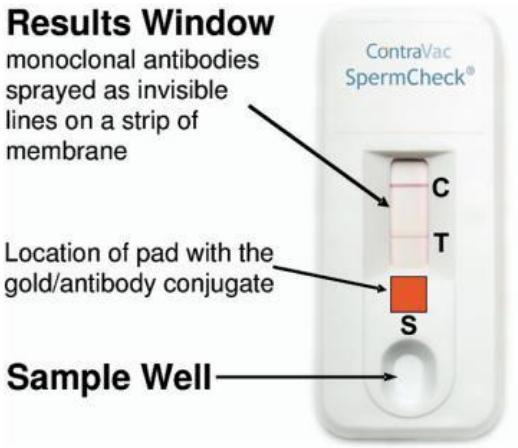
Brand	Information and data
SpermCheck®	Peer reviewed published data through a prospective, noncomparative observational study. Claims to be 98% accurate. Available for £29.99.
Trak male fertility	No public data available. Internal study suggests dynamic range of results and precise coefficient of variation. Available for $199.99.
SwimCount	Internal data claims calibration to the current WHO standard. Available for £39.99

Over the counter sperm analysis kits. Public validation data is only available for SpermCheck®.

SpermCheck® is currently one of the commercially available devices based on the peer reviewed articles of Herr and colleagues in the 90's (Herr *et al, 1990a, 1990b, 1999)*. This is a immunodiagnostic device capable of determining the sperm concentration in a semen ejaculate as operated by the user in the privacy of their home. The SpermCheck test procedure is relatively simple; following liquefaction, a small quantity of semen is removed and added to a buffer solution on the device. The sample then

begins to flow in a lateral direction and it passes through a permeable pad where SP-10 protein binds to its complementary monoclonal antibody. 2 tests lines indicate successful capture of SP-10 antigen and normal fluid flow respectively (Klotz *et al* 2008).

Figure 12: SpermCheck® results window



Outlines the test (T) and control (C) lines on the SpermCheck® device, as demonstrated by Klotz et al, (2008). Absence of C line indicates insufficient flow of the solution inside the device.

1.15 Null and alternative hypothesis

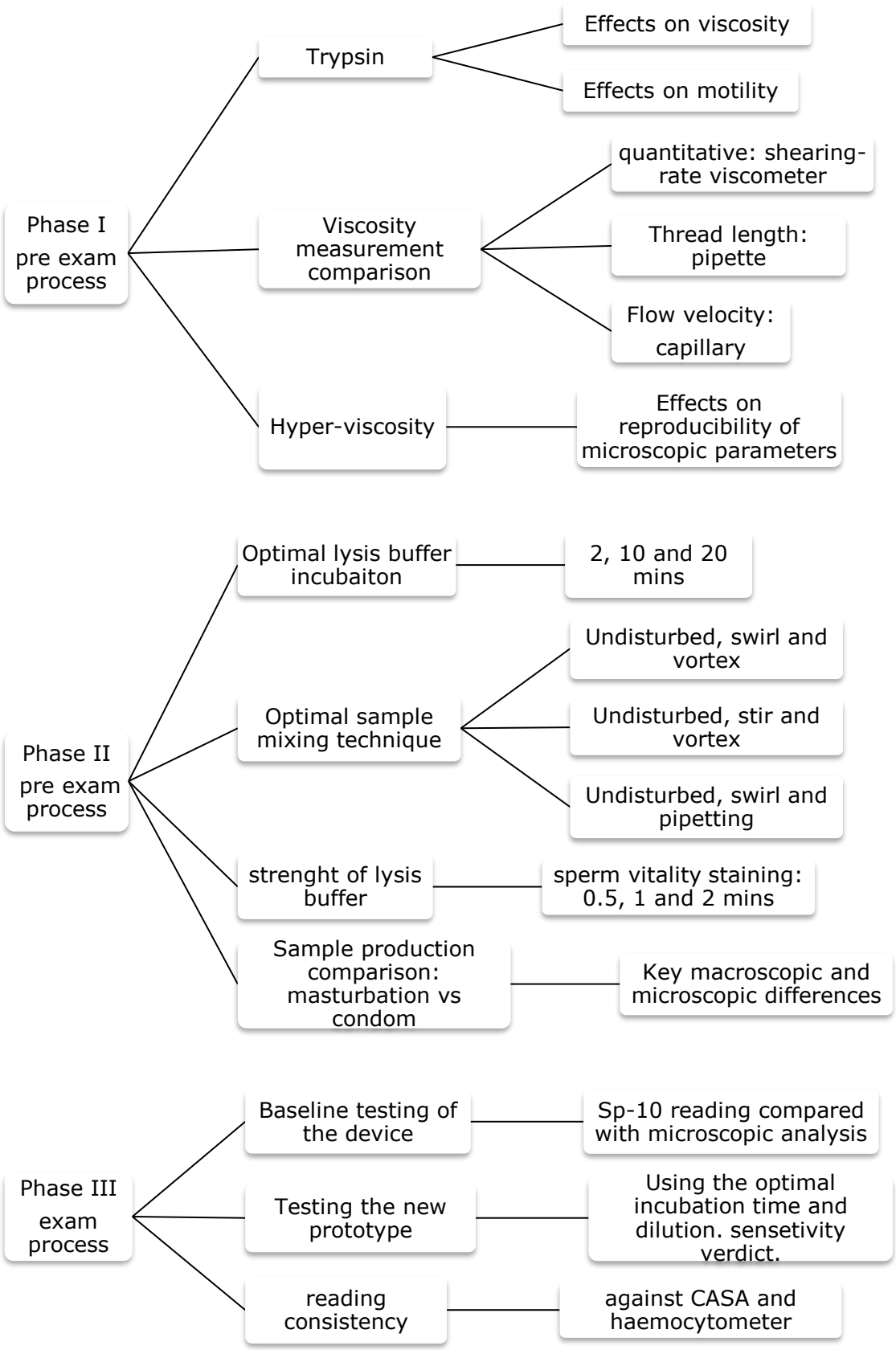
Null hypothesis: Sperm concentration count using the novel immunoassay is not an accurate method of analysis and it is inferior to microscopic methods of concentration assessment.

Alternative hypothesis: The novel immunoassay has the potential to accurately count sperm without biological and subjectivity restrictions of manual and CASA counts.

1.16 Aims and objectives

The aim of this study was to validate a novel sperm counting device for indirect sperm concentration count by detecting SP-10 in semen samples. In order to validate the device, a 3 phase plan (figure 13) was designed to address the preliminary challenges posed by the impact of inadequate mixing and hyper-viscosity. The device was then directly examined against CASA for performance assessment.

Figure 13: The 3 phase plan for validation of the device



The primary and secondary objectives of the project.

2.0 Methods and materials

2.1 Study population

All the specimens analysed for the purpose of this study were produced by consenting donors or patients attending the Andrology Clinic at Queen's Medical Centre, Nottingham. The samples were either produced by means of masturbation onsite or collected at home using a non-spermicidal condom. All the samples were collected in non-toxic sterile polypropylene containers (Alpha labs, Eastleigh UK).

The collection, delivery and initial incubation of semen prior to analysis were carried out in accordance with the Latest WHO (2010) guidelines. Patients and donors kept a period of between 2-7 days of sexual abstinence ahead of producing a sample for analysis. Upon acceptance of samples at the andrology laboratory, the samples were incubated at room temperature for a minimum of 20 minutes to achieve liquefaction. All the tests were done within 1 hour of sample production; for time sensitive experiments, the samples were analysed within 20-30 minutes of sample collection. The number of samples analysed for each experiment is outlined in the results section.

2.2 Manual semen analysis

Manual sperm concentration count was performed using either an improved Neubauer haemocytometer (Hausser scientific. Horsham, PA) or a

disposable haemocytometer (Cell-vision. Heerhugowaard, Netherlands). The concentration analysis analysis was carried out as per instructed by WHO (2010). Initially, a wet-prep slide was analysed under X40 magnification using phase contrast microscopy, in order to determine the most suitable dilution factor. The dilution factors used were 1:2, 1:5, 1:10 and 1:20 (the exact amount of semen and diluent used to make up said dilutions varied in response to the solution volume required for each test).

The chosen diluent for manual sperm concentration count was a sodium bicarbonate formalin-based solution as recommended by the WHO (2010). After determining the dilution factor, semen was added to the diluent in a micro-centrifuge tube (Membrane Solutions, Kent, WA) and vortexed for 10 seconds to mix thoroughly. 2 x 10µl of the solution were then removed independently of one another using a M10 positive displacement pipette (Gilson Scientific UK. Bedfordshire, UK) and were added to each gridded platform on the haemocytometer. In order to let the solution settle in the haemocytometer and appear in one plain under the microscope, the haemocytometer was placed in a damp chamber for a minimum of 5 minutes. Once the solution was ready for analysis, a minimum of 200 sperm were counted on each side of the haemocytometer under X40 magnification using phase contrast microscopy (the same number of rows were counted on each side of the haemocytometer). The number of sperm counted on each side must be close enough within an 'acceptable difference' range, for the data to be considered valid.

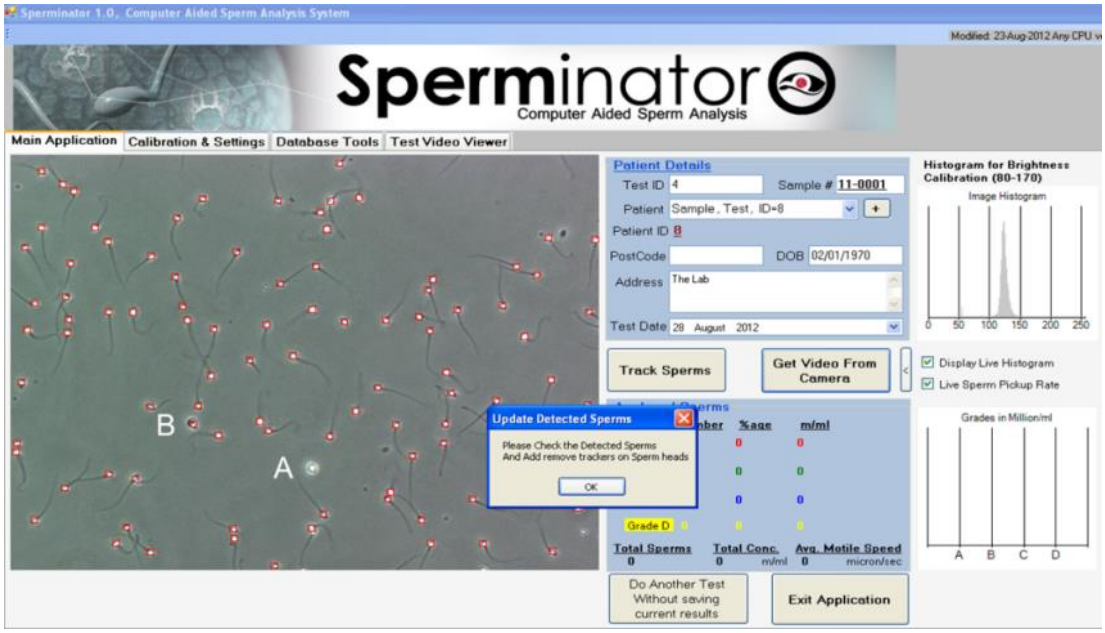
2.3 CASA semen analysis

The CASA software uses automated sperm detection using thresholds which ensures the identified objects are limited to sperm and other similar size cells and debris are not highlighted. In addition, CASA allows on screen editing of the highlighted sperm in the field of view. This property enables the user to highlight any sperm that was not originally captured by the software results in a more accurate sperm count. In order to comment on the motility status of the sample, this program uses a technology named 'automated tracking' which is capable of tracking multiple targets at once and determine the velocity at which sperm are moving during a 1 second period (as well as making note of non-progressive and immotile sperm)(Tomlinson *et al* 2010).

The CASA software was used as per described by the developer's guideline (Tomlinson *et al* 2010). The equipment used for automated semen analysis included an optical Olympus (BX-50) microscope with phase settings, Fire-I 580 Fire wire camera (Unibrain. Athens, Greece) and a PC with windows 7 operating system capable of running the CASA software and receive images as well as live feed as mediated by the camera from the microscope.

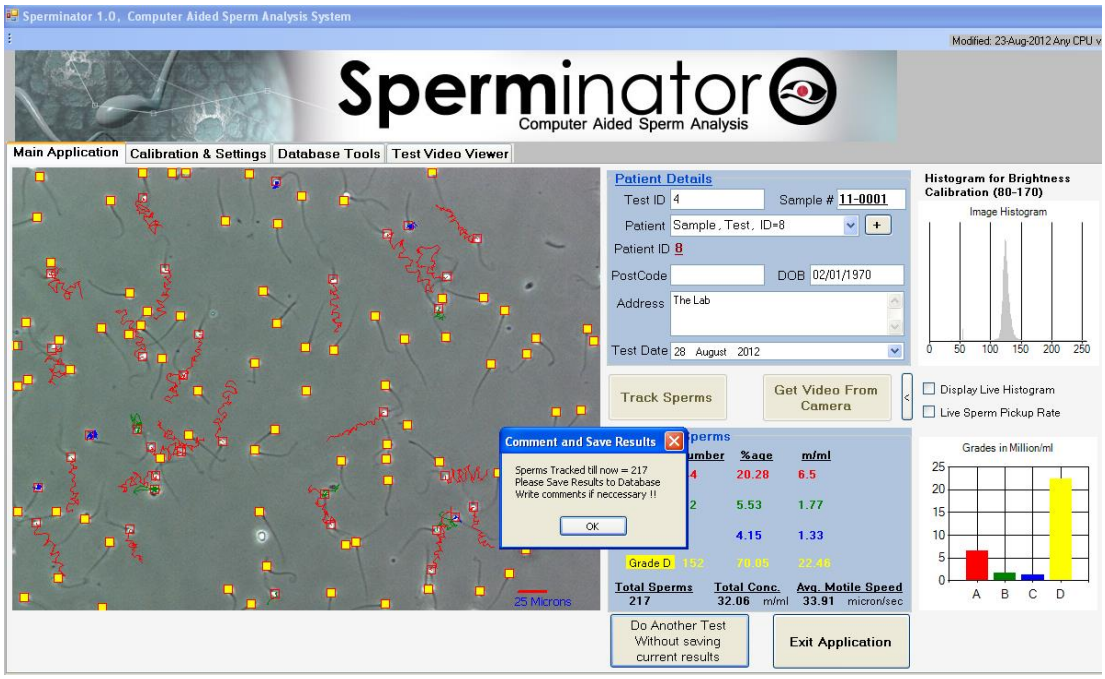
To carry out the automated analysis, 20 micron deep, 4 chamber leja (Leja®, Netherlands) slides were used. The following 2 figures, demonstrate the CASA environment.

Figure 14: Sperm recognition by the Sperminator software



Recognised sperm heads by the device are highlighted in red.

Figure 15: Sperm tracking by the Sperminator software



Sperm as tracked by CASA during a 1 second period. Red trails track the motion of motile sperm heads. Blue dots show the non-progressive sperm heads and yellow squares indicate immotile sperm heads.

2.4 Proteolytic enzyme, lysis buffer and diluent

2.4.1 Trypsin

In order to overcome the challenge of accurate semen analysis for hyper-viscous samples, it was decided to assess the feasibility of using proteolytic enzymes to reduce the viscosity. The effect of proteolytic enzymes on semen viscosity and sperm motility were then assessed. Trypsin was chosen as the ideal enzyme due to its readily availability, suitable pH (~7.6) and range of clinical applications. Trypsin-EDTA solution 1X (Gibco® Trypsin. Grand Island, NY) contains 0.25% trypsin, 0.02% EDTA in Hank's balanced salt solution. The container was sterile-filtered and was stored at -20°C to prevent autolysis (SigmaAldrich.com 2017a). Aliquots of the trypsin solution were thawed accordingly and reached room temperature before being used for the experiment.

The dilution factor of 1:20 was chosen to examine the effect of trypsin on semen viscosity (50µl semen + 0.95ml 0.25% trypsin). Samples were also diluted in the buffer using the same dilution factor (50µl semen + 0.95ml lysis buffer). A combined dilution was also prepared (50µl semen + 0.475ml trypsin + 0.475ml lysis buffer) to determine whether presence of both dilutions has a more significant impact on viscosity. The solutions were incubated at room temperature for 20 mins. The viscosity of neat semen and the prepared solutions were then determined using a shearing rate viscometer.

2.4.2 Lysis buffer

As per described by Herr *et al* (1990a) SP-10 is displayed in the acrosomal region of the sperm head in association with the inner acrosomal membrane. For the device to be able to detect the concentration of SP-10 accurately, a detergent was used to lyse the sperm acrosomal membrane and subsequently release SP-10 into the solution. The lysis buffer used was prepared by the assay team at SPD. The following table demonstrates the contents of the buffer and highlights their significance.

Table 9: Properties of the lysis buffer components

Component	Concentration	Application
Triton X-100 $C_{34}H_{62}O_{11}$ **(for x=10)**	0.5%	A non-ionic detergent used for solubilising membrane proteins. The most widely used detergent to lyse cells and extract proteins. (Sigmaaldrich.com 2017b)
PBS (phosphate buffered saline)	0.01M	Common buffer used in immunoassays with a neutral pH.
Sodium azide NaN_3	0.05%	Probe reagent and preservative.

Major components of the lysis buffer, concentration and application.

2.4.3 Diluent

In order to immobilise the entire sperm population in samples for manual concentration analysis, they were diluted in a diluent solution. The chosen buffer was DPBS 0.4% PFA which instantly stops sperm motility altogether but does not disintegrate any of the cells. The dilution factor of 1:20 was used for all the manual concentration analysis unless stated otherwise.

2.5 Sperm VitalStain™

In order to gain a more detailed understanding of the effect of the lysis buffer on sperm, vitality analysis was carried out. For SP-10 to be released from the sperm acrosome, the lysis buffer must be able to compromise the membrane on the entire sperm population. A 100% non-viable result of vitality assessment on a sample exposed to lysis buffer would indicate that the chosen concentrations of the lysis components and the dilution factor by which it was added to semen are highly satisfactory. Sperm VitalStain™ (NidaCon International AB. Mölndal, Sweden) is a single step saline solution containing eosin and nigrosine dyes, was used for this purpose.

The sperm VitalStain™ bottle was well shaken before use. 30µl of the stain medium was extracted using a M10 positive displacement pipette (Gilson scientific UK. Bedfordshire, UK) and added to 30µl of semen in a micro-centrifuge tube (Membrane solutions. Kent, WA) and mixed well by pipetting. Subsequently, the sample was incubated at room temperature for 30 seconds. 20µl of the solution was then removed and placed on a glass slide (CellPath Ltd. Wales, UK) to create a smear. The smear was made by placing a second slide on top of the initial slide. Once the droplet was evenly spread, the second slide was horizontally pulled apart from the first slide. This method created two suitable evenly spread slides for analysis. The smear was then given time to airdry and was examined at X1000 microscopy using immersion oil. A total of 200 sperm were counted on each slide and scored accordingly. The white (unstained) sperm were classified as alive and the red (or pink) sperm were classified as dead.

Key components of Sperm VitalStain™ are sodium chloride, eosin Y, nigrosine, formalin and water (nidacon.com 2015).

2.6 Viscosity measurement

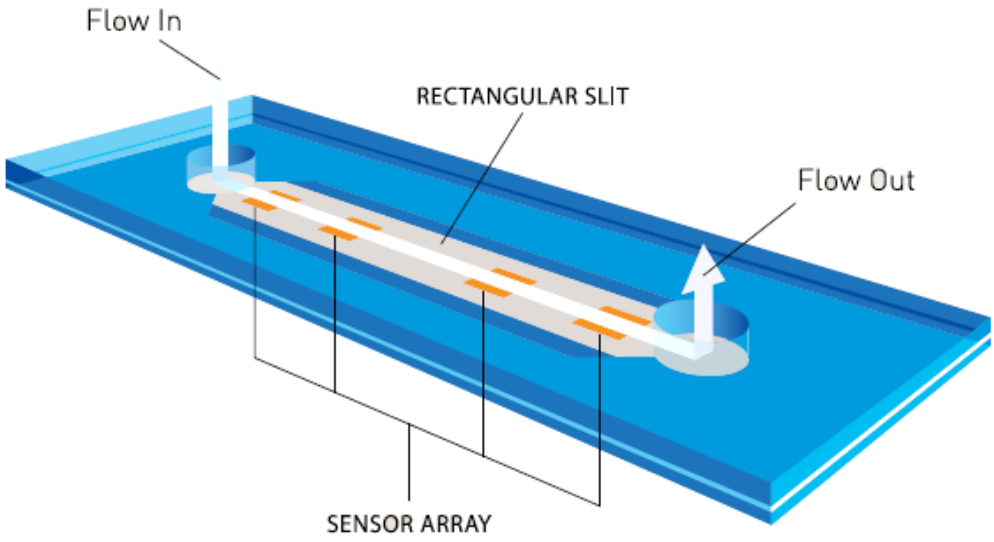
To measure semen viscosity, WHO (2010) literature recommends the aspiration of semen in a disposable plastic pipette and gently letting semen to drip using gravity. Semen which forms droplets or strings with estimated length of 2cm or shorter are considered to have normal viscosity and longer threads indicate high viscosity.

As described by Douglas-Hamilton *et al* (2005), the second method of viscosity measurement chosen was the time it takes (in seconds) for semen to fill a cover-slip fixed chamber. 5µl of semen was aspirated using a M10 positive displacement pipette (Gilson scientific UK. Bedfordshire, UK) and injected in a leja slide chamber at 45° angle. To measure the time taken for semen to fill the chamber, an electronic stop watch was used to keep the time it took samples to fill the leja chambers. Using the stop-watch was due to reducing human error and accurate time keeping.

The last method to measure viscosity was using a shearing rate viscometer. A viscometer can provide an accurate viscosity value in centipoise (1 cP = 1 mPa.s). *micro*VISC (RheoSense, San Ramon, California, United States) was used for this purpose. It uses laminar flow in a rectangular slit capillary made of borosilicate glass to determine the viscosity of a fluid. *micro*VISC

uses a pumping system to introduce the fluid to the pressure sensors at a specific and controlled flow rate. The resistance of the fluid to flow is the very definition of viscosity (RheoSense.com 2017).

Figure 16: Mechanism of flow rate measure in the viscometer



Schematic of rectangular slit microfluidic cell and the position of the sensory arrays in the Viscometer (RheoSense.com 2017).

2.7 Novel 'home sperm test kit' device

The prototype of the device used for this study resembled the appearance of pregnancy tests used every day by thousands of women. Based on the same lateral flow analytical architecture, the underlying mechanism of SP-10 detection by the device is similar to that of pregnancy sticks detecting human chorionic gonadotrophin (hCG) levels in urine. The use of an immune-chromatographic assay for sperm concentration detection has notable advantages; it's simple to use, relatively inexpensive, and over 98% accurate as reported by Herr *et al* (1999).

Figure 17: The early prototype of the novel device

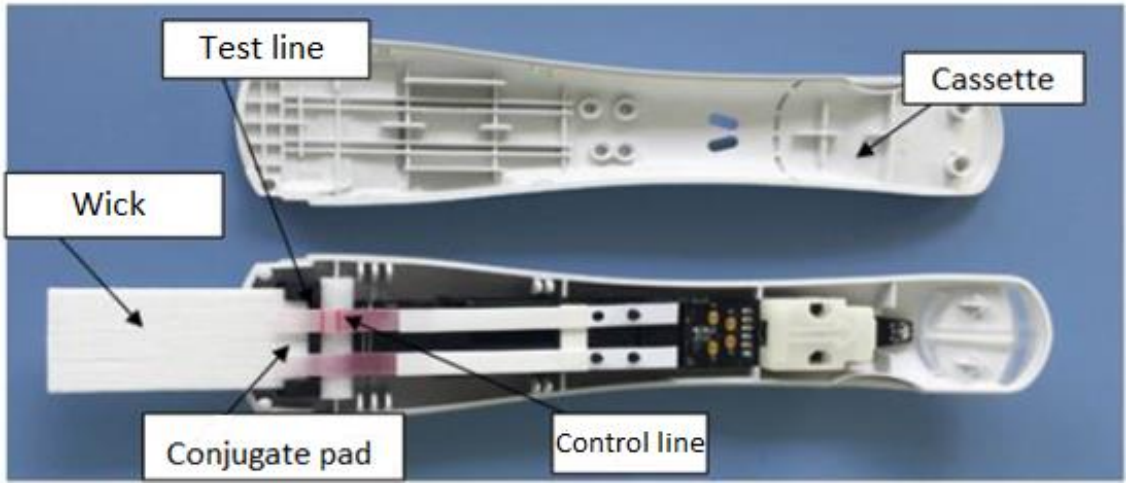


Figure above outlines the major components inside the device including the two sensitivity strips.

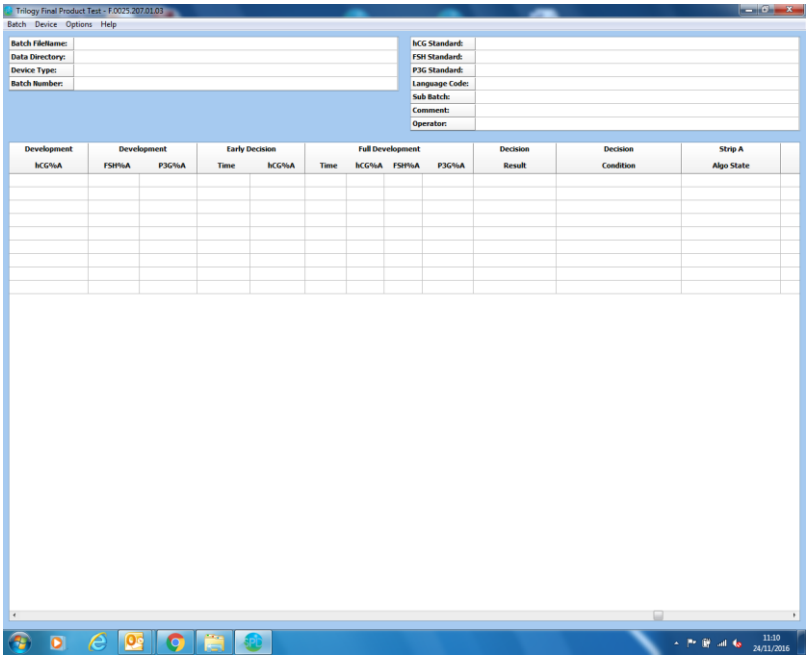
The wick is a rigid pad with a polyester core, covered with a polyethylene skin sintered together. It takes around 5 seconds for the entire surface of the pad to get wet following the addition of 1 ml of the sample solution. The sample pad should be large enough so patients would find it easy to pour the sample on it. The device is activated as 1 ml of the sample is introduced to the wick. The fluid then flows laterally to the conjugate pad.

The conjugate pad holds latex in dry state and contains gold nanoparticles. As the sample flows through the conjugate pad, antibodies are conjugated to the colloidal gold particles forming a "sandwich assay". The number of complexes formed at this stage of the test determines the outcome of the analysis. The newly formed SP-10-gold complex then flows through the test membrane.

Followed by the conjugate pad, the device contains two antibody lines referred to as the test line and control line. Higher concentration of SP-10 results in a more intense test line. The test line is plotted with anti-SP-10 antibody therefore can detect the presence and concentration of SP-10. The presence of a control line is vital as it indicates that the device has functioned correctly and a sufficient amount of the test fluid has flowed over the test line.

In order to validate the specificity and sensitivity of the device, a small specialised laboratory jig was used as provided by SPD. The jig detected the reaction of the device to the test fluid and translated it into algorithms by a software named Thor that was also developed by the manufacturer. Figure 18 demonstrates the environment of the software used.

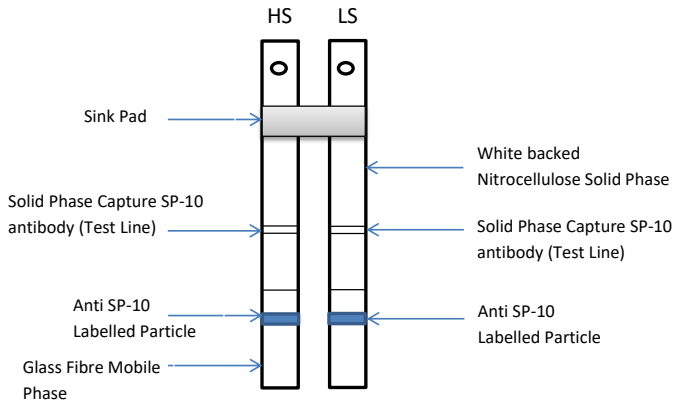
Figure 18: Thor software environment



The jig signalled the concentration of antibody bound SP-10 in the sample. The concentration of SP-10 appeared in algorithms on the screen and subsequently translated into million per ml using a statistical software.

The 3rd phase of the experiment was done using a revisited prototype of the device. There were some minor changes made to make the device more sensitive and specific for SP-10 detection. Figure 19 demonstrates the arrangement of the high sensitivity (HS) and low sensitivity (LS) of this prototype. The strips have the same chemistry and formulation and therefore are expected to yield similar readings.

Figure 19: The internal components of the new prototype



The parallel arrangement of the high and low sensitivity strips inside the device.

2.8 Four well testing

Routine seminal fluid analysis is performed by examination of a small volume of the sample to represent the characteristics of the entire ejaculate. The result of the analysis therefore rely entirely on achieving homogeneity by ensuring that the sample is mixed well. similarly, the SP-10 assay also relies on the homogeneity of the sample for accurate analysis. To assess the effect of various semen mixing techniques on sample quality, the four well testing model was chosen. This test was done by dividing the bottom of the analysis pot into 4 distinct zones (1 to 4) by marking the outside of the pot with a marker pen. 4 extracts for undisturbed semen as

well as mixed semen using different methods were removed independently. 5µl of each extract was injected in a leja chamber (Leja®, Netherlands) for CASA analysis.

2.9 Mixing techniques

As previously described seminal fluid may retain its viscous characteristic post liquefaction. Semen viscosity makes extracting a representative sample of semen for routine analysis problematical. In addition, the viscosity of semen withstands flow and resists migration laterally, therefore proves challenging for device analysis (WHO 2010; Vasan 2011).

In an attempt to increase the reproducibility of sperm count data and study the negative impact inserted on sperm motility due to mechanical stress of mixing methods, the following mixing techniques were carried out (table below). Samples chosen for this phase of the experiment were all produced on site to minimise the effects of external factors such time, temperature and unintended mixing during transport.

Table 10: Semen mixing techniques

Technique	Procedure
Undisturbed	Kept undisturbed at room temperature for the period of 20-30 mins. Immediately followed by four well analysis.
Stirring	The sample was gently stirred 10 times using a pasture pipette (Sterilin. Newport, UK), followed by four well analysis.
Swirling	The sample container was gently swirled for 10 seconds, followed by four well analysis.
Pipetting	The sample was gently pipetted 10 times using a pasture pipette (Sterilin. Newport, UK), followed by four well analysis.
Vortexing	The sample was vortexed for 10 seconds, followed by four well analysis.

The mixing techniques were compared in terms of reproducibility and impact on motility to identify the optimal technique for device analysis.

2.10 Sample collection at home vs onsite

In an effort to examine whether the sample produced at the clinic truly represents the man's fertility status, this study was designed. This trial required the patients to produce a sample onsite by masturbation and produce a second sample at home by intercourse in a non-spermicidal condom. An information sheet was written to provide the patients with the basic knowledge regarding the aim of the analysis. It was also explained to the patients that taking part in this experiment is entirely voluntary and opting out at any point will not affect their diagnostic tests or treatment in any way. The information sheet asked the patients to abstain from any sexual activity for a minimum of 48 hours before producing each sample and to collect the entire ejaculate in both the sample pot and condom. For the sample that was produced at home, the patients were told to bring it to

the andrology laboratory ideally within 1 hour of sample production. They were also given instruction on keeping the sample close to their body (i.e. a pocket inside of the jacket) during transport in order to minimise the effect of environmental temperature on the sample. The information sheet also asked the patients to carefully take off the condom after producing the home sample, tie the end of it to avoid leakage and place it in the sterile pot that was also provided from the laboratory.

Patients were asked to complete a questionnaire upon sample delivery for both samples. The questions were as follow:

- Have you collected the entire sample inside the pot?
- Has the sample been tampered with at any point from production to delivery?
- How many days has it been since you r last ejaculation?
- Approximately how many days a week do you ejaculate?
- Approximately how long did it take from arousal to ejaculation at home?
- Approximately how long did it take from arousal to ejaculation onsite?
- How would you describe your ethnicity?

The condom used for this experiment was Pasante unique (Pasante healthcare Ltd, UK) which had been previously tested for sperm toxicity (Tomlinson *et al* 2012). The specimen containers provided for the patients was 60ml CE marked specimen containers (Alpha labs Ltd, UK). Volume of the condom ejaculate was converted from the ejaculate weight. To accurately determine the weight of the ejaculate, the following mean weight (g) of condoms and specimen pots were created:

Table 11: Mean weight for samples produced in condoms

Container	Mean weight (g)
Condom only	0.565
Specimen pot (with label)	14.78
Condom in labelled pot	15.37

Mean weight (g) for condoms, specimen pots used to calculate the volume of the ejaculates.

Once the samples were weighted on a balance, weight of the ejaculate was then converted into volume (ml) in accordance with the WHO (2010) guidelines. The analysis of the samples were carried out as explained in 2.2 and 2.3 manually and using CASA.

2.11 Statistical analysis

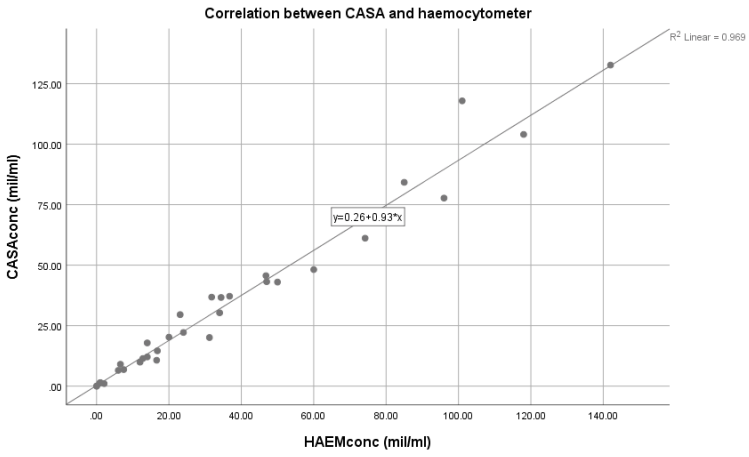
The preliminary correlation analysis (with Pearson's and Spearman's option 2-tailed with 95% confidence limits) and scatter plots were done using IBM SPSS statistics software (1.0.0.781; 64-bit ed. Trial subscription). The device testing data were analysed by a combination of JMP ver11 and SAS ver9.3. JMP was used to produce the general scatter plots with an ellipse of 95% confidence. For the device analysis, SAS was used to calculate bias corrected Pearson's correlation estimates with 95% confidence limits calculated by Fishers method using Proc Corr with the Spearman's option. The data from the correlation analysis were used to create variable comparison graphs of high and low sensitivity strips produced by SAS using the output data. For mixing method experiments, JMP was used due to its variability analysis feature.

3.0 Results

3.1 Haemocytometer and CASA data correlation

To ensure the concentration analysis using both CASA and haemocytometer were in harmony. Samples (32) were analysed using both methods. Haemocytometer is the gold standard recommendation by the WHO (2010) for sperm count. However, analysing multiple samples using CASA is more time effective. In addition, CASA can provide velocity of motile sperm which may be significant for analysing the effect of hyper-viscous samples on 'sluggish' sperm. Based on time efficiency, CASA counting was chosen as a more preferable method of analysis. It was however important to validate the results of CASA against the WHO (2010) recommended method. This section of the thesis therefore aimed to find a significant correlation between haemocytometer and the CASA counting. The analysis showed a significant relationship between the two methods ($p < 0.0001$). The following graph compares the correlation between CASA readings and the haemocytometer in form of a scatterplot.

Figure 20: Correlation between CASA and haemocytometer



Direct correlation between the two counting methods is seen (n=32). Stronger relationship is observed for lower concentrations.

According to the plot, at lower concentrations the readings are generally closer, although significant correlation is observed throughout the training data as the mean concentration of CASA counts is 34.17 ml/mil and haemocytometer is 36.43 mil/ml.

3.2 Viscosity measurement of semen

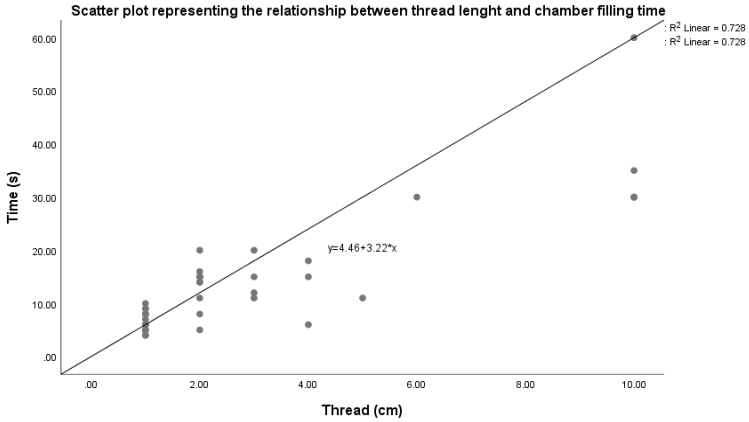
This section of the project aimed to compare the 3 chosen methods of viscosity measurement with one another in an effort to create a normative scale of semen viscosity. These methods were as follows:

- Measurement of the thread length (cm) of semen as it is dripping from a pasture pipette using gravity. This method is recommended by the WHO (2010).
- Measuring the time (s) it takes for 5µl of semen to fill a leja chamber.
- Measuring the viscosity (mPa.s) using a sheering rate viscometer.

3.2.1 Thread (cm) measure vs time (s) to fill the chamber

This section analysed the velocity (s) of 5µl of semen to fill a leja chamber compared with the thread length formed for dripping the sample from a pasture pipette (WHO) for 41 samples. Correlation between thread length and filling chamber time was significant ($p < 0.0001$). The following graph illustrated the correlation in form of a scatterplot for visual demonstration. The data set in figure 21 is discrete in nature and appears to contain a few outliers particularly at high viscosity end of the spectrum.

Figure 21: Correlation between thread length and chamber filling time

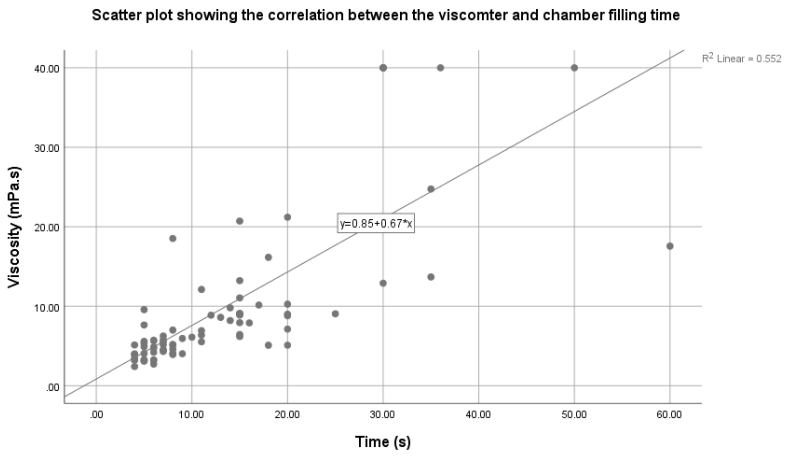


Stronger correlation is observed for lower viscosities. (n= 41)

3.2.2 Viscometer (mPa.s) vs time (s) to fill the chamber

This section of the analysis aimed to outline the relationship between viscosity of semen as determined by the viscometer and the time taken for 5µl of semen to fill a leja chamber. A total of 78 patient samples were analysed for this section. The following table illustrates the Pearson correlation between mPa.s and s. There is a significant correlation between these 2 methods of semen viscosity measurement ($p < 0.0001$). To visually demonstrate this relationship, the following scatter plot was constructed.

Figure 22: Correlation between the viscometer and chamber filling time

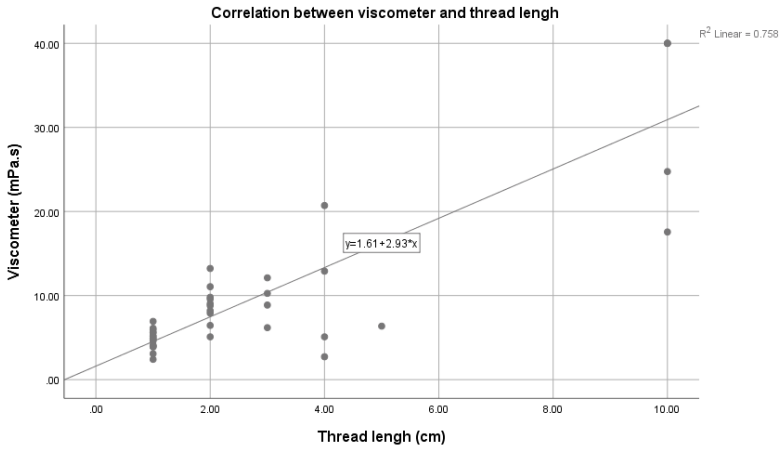


Stronger relationship is observed for lower viscosity readings (n= 78).

3.2.3 Viscometer (mPa.s) vs thread length (cm)

This section of the results, aims to determine the relationship between viscometer reading of semen viscosity and the recommended WHO (2010) method thread length (cm) measurement. A strong correlation was observed ($p < 0.0001$) for 41 patient samples by Pearson's correlation analysis. This correlation was then demonstrated on the following scatter plot.

Figure 23: Correlation between viscometer and thread length



Correlation is generally stronger for the lower viscosity measurements (n= 41)

3.2.4 Normative scale of viscosity

Following the determination of how different methods of viscosity measurement correlate with one another, table 18 was created to demonstrate a normative scale of semen viscosity using all three methods of measurement. The table aims to classify semen motility into 3 groups; normal, slightly viscous and viscous. Apart from a few outliers, the majority of samples were in the same category using all 3 methods of viscosity measure.

Table 12: The normative scale of viscosity

Viscosity class	Thread length (cm)	Filling time (s)	Viscometer (mPa.s)
Normal	<2	10	1-7
Slightly viscous	2-6	10-20	7-12
Viscous	>6	20-60	>12

The viscosity classification for thread length (cm), chamber filling time (s) and quantitative viscometry (mPa.s).

3.3 Trypsin

3.3.1 Effect of trypsin on viscosity

To determine the effect of a proteolytic enzyme on reduction of semen viscosity, a total of 52 samples were analysed. Additionally, the effect of the lysis buffer on semen viscosity was analysed on the same set of samples. This section aimed to determine whether in addition to the lysis buffer, the use of a proteolytic enzyme such as trypsin is required to effectively reduce semen viscosity. The viscosity of neat semen, lysis solution and the enzyme solution were measured by the shearing rate viscometer and the time (s) it took for the solution to fill a leja chamber. Both solutions were incubated at room temperature for 20 minutes before viscosity reading and semen was diluted by the factor of 1:20 in both diluents. The average viscosity for neat semen of 52 samples was calculated as 10.69 mPa.s. Diluting semen in the lysis buffer and the trypsin solution reduced the viscosity to 1.48 mPa.s and 1.36 mPa.s respectively. The chamber filling time was also reduced significantly from the average of 15.5 seconds to 2 seconds for both diluents. There was no significant difference observed between using either diluent in terms of reducing the semen viscosity as they were equally effective. Non-parametric correlation

(Spearman's) analysis found a strong relationship ($P < 0.0001$) between viscosity of neat semen compared with 1:20 dilution in trypsin for 52 semen samples.

A second method of viscosity measurement was also used for this experiment. The time (s) it takes for 5µl of semen to fill a leja chamber was recorded and compared with the time (s) it takes for the semen-trypsin solution to fill the chamber. On average, it took about 15s for the neat semen of the 52 samples to fill the chamber. This time was reduced to 2s after 1:20 dilution of semen in trypsin (Table below). The results of this experiment showed significance viscosity reduction after addition of trypsin.

Table 13: Effect of trypsin on semen viscosity

Function	Neat visc (mPa.s)	Neat visc (s)	Smn+tryp (mPa.s)	Smn+tryp (s)
Mean	10.69	14.48	1.35	2.04
SD	9.91	10.46	0.50	0.19

Significant reduction of viscosity is observed after addition of trypsin. Mean viscosity of 1.35 mPa.s is almost the viscosity of water.

3.3.2 Effect of trypsin on Motility

29 semen samples were analysed for determination of the effect of trypsin on motility. At 0.25% concentration, trypsin reduced semen viscosity by almost 26%.

Table 14: Effect of trypsin on sperm motility

Function	% Mot neat	% Mot trypsin	% Mot reduction
Mean	42.68	17.21	25.46
SD	17.41	10.85	12.27

Shows the impact of trypsin on sperm motility at 0.25% concentration. 25.46% motility reduction was observed. n=29

This section of experiment also determined the impact of trypsin on sperm membrane. The results indicate that the membrane of some sperm are impaired but at 0.25%, trypsin doesn't impair the membrane of the entire sperm population.

3.4 lysis buffer

3.4.1 Optimal lysis buffer dilution factor

63 samples were diluted at 1:2, 1:5, 1:10, 1:15 and 1:20 and incubated at room temperature for 20 minutes. The effect of lysis buffer on semen viscosity was then recorded using the viscometer. The following table outlines the amount of semen and lysis buffer used for each dilution as well as the mean viscosity for each dilution.

Table 15: Serial dilution of semen in lysis buffer

Dilution	Semen vol (µl)	Lysis buffer vol (µl)	Total vol (µl)	Mean viscosity (mPa.s)
1:2	200	200	400	2.75
1:5	100	400	500	1.68
1:10	40	360	400	1.39
1:15	40	560	600	1.31
1:20	50	950	1000	1.26

Semen and lysis buffer volumes used for determination of effects of dilution factors on viscosity (n=63).

The mean viscosity of neat semen was determined to be 10.83 mPa.s. The results show that even at 1:2 dilution, the viscosity is significantly reduced. As per expected, the 1:20 dilution has the lowest viscosity out of all the dilution factors.

3.4.2 Optimal lysis buffer incubation period

The following table illustrates the initial viscosity of semen as well as the viscosity of semen after introduction of lysis buffer at 3 time points for 70 samples. It appears that semen viscosity is significantly reduced after 2 mins of incubation in the lysis buffer at 1:20 dilution at room temperature.

Table 16: Viscosity of semen in lysis buffer at 3 incubation time points

Function	Initial visc (mPa.s)	2 mins visc (mPa.s)	10 mins visc (mPa.s)	20 mins visc (mPa.s)
Mean visc (mPa.s)	10.31	1.29	1.30	1.28
SD	10.23	0.18	0.19	0.18

Viscosity was significantly reduced as early as 2 mins of incubation in lysis buffer (n=70).

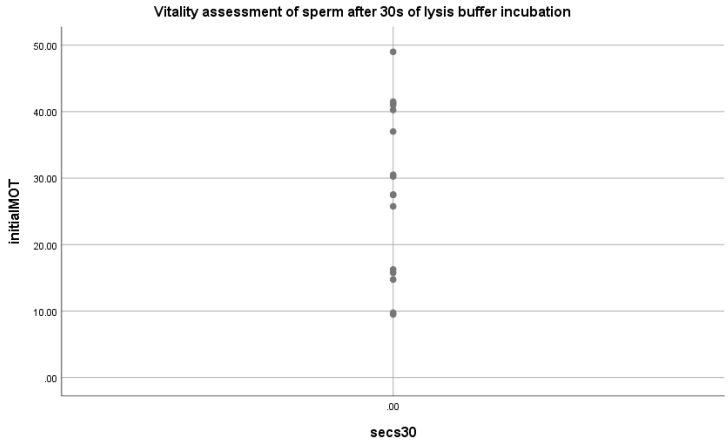
The impact of lysis buffer on viscosity of semen appears to be significant regardless of period of incubation at 1:20 dilution. The 3.7 section of the results analysed the accuracy of SP-10 detection at different incubation periods in lysis buffer.

3.4.3 Vitality

The aim of this analysis was to determine the time it takes for the lysis buffer to impair the acrosomal membrane of the entire sperm population. Semen was diluted at 1:20 dilution in the lysis buffer at room temperature. The solution was incubated for 30s, 1 min and 2 mins before the vitality analysis was carried out. 2x200 sperm was counted for each analysis. Results showed that 100% of sperm in all samples were impaired for all 3 time points regardless of the initial motility of the sample. The following

graph demonstrates the 100% impairment of sperm membranes for 16 samples after 30s of incubation in lysis buffer.

Figure 24: Vitality assessment of sperm after 30 secs of incubation in lysis buffer



Total impairment of acrosomal membrane after 30 seconds regardless of sample motility (n=16).

3.5 Mixing results

Semen mixing was categorised into the following 3 groups:

- Undisturbed, swirled and vortexed samples
- Undisturbed, stirred and vortexed samples
- Undisturbed, swirled and pipetted samples

3.5.1 Undisturbed, swirled and vortexed samples

Table 17: Undisturbed, swirled and vortexed summary table

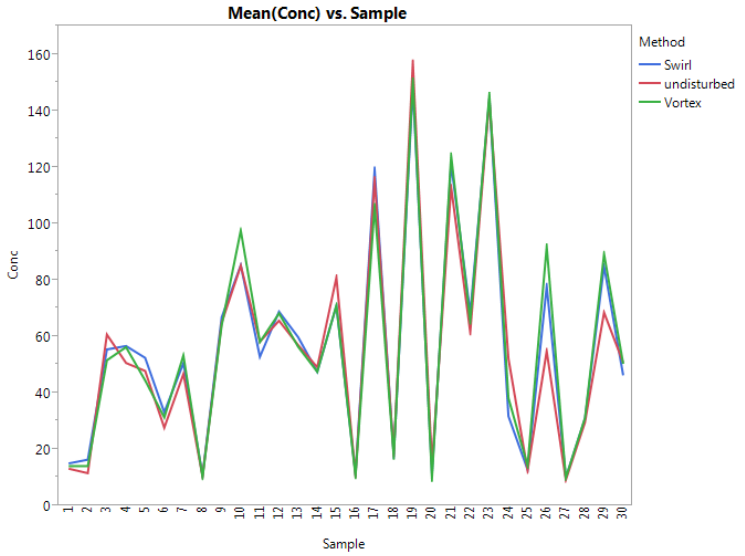
Number of the samples	30
Mixing methods	undisturbed, swirled, vortexed
No of wells tested for each method and each sample	4
Total No of tests	360

Summary of the concentration and motility analysis for undisturbed, swirl and vortex mixing methods.

3.5.1.1 Concentration

To investigate whether a correlation exists between the mean concentration of each mixing method, the following graph was produced. The graph shows consistency among undisturbed, swirled and vortexed methods.

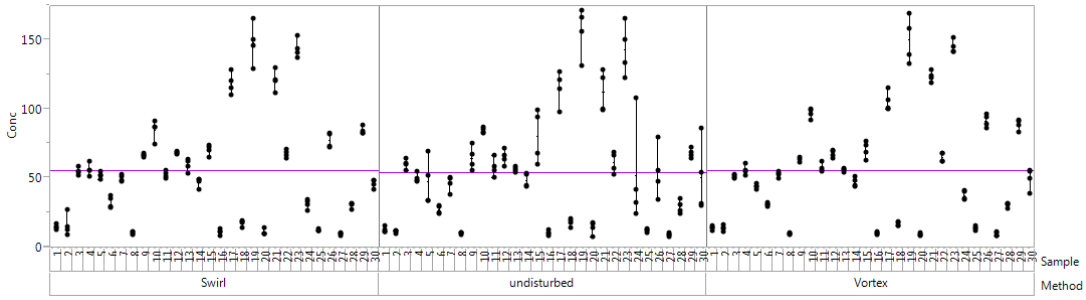
Figure 25: Correlation between undisturbed, swirled and vortexed concentration counts



The mean sperm concentration (mil/ml) for each sample with swirled, undisturbed and vortexed methods.

The following plot aims to show the individual results of the tests grouped by sample numbers and methods. The purple line represents the mean sperm concentration for each method.

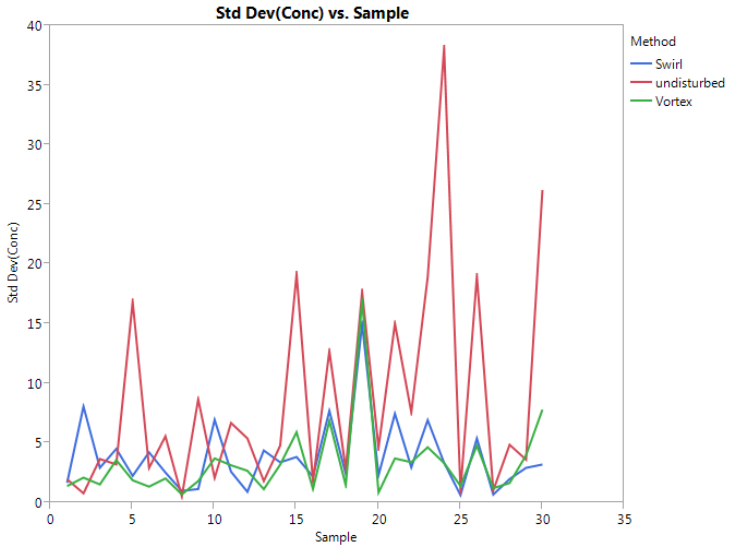
Figure 26: Distribution of 4 well testing for each mixing method against concentration



Vortex group shows higher reproducibility as the dots on each vertical line are closer to one another. The mean sperm concentration (mil/ml) for each method is highlighted by purple lines.

The following graph, demonstrates the standard deviation for each samples sperm concentration value. This graph shows great variability from the 4 well testing of undisturbed samples. Particularly samples 5, 15, 24, 26 and 30.

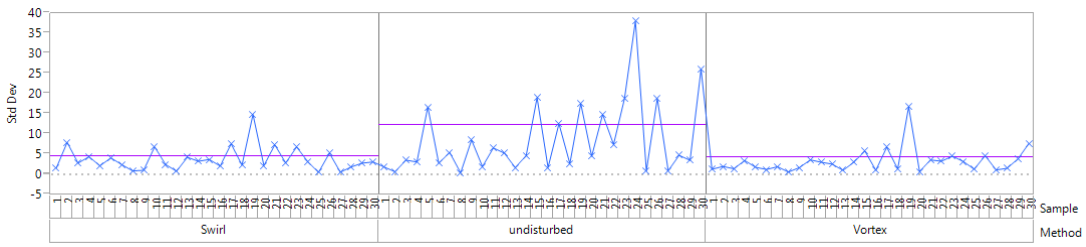
Figure 27: Standard deviation of undisturbed, swirled and vortexed concentration counts



Standard deviation for each sample with each method shown using a different colour. High variability from the undisturbed (red) samples is observed.

The plot below demonstrates standard deviation within each sample split by method. The purple line represents the average standard deviation for that method. The highest variation is observed when the samples are left undisturbed and the least after vortexing.

Figure 28: Standard deviation of sperm concentration for each group



The vortex group expresses the highest level of reproducibility. The purple lines are the average standard deviation for each method.

Table below shows value of the average standard deviation for each method (as highlighted by the purple line by figure 29). Vortexing method had the lowest average standard deviation of 4.5 mil/ml. The swirl method had a slightly larger standard deviation by 0.4 mil/ml and the average standard deviation for the undisturbed samples was higher by 7.9 mli/ml.

Table 18: Average variance for each mixing method

Method	Mean	Average Variance	Average SD	CV
Swirl	56.0	23.7	4.87	8.70%
Undisturbed	54.8	153.3	12.38	22.60%
Vortex	56.5	20.5	4.53	8.00%

The lowest average variance was observed for the vortex method, closely followed by swirling. Undisturbed category expressed a much higher average variance.

In order to show the source of variability, variance components were calculated. Table below shows that 4.3% of the variation is due to the 4 positions (4 wells) for a given sample and method. 94.1% of the variability is as a result of the range of sperm concentration for each sample in this group (the range varied from oligozoospermic samples to samples with concentration of over 100mil/ml). The high percentage of variability is to be expected between the samples, as no excluding criterion was set on the sperm concentration. 1.6% of the total variance is due to the different mixing methods used.

Table 19: Sources of variation: methods, concentrations and 4 well testing

Component	Var Component	% of Total	Plot%	Sqrt(Var Comp)
Method	24.4819	1.6		4.948
Individual conc	1446.8546	94.1		38.038
4 well testing	65.5775	4.3		8.098
Total	1536.9141	100.0		39.203

The largest source of variation is due to individual sperm concentration of the test samples (n=30).

3.5.1.2 Motility

To analyse the data in a greater depth, the velocity of sperm motility was also measured. Details of velocity classifications as measured by CASA are as follow:

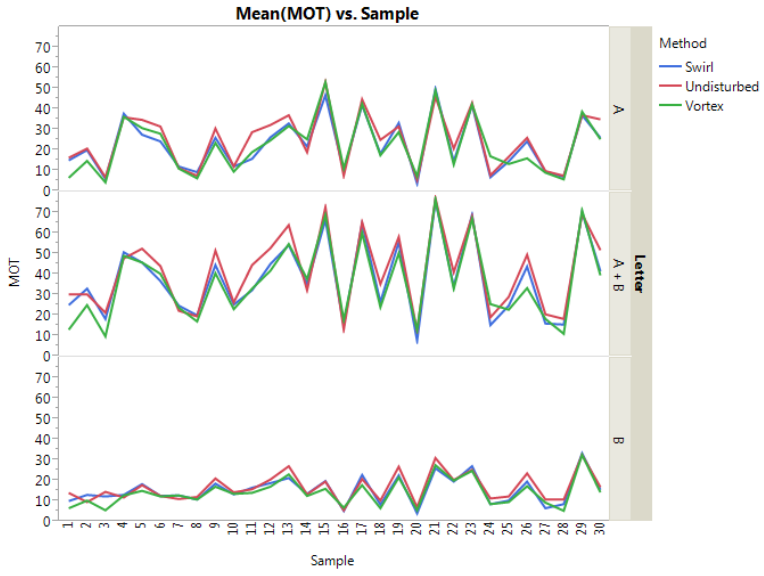
Table 20: Velocity categories of sperm

Classification	velocity (µm)
A	>25 µm
B	5-25 µm
A+B	Overall

The 3 velocity classification of sperm motility. Only applies to progressive motile sperm in a sample.

The following graph shows the mean for each sample's percentage motility for the different methods. As appears on figure 30, the mean percentage motility for all three methods track well and are consistent across all the velocity categories.

Figure 29: Correlation between undisturbed, swirled and vortexed motility analysis

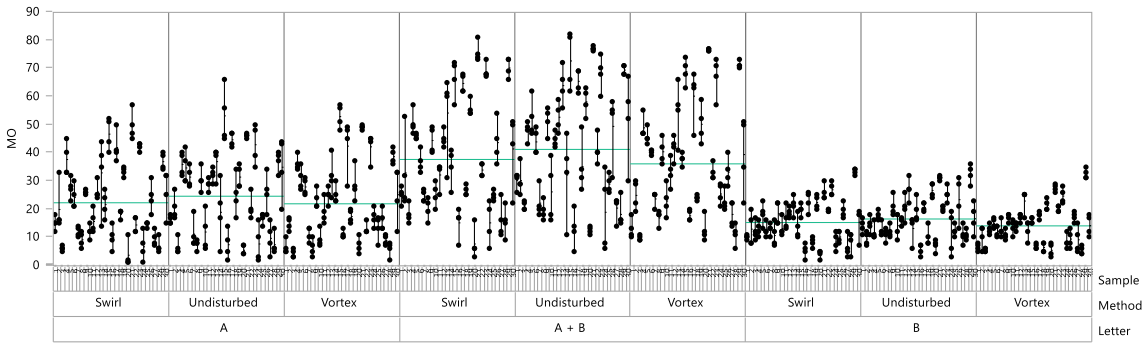


The plot highlights %MOT of each sample for different velocity categories. Different mixing techniques are represented by different colours. The trend is generally followed by all mixing techniques.

The following plot shows the individual %MOT of the tests grouped by sample number, mixing method and velocity category. 4 dots appear on

each vertical line for every sample. These dots are indicative of the results of the 4 well analysis. The average percentage motility is highlighted by the green lines and the undisturbed average is higher than the mixed methods in each velocity category as well as combined.

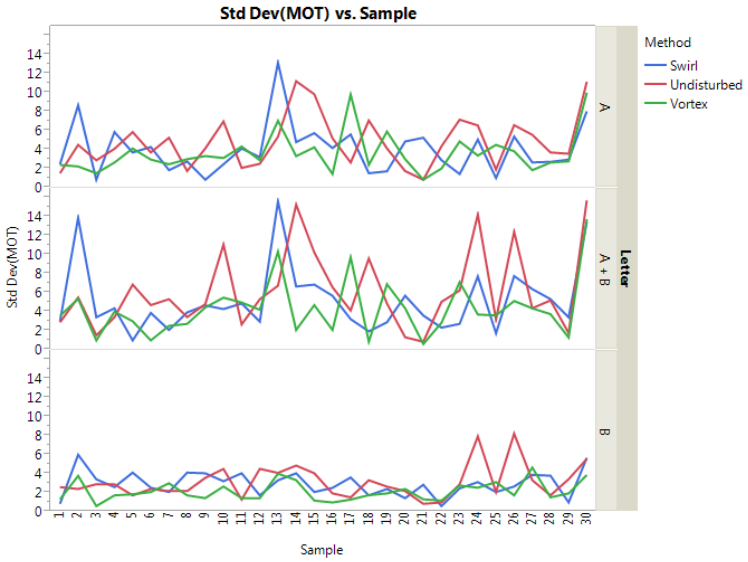
Figure 30: Distribution of 4 well testing for each mixing method against sperm motility



Mean %MOT for undisturbed, swirled and vortexed. 4 dots on each vertical line represent the 4 well testing in each velocity category.

The following figure shows the standard deviation for each sample's motility. The 3 sections in the figure illustrate the 3 velocity categories. It can be seen from this graph that there is clear difference in standard deviation of motility readings between samples.

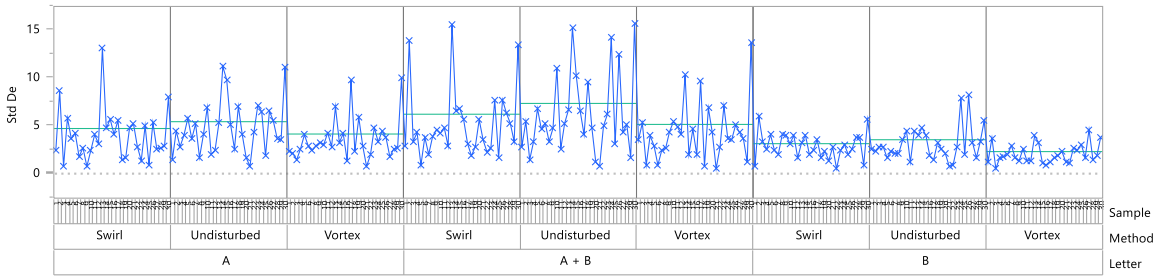
Figure 31: Standard deviation of undisturbed, swirled and vortexed motility analysis



The standard deviation of MOT readings for each sample using different mixing methods. Categories A and A+B show many more outliers compared with category B.

Figure 33 demonstrates standard deviation within each sample split by method. This graph shows that the highest mean of standard deviation is observed for undisturbed samples and the lowest mean is for the vortexed samples, although this difference is small. There appears to be a larger difference in variation depending on the velocity category as B category showing the lowest average standard deviation. The average motility is highlighted by green lines for undisturbed and mixed groups of samples.

Figure 32: Standard deviation of sperm motility analysis for each mixing method



Motility standard deviations grouped by Sample number, method and velocity category. Horizontal green lines represent the mean value in each sub-section. The vortex method expresses the highest reproducibility in each velocity category whilst the lowest is for undisturbed.

In the table below, the average standard deviation column represents values for the green lines from the figure above. Within the measurements of the %A, %B and %A+B, the method with the lowest average standard deviation was the vortex method. For %A MOT the vortex method has an average standard deviation of 4.2 percentage points. For %B category the vortex method has an a average standard deviation of 2.4 percentage points and 5.2 percentage points for %A+B.

Table 21: Standard deviation for motility in numbers

Letter	Method	N Rows	Mean(Variance(MOT))	Average SD
A	Swirl	30	22.7	4.8
A	Undisturbed	30	30.0	5.5
A	Vortex	30	17.6	**4.2**
A + B	Swirl	30	39.1	6.3
A + B	Undisturbed	30	54.5	7.4
A + B	Vortex	30	27.0	**5.2**
B	Swirl	30	10.1	3.2
B	Undisturbed	30	12.9	3.6
B	Vortex	30	5.6	**2.4**

The average standard deviation for every velocity category and mixing method. Vortex method expresses the lowest value in each velocity category.

Variance components were calculated as table below shows. The table highlights the source of variability; 67% of the total variance is coming from between samples, a high variability value is expected as there was no excluding criterion set on the range of sperm motility in the analysed samples (the motility ranged from asthenozoospermic samples to over 70% progressive motility). 0.77% of the total variance is coming from between methods and 8.7% of the variation is coming from the readings of the 4 wells analysis for a given sample and method.

Table 22: Sources of variation: methods, concentrations and 4 well testing

Component	Var Component	% of Total	Plot%	Sqrt(Var Comp)
Velocity	64.69903	23.1		8.044
Mixing method	2.16035	0.772		1.470
Individual motility	188.65092	67.4		13.735
4 well testing	24.33005	8.7		4.933
Total	279.84035	100.0		16.728

Shows the variance components, highlights the sources of variation by percentages.

3.5.2 Undisturbed, stirred and vortexed samples

Details of the analysis for this part are as follow:

Table 23: Undisturbed, stirred and vortexed summary table

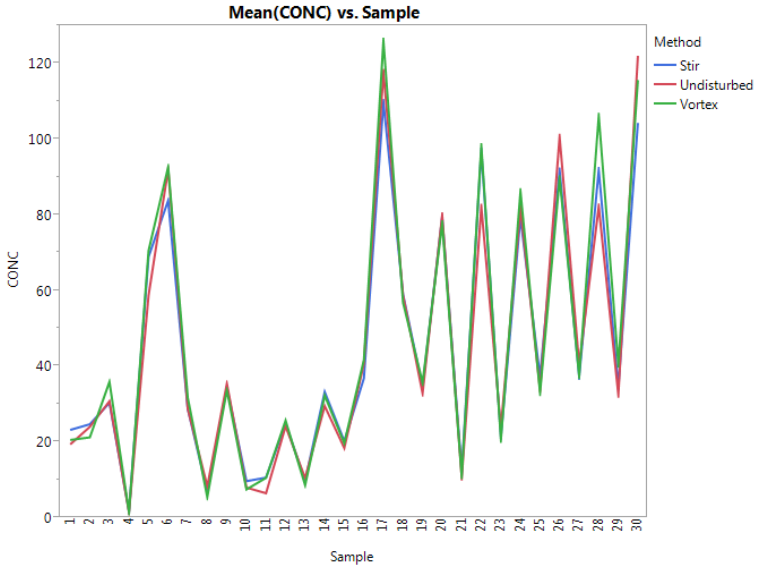
Number of the samples	30
Mixing methods	undisturbed, stirred, vortexed
No of wells tested for each method and each sample	4
Total No of tests	360

Summary of the concentration and motility analysis for undisturbed, stir and vortex mixing methods.

3.5.2.1 Concentration

The plot below shows the mean sperm concentration for each method for every sample. The graph shows that the mean concentration for all 3 methods track well and is consistent among the methods.

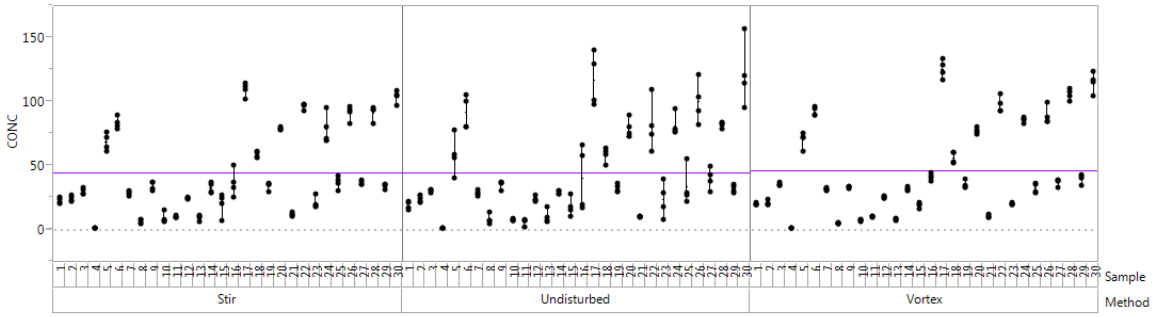
Figure 33: Correlation between undisturbed, stirred and vortexed concentration counts



The mean sperm concentration (mil/ml) for each sample with stirred, undisturbed and vortexed methods.

Figure 35 shows the individual results of the analysis grouped by sample and method. The mean sperm concentration (mil/ml) is highlighted by purple lines.

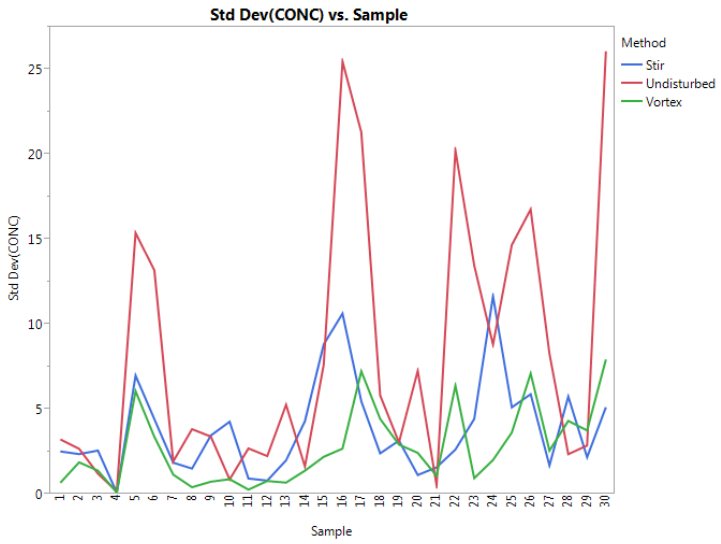
Figure 34: Distribution of 4 well testing for each mixing method against concentration



Vortex group shows higher reproducibility as the dots on each vertical line are closer to one another. The mean concentration (mil/ml) value for each method is highlighted by the purple lines.

The following graph shows the standard deviation for each sample. This plot highlights that the standard deviation of sperm concentration shows more variability for undisturbed samples. Particularly samples 5, 16, 22, 26 and 30.

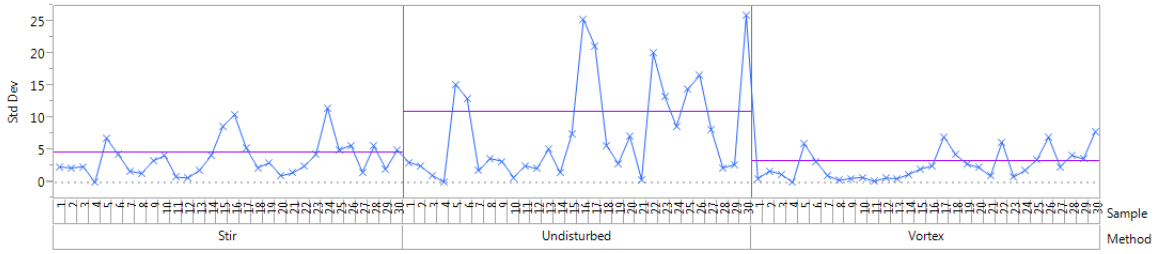
Figure 35: Standard deviation of undisturbed, swirled and vortexed concentration counts



The standard deviation for each sample with each method shown using a different colour. High variability from the undisturbed samples are evident.

The graph below demonstrates the standard deviation within each sample split by method. The graph shows the most variation for the undisturbed samples and least for vortexed samples.

Figure 36: Standard deviation of sperm concentration for each group



The standard deviation of sperm concentration readings grouped by sample number and methods. The vortex group expresses the highest level of reproducibility. The purple lines highlight the average standard deviation for each method.

The following table gives the values for the average standard deviation for each method (as highlighted by the purple lines in the figure above). The vortex method appears to have the lowest average standard deviation with a value of 3.5 mil/ml. The stir method has a larger average standard deviation by 1.3 mil/ml and the undisturbed method, averages 11.1 mil/ml, peaking 7.6 mil/ml higher.

Table 24: Average variance for each mixing method

Method	Mean	Average Variance	Average SD	CV
Stir	44.6	22.7	4.76	10.70%
Undisturbed	44.6	123.2	11.1	24.90%
Vortex	46.5	12.4	3.53	7.60%

The lowest average variance was observed for the vortex group, followed by stirring. The undisturbed category expressed a much higher average variance.

To highlight the source of variability, variance components were calculated. Table below shows that 94.0% of the total variance is coming from between samples. The high percentage is to be expected since no excluding criterion was set on the sperm concentration and the study subjects ranged from oligozoospermic to concentrations of over 100 mil/ml. 1.6% of the total

variance is coming from between methods and 4.4% of the variance is as a result of the 4 well testing for a given sample and method.

Table 25: Sources of variation: methods, concentrations and 4 well testing

Component	Var Component	% of Total	Plot%	Sqrt(Var Comp)
Method	19.1554	1.6		4.377
Individual conc	1127.5465	94.0		33.579
4 well testing	52.3766	4.4		7.237
Total	1199.0785	100.0		34.628

The largest source of variation is due to individual sperm concentration of the test samples (n=30).

3.5.2.2 Motility

Details of velocity classifications as measured by CASA are as follow:

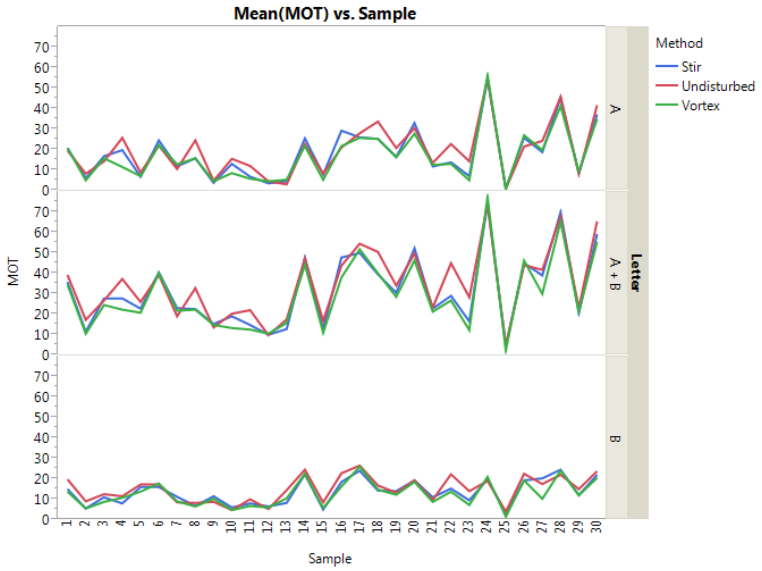
Table 26: Velocity categories of sperm

Classification	velocity (µm)
A	>25 µm
B	5-25 µm
A+B	Overall

Motility classifications (expressed in percentages) and their respective velocity categories. These classifications only apply to progressive motile sperm in a sample.

The following figure shows the mean percentage motility for each sample. As the plot demonstrates, the mean percentage for all mixing methods track well and are consistent.

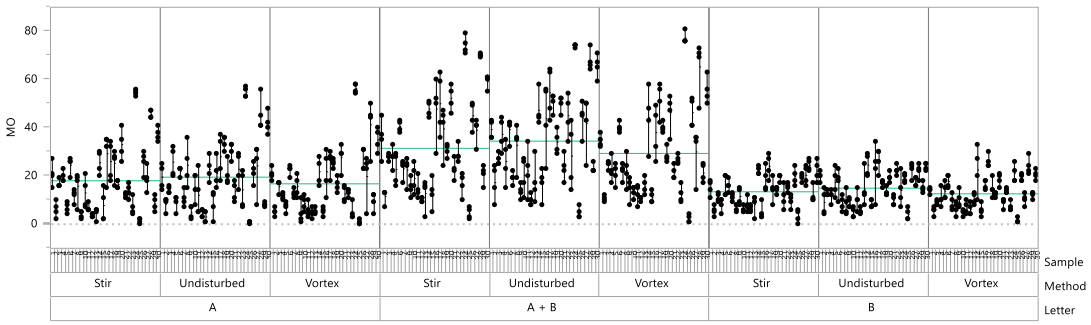
Figure 37: Correlation between undisturbed, stirred and vortexed motility analysis



The plot highlights %MOT of each sample for different velocity categories. Different mixing techniques are represented by different colours. The trend is generally followed by all mixing techniques.

The following plot shows the individual %MOT of the tests grouped by sample number, mixing method and velocity category. 4 dots appear on each vertical line for samples represent the range of the 4 well analysis. Green lines represent the mean (%MOT) for each group. Mean percentage motility appears to be higher than both mixed groups in every motility category.

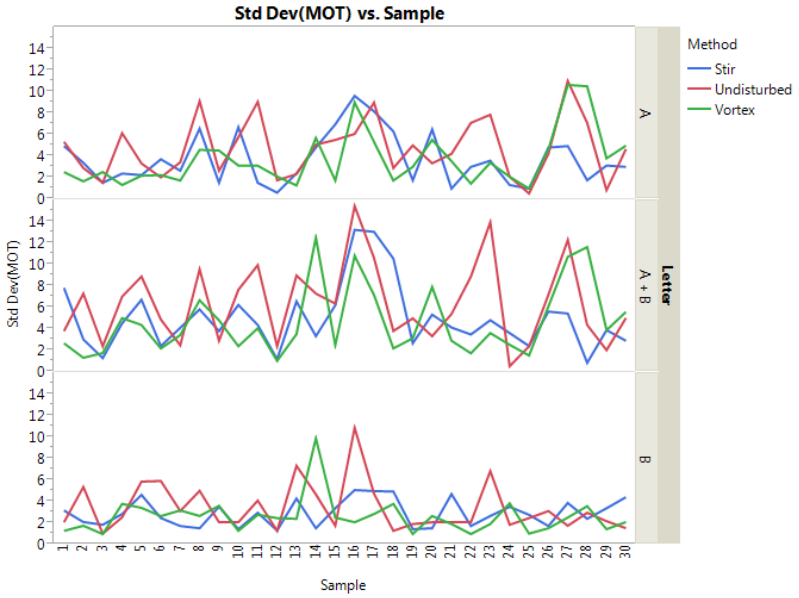
Figure 38: Distribution of 4 well testing for each mixing method against sperm motility



Mean %MOT for undisturbed, swirled and vortexed. 4 dots on each vertical line represent the 4 well testing in each velocity category.

The plot below shows the standard deviation for each sample and for A, B and A+B categories. it can be seen from this graph that there is clear difference in standard deviation of motility reading between samples.

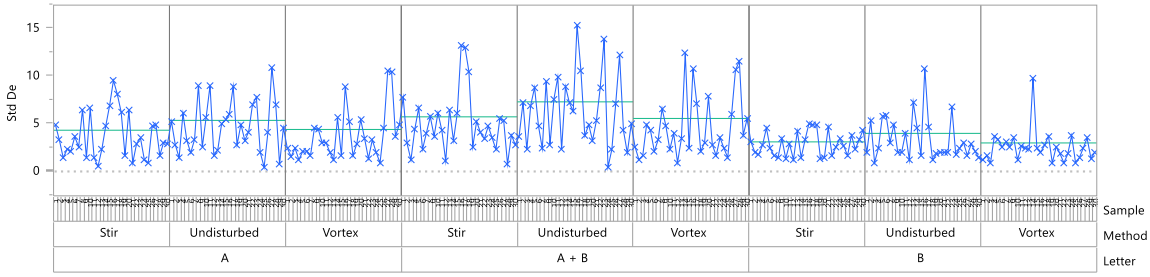
Figure 39: Standard deviation of undisturbed, stirring and vortexed motility analysis



The standard deviation of motility readings for each sample using different mixing methods. Categories A and A+B show more outliers compared with category B.

Figure 41 demonstrates standard deviation within each sample split by method. This graph shows the highest standard deviation reading for undisturbed samples and least when using Vortex. There appears to be a larger difference in variation depending on velocity with B showing the lowest average standard deviation. Green lines represent the mean standard deviation for each group.

Figure 40: Standard deviation of sperm motility analysis for each mixing method



MOT standard deviations grouped by Sample number, method and velocity category. Horizontal green lines represent the mean value in each sub-section. The vortex method expresses the highest reproducibility in each velocity category whilst the lowest is for undisturbed.

In the table below, the average standard deviation column represents values for the green lines in the figure above. For %A category, the stir method has an average SD of 4.4 percentage points while the vortex method's average is 0.1 percentage points greater, followed by another increase of 0.1 percentage point for the undisturbed samples. For %B and %A+B categories, the lowest average SD is observed for the vortexed samples (3.0 and 5.6 percentage points respectively). The values are increased for stir category and even greater for the undisturbed samples for both %B and %A+B categories.

Table 27: Standard deviation for motility in numbers

Test	Method	N Rows	Mean(Variance(MOT))	Average SD
A	Stir	30	19.2	**4.4**
A	Undisturbed	30	29.3	5.4
A	Vortex	30	19.9	4.5
A + B	Stir	30	33.5	5.8
A + B	Undisturbed	30	54.1	7.4
A + B	Vortex	30	31.5	**5.6**
B	Stir	30	10.0	3.2
B	Undisturbed	30	16.4	4.0
B	Vortex	30	9.3	**3.0**

Shows the average standard deviation for every velocity category and mixing method. Vortexing has produced the lowest average for each velocity category.

Table below demonstrates the calculated values of variance components in order to highlight the source of variability. 70.7% of the total variance is coming from between samples. A high percentage value is to be expected as no excluding criterion was set on sample motility and the study subjects ranged from asthenozoospermic to %A+B of over 70%. 0.83% of the total variance is coming from between methods and 10.7% of the variation is as a result of the 4 well testing.

Table 28: Sources of variation: methods, concentrations and 4 well testing

Component	Var Component	% of Total	Plot%	Sqrt(Var Comp)
Velocity	41.06732	17.8		6.408
Mixing method	1.92584	0.8336		1.388
Individual motility	163.30037	70.7		12.779
4 well testing	24.73030	10.7		4.973
Total	231.02383	100.0		15.199

Highlights the sources of variability by showing the percentages of variance components.

3.5.3 Undisturbed, swirled and pipetted samples

Details of the analysis for this part are as follows:

Table 29: Undisturbed, swirled and pipetted summary table

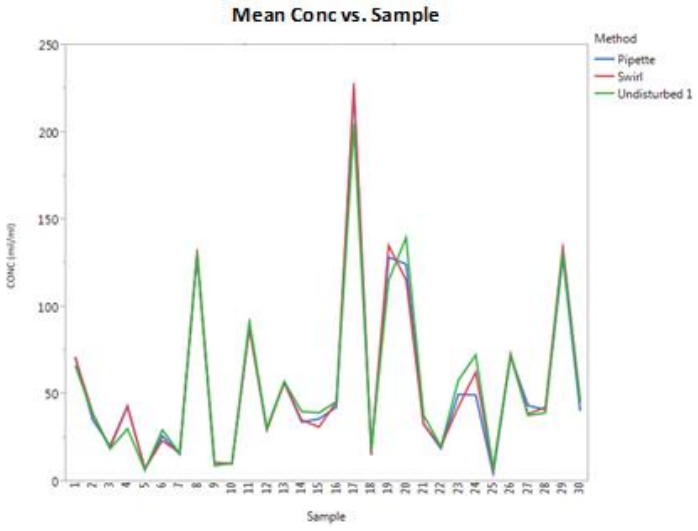
Number of the samples	30
Mixing methods	undisturbed, swirled, pipetted
No of wells tested for each method and each sample	4
Total No of tests	360

Summary of the concentration and motility analysis for undisturbed, swirl and pipette mixing methods.

3.5.3.1 Concentration

The plot below shows the mean sperm concentration for each method for every sample. The graph shows that the mean concentration for all 3 methods are consistent between different methods.

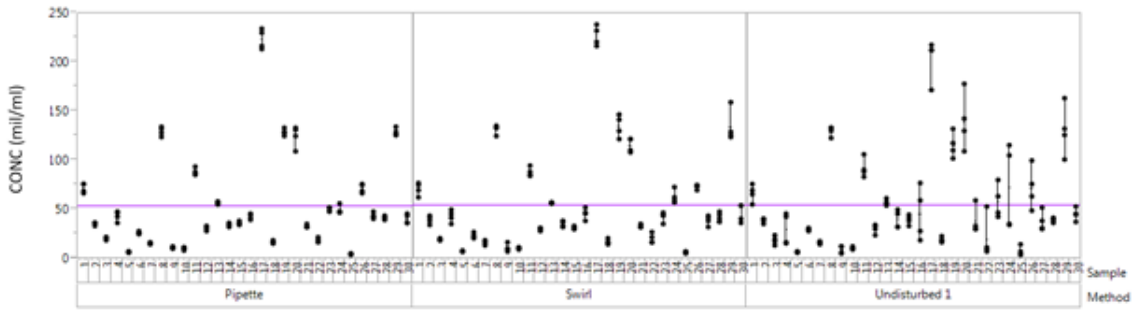
Figure 41: Correlation between undisturbed, pipetted and vortexed concentration counts



Mean sperm concentration (mil/ml) for each sample with undisturbed, swirled and pipetted methods.

The plot below shows the individual concentration results of the analysis grouped by sample and method. The 4 dots on each vertical line represent the range of the 4 well analysis for each sample. Purple lines outline the mean concentration (mil/ml) for each category.

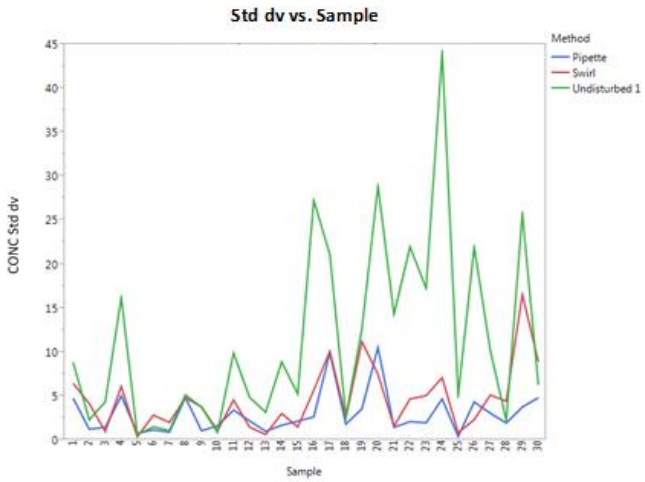
Figure 42: Distribution of 4 well testing for each mixing method against concentration



Concentration for undisturbed, swirled and pipetted. Range of 4 well analysis shown by the dots on each vertical line. The pipetted group shows higher reproducibility as the dots appear closer to one another.

Figure 44 outlines the standard deviation for each sample concentration. The plot shows that the standard deviation of concentration for undisturbed samples express a wide range particularly for samples 4, 16, 20,21, 22, 23, 24, 26 and 29.

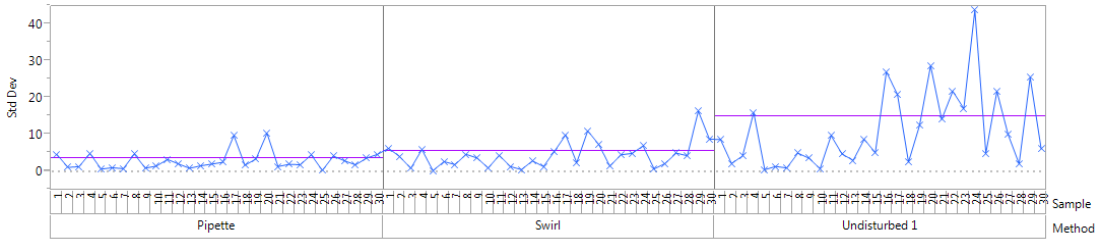
Figure 43: Standard deviation of undisturbed, swirled and vortexed concentration counts



Standard deviation for each sample using each method. High variability from the undisturbed (green) is evident.

Figure 45 demonstrates the standard deviation within each sample split by method. The graph shows the most variation for the undisturbed samples and least for pipetted samples. Purple lines represent the mean value for each group.

Figure 44: Standard deviation of sperm concentration for each group



Standard deviation of sperm concentration readings grouped by sample number and methods. Pipette group expresses the highest level of reproducibility.

The following table gives the values for the average standard deviation for each method (as highlighted by the purple lines in the figure above). The pipetting method appears to have the lowest average standard deviation with a value of 3.84 units. For swirling method, average standard deviation was higher by almost 2 units. Finally, the undisturbed method averaged by 9.6 units of standard deviation higher than swirling.

Table 30: Average variance for each mixing method

Method	Mean	Average Variance	Ave SD	Ave CV
pipette	54.63	14.77	3.84	7.0%
Swirl	55.3	33.78	5.81	10.5%
Undisturbed	55.5	236.53	15.38	27.7%

The lowest average variance was observed for the pipette method, followed by swirling. Undisturbed category expressed a much higher average variance.

As per previous parts, variance components were calculated. Table below shows that 94.4% of the total variance is coming from between samples. The high percentage is to be expected as there was not any excluding

criterion set on the sperm concentration and the samples ranged from oligozoospermic to concentrations of over 200 mil/ml. 1.6% of the total variance is coming from between methods and 4.0% of the variance is as a result of the 4 well testing for a given sample and method.

Table 31: Sources of variation: methods, concentrations and 4 well testing

Variance Components

Component	Var Component	% of Total	20 40 60 80	Sqrt(Var Comp)
Method	37.5534	1.6		6.128
Sample[Method]	2229.0775	94.4		47.213
Within	94.3290	4.0		9.712
Total	2360.9599	100.0		48.590

The largest source of variation is due to individual sperm concentration of the test samples (n=30).

3.5.3.2 Motility

Details of velocity classifications as measured by CASA are as follows:

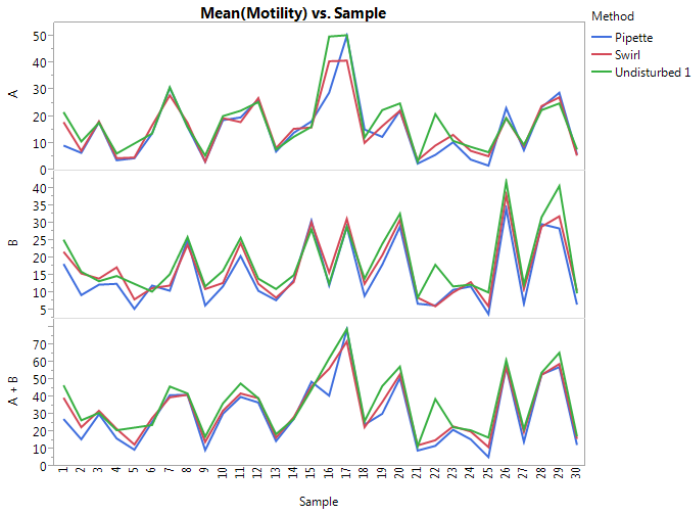
Table 32: Velocity categories of sperm

Classification	velocity (µm)
A	>25 µm
B	5-25 µm
A+B	Overall

Motility classifications (expressed in percentages) and their respective velocity categories

The following figure shows the mean percentage motility for each sample. As the plot demonstrates, the mean percentage for all mixing methods correlate well and are generally consistent.

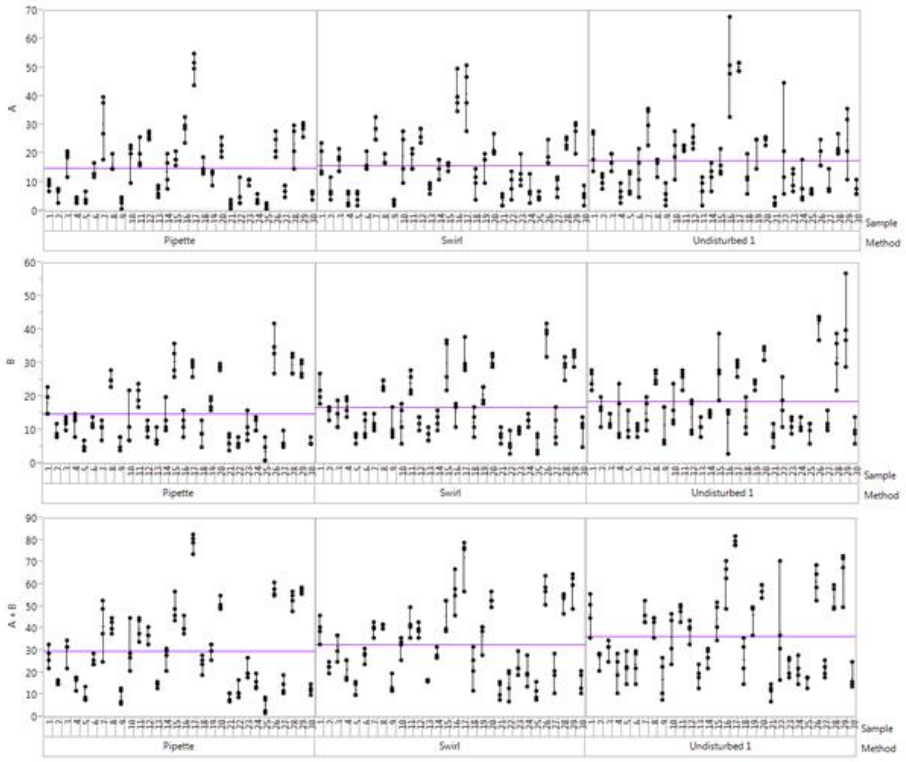
Figure 45: Correlation between undisturbed, swirled and pipetted motility analysis



The plot highlights %MOT of each sample for different velocity categories. Different mixing techniques are represented by different colours. The trend is generally followed by all mixing techniques.

The following plot (figure 47) shows the individual %MOT of the tests grouped by sample number, mixing method and velocity category. 4 dots appear on each vertical line for every sample representing the results of the 4 well analysis. The average motility of undisturbed samples appear to be higher than both swirl and pipette groups. The mean value is shown by purple lines for each category.

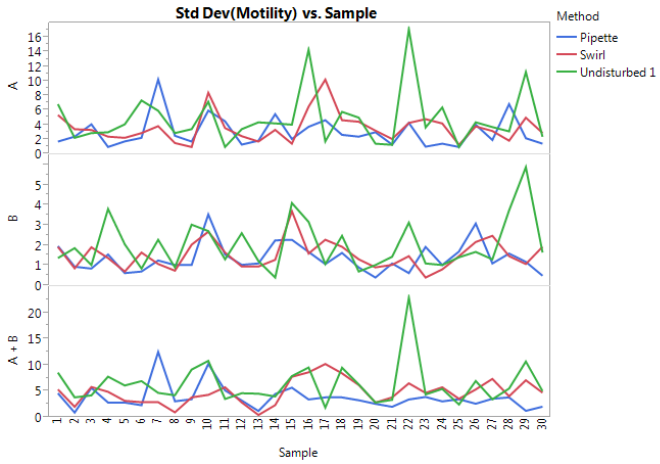
Figure 46: Distribution of 4 well testing for each mixing method against sperm motility



Motility analysis for each velocity category, coupled with sample numbers and mixing methods. The 4 dots on each vertical line represent the 4 well testing for each sample. Pipette sub-section demonstrates the most reproducible data for each velocity category.

The plot below shows the standard deviation for each sample and for A, B and A+B categories. Casual observation of this graph offers little in terms of high and low variability between different methods of mixing. A few spikes from the undisturbed category however seem to be significantly higher compared to swirled and pipetted samples.

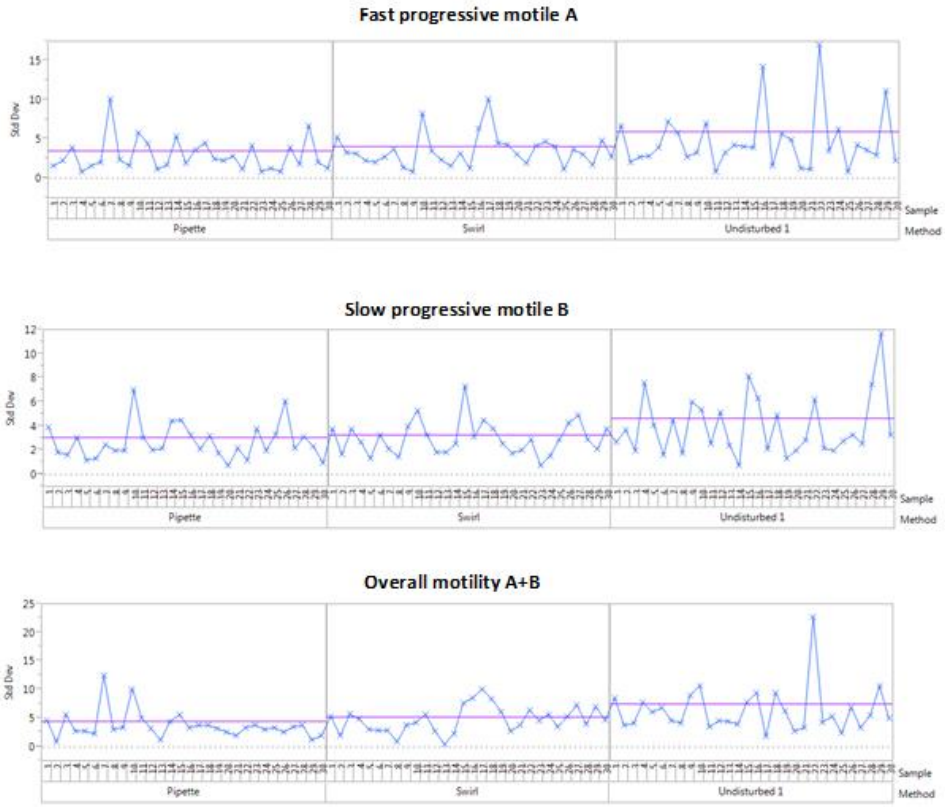
Figure 47: Standard deviation of undisturbed, swirled and vortexed motility analysis



The standard deviation of MOT readings for each sample using different mixing methods. Categories A and A+B show more outliers compared with category B.

Figure 49 consists of 3 sections, dedicated to fast motile (A), slow progressive (B) and overall motility (A+B). This plot demonstrates standard deviation within each sample split by mixing method. For every velocity category, the highest standard deviation mean is observed for undisturbed samples and the lowest for pipetted samples. Purple lines represent the mean value for each group.

Figure 48: Standard deviation of sperm motility analysis for each mixing method



Motility standard deviations grouped by Sample number, method and velocity category. Horizontal green lines represent the mean value in each sub-section. The pipette method expresses the highest reproducibility in each velocity category whilst the lowest is for undisturbed.

Table 40, the average standard deviation column represents values for the purple lines in figure 49. The lowest average value of standard deviation was observed for pipetted samples in each velocity category, whilst the highest was for the undisturbed samples. The table demonstrates that the data from the pipetted samples are more reproducible.

Table 33: Standard deviation for motility in numbers

Test	Method	Mean	Ave variance	Ave SD	Ave CV
A	**Pipette**	15.11	13.26	**3.64**	24.1%
A	Swirl	15.92	17.24	4.15	26.1%
A	Undisturbed	17.76	36.93	6.08	34.2%
A + B	**Pipette**	30.05	20.27	**4.50**	15.0%
A + B	Swirl	32.96	28.73	5.36	16.3%
A + B	Undisturbed	36.58	56.4	7.51	20.5%
B	**Pipette**	14.94	9.62	**3.10**	20.8%
B	Swirl	17.04	11.29	3.36	19.7%
B	Undisturbed	18.82	22.28	4.72	25.1%

The average standard deviation for every velocity category and mixing method. Pipetting has produced the lowest average standard deviation for each velocity category.

Table below demonstrates the calculated values of variance components. These values highlight the sources of variability. On average, 84.8% of the total variance is coming from between samples. A high percentage value is to be expected as no excluding criterion was set on sample motility and the study subjects ranged from asthenozoospermic to %A+B of over 80%. On average 0.92% of the total variance is coming from between different mixing methods and the average of 14.2% of the variation is as a result of the 4 well testing.

Table 34: Sources of variation: methods, concentrations and 4 well testing

Variance Components

	Component	Var Component	% of Total	20 40 60 80	Sqrt(Var Comp)
A	Method	2.12644	1.6		1.458
	Sample[Method]	106.76428	81.4		10.333
	Within	22.31066	17.0		4.723
	Total	131.20138	100.0		11.454
	Component	Var Component	% of Total	20 40 60 80	Sqrt(Var Comp)
B	Method	0.985089	1.0		0.9925
	Sample[Method]	79.729518	83.8		8.9291
	Within	14.396296	15.1		3.7942
	Total	95.110903	100.0		9.7525
	Component	Var Component	% of Total	20 40 60 80	Sqrt(Var Comp)
A+B	Method	0.53607	0.1618		0.732
	Sample[Method]	295.70655	89.2		17.196
	Within	35.13148	10.6		5.927
	Total	331.37410	100.0		18.204

The sources of variability by the percentages of variance components. The table comprises of 3 sections each representing A, B and A+B velocity categories.

3.5.4 Summary of semen mixing results

The undisturbed samples showed the highest mean percentage for motility compared with all other mixing techniques across the board for all motility categories (A, B and A+B). As per hypothesised, undisturbed samples also had the highest mean variance compared to every method of sample mixing used in this trial resulting in less reproducibility and lack of accuracy. The mean variance of 55 was recorded after analysing 90 patient samples for total motility (A+B) when analysed undisturbed. This figure was significantly higher than all other mixing techniques: pipetting (20.27 for 30 patients), stirring (33.5 for 30 patients), swirling (34 for 60 samples) and vortexing (29.25 for 60 samples). In addition to calculating the mean variance for each mixing technique, a table of variance component was created. The result of the variance component table was expectedly high as there was no excluding criterion set for sperm motility amongst the samples, therefore the majority of variance was due to the wide range of sperm motility for patients in each group. Nonetheless, 10% of the total variance in 90 patients were due to the effect of homogenisation methods used on each sample. The 10% figure is statistically significant and therefore confirms the findings of published experiments on lack of standardisation in preparation for sperm motility analysis.

For progressive motility, 15%CV threshold was chosen to determine the reproducibility of the results. It's very challenging to set the threshold at 10%CV for motility analysis as it naturally differs frequently from one field of view to another under the microscope. At 15%CV, undisturbed samples

resulted in 43%, stirring 47%, swirling 63% and vortexing 67% reproducibility. The results indicated that the most accurate data was produced after vortexing the sample, whilst the poorest set of data were showed for undisturbed samples. Motility was reduced after all methods of mixing in comparison with the undisturbed readings. highest motility reduction (in the categories that vortexing was done) was observed after vortexing (averaged at 26% for 60 samples).

A plot of standard deviation for concentration analysis of each category showed many outliers for the undisturbed samples. The trend of the results clearly pointed out that undisturbed samples are not fully homogenised throughout and extraction of a small amount of the sample will not accurately represent the entire ejaculate. Undisturbed samples produced the poorest data in comparison with all other methods; the average SD of undisturbed samples for 90 patients was 12.96 mil/ml. Expectedly, the average SD for vortexed samples were lower than other methods (4 mil/ml for 60 patient samples). 3.5.3 section of the experiment, assessed pipetting the sample instead of vortexing. The average SD of 3.84 mil/ml for pipetting for 30 patients was the lowest in that category.

Acceptable reproducibility was taken as CV of <10 for sperm concentration analysis. Not mixing the samples at all provided only 53% acceptable results, stirring 73%, swirling 83% and vortexing provided 95% reproducibility.

3.6 Lysis buffer time analysis: device reaction

A total of 20 patients took part in this part of the experiment. In order to determine the optimal incubation period for the semen and lysis buffer solution, 3 incubation periods were chosen for each sample: 2 mins, 10 mins and 20 mins (samples were incubated at room temperature). Semen was diluted (1:20) in lysis buffer. The following table outlines the shorthand words and acronyms used in texts and tables for this section of the results.

Table 35: Acronyms for section 3.6

Acronym	Definition
Conc	Sperm Concentration
High	High Sensitivity Strip
Low	Low Sensitivity Strip
Spearman's	Spearman's Rank Correlation
LCL	95% Lower confidence limit (Calculated using Fisher's Method)
UCL	95% Upper confidence limit (Calculated using Fisher's Method)
N	Number of Observations

The acronyms and shorthand words used in text and subtext sections of this part of the analysis.

The following table outlines the correlation analysis between concentration of semen as determined using CASA and concentration of SP-10 as detected by the high and low sensitivity strips of the device.

Table 36: Correlation between CASA counting and device results at 3 time points

Incubation time	Tested	Spearman's	LCL	UCL	p-value
2 mins	Conc vs. high	0.69	0.35	0.87	0.0004
	Conc vs. low	0.81	**0.57**	0.92	<0.0001
	High vs. Low	0.71	0.38	0.88	0.0002
10 mins	Conc vs. high	0.79	**0.54**	0.91	<0.0001
	Conc vs. low	0.74	0.45	0.89	<0.0001
	High vs. Low	0.90	**0.77**	0.96	<0.0001
20 mins	Conc vs. high	0.90	**0.75**	0.96	<0.0001
	Conc vs. low	0.89	**0.74**	0.96	<0.0001
	High vs. Low	0.83	**0.62**	0.93	<0.0001
Overall	Conc vs. high	0.81	**0.70**	0.88	<0.0001
	Conc vs. low	0.81	**0.70**	0.88	<0.0001
	High vs. Low	0.82	**0.71**	0.89	<0.0001

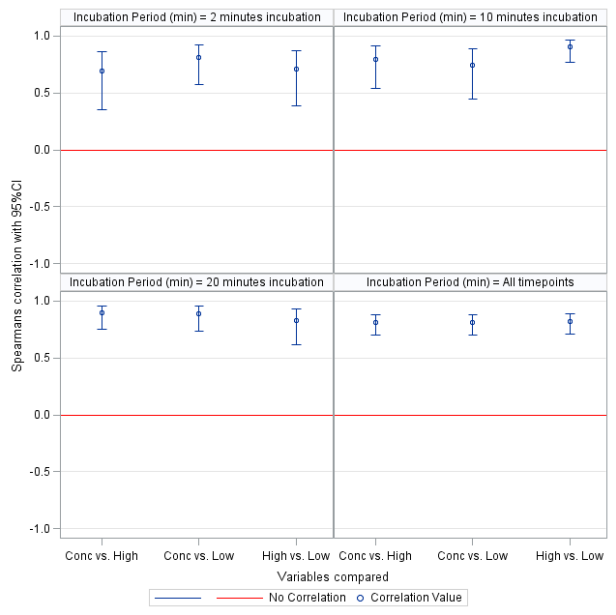
The statistical tests in order to find a correlation between semen concentration (CASA) and concentration of SP-10 detected using the device following 2 mins, 10 mins, 20 mins of incubation as well as the overall data.

The lower confidence limit is above 0.5 for variables conc vs. Low indicating that at 2 minutes it is likely that there is at least moderate correlation between the variables. However, the data in the above table do not offer sufficient evidence that there is at least moderate correlation between conc vs. High and High vs. Low after 2 minutes of incubation.

After 10 minutes of incubation, the lower confidence limit is above 0.5 for variables conc vs. high and high vs. low. This indicates that at 10 minutes of incubation, it is likely that there is at least moderate correlation between there variables. However there is not enough evidence indicating that there is at least moderate correlation between conc vs. low variables. At 20 minutes (and the overall data), the lower confidence limit is above 0.5 for all variables tested, indicating that there is at least moderate correlation between all the variables.

The following plots are the graphic representations of the correlation between conc vs high, conc vs. low and high vs. low by incubation period ad variables tested. Figure 50 shows, at 20 mins the likelihood of at least moderate correlation for conc vs. high sensitivity strip, conc vs. low sensitivity strip and high vs. low strips. Finally, using all time points it's likely that there's at least moderate correlation between all the variables.

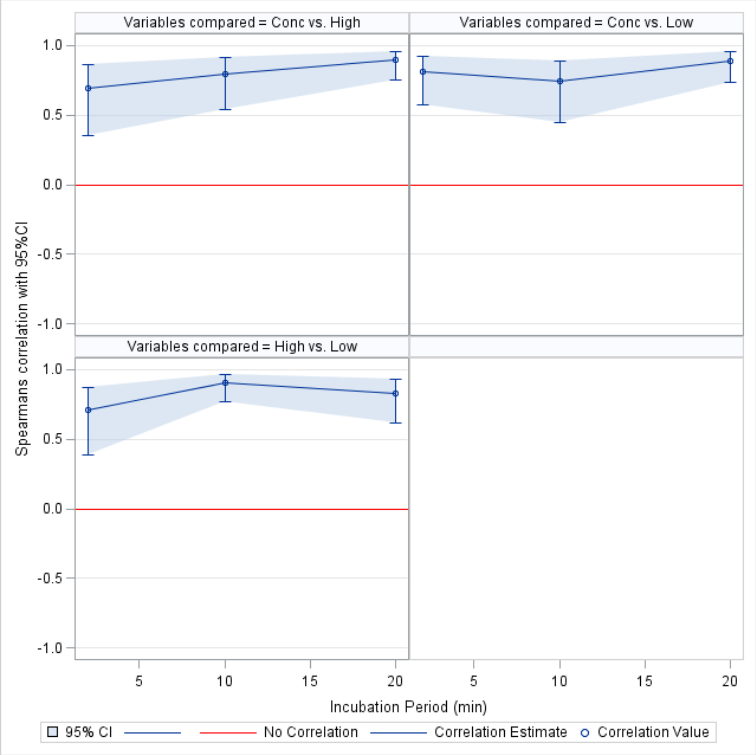
Figure 49: Spearman's correlation of high and low strips vs CASA concentration



Graphic representation of the Spearman's correlation with the compared variables, by incubation period. After incubation of 2 mins, 10 mins, 20 mins and overall.

Figure 51 demonstrates that the lower confidence limit indicates at least moderate correlation for 10 mins and 20 mins time points between concentration and the high sensitivity strip. The lower confidence limit for concentration against the low sensitivity strip yielded in at least moderate correlation at 2 and 20 mins of incubation. Finally, the lower confidence limit of high and low sensitivity strips has at least moderate correlation at 10 mins and 20 mins.

Figure 50: Spearman's correlation of strips function compared with CASA concentration



Graphic representation of the Spearman's correlation with the compared variables, by variables tested.

Overall possible trend suggests that it is possible that the longer the incubation period the better the correlation. This claim needs further number of study subject to provide stronger evidence suggesting this claim.

3.7 Device analysis

The following table summarises the acronyms used in this part of the results section.

Table 37: Acronyms for section 3.7

Acronym	definition
CI	Confidence Interval
Conc	Concentration
Sensitivity	Proportion of participants with normal sperm concentration that the device calls normal levels.
Specificity	Proportion of participants with low sperm concentration that the device calls low levels.
PPV	Positive Predictive Value: Proportion of participants which the device calls normal levels of sperm concentration who have normal levels of sperm concentration.
NPV	Negative Predictive Value: Proportion of participants which the device calls low levels of sperm concentration who have low levels of sperm concentration.
ULA	Upper limit of agreement
LLA	Lower limit of agreement
mill/ml	Million per millilitre
%A	Percentage absorbance – quantitative measure of the test line signal intensity
Accuracy (%)	(Number of correct results/ total number of results) x 100

Outlines the acronyms and their respective definitions for this section of the analysis

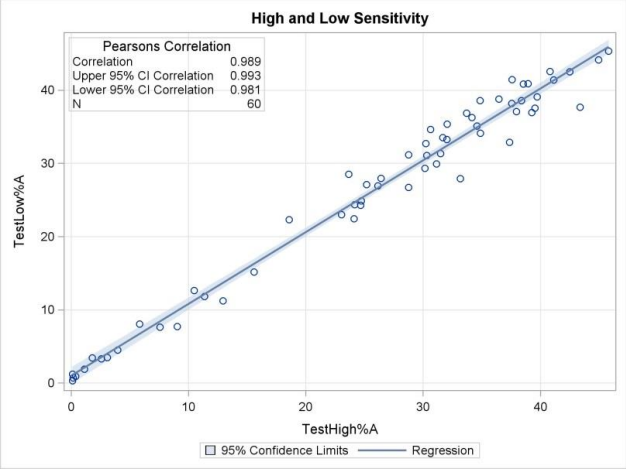
30 patient samples were analysed for this part of the experiment. Having already determined the most efficient protocol, the following 30 samples were analysed after 10 minutes of incubation in lysis buffer at a 1:20 dilution. 23 patients produced the sample onsite and the remaining 7 brought the sample from home. This section of the results used the new prototype of the device (components and the internal arrangement of the strips are demonstrated in figure 19). The sensitivity of the strips were altered according to the previous findings to optimise the sensitivity strips. Although still referred to as high and low sensitivity strips, the strips for the

new prototype were the same in terms of their positioning inside the device. The strips continued utilising a typical sandwich immunoassay employing antibodies specific for SP-10 and used chromatographic principles to separate bound and free coloured labels.

3.8 High and low sensitivity strips: a comparison

In order to establish an agreement between the high and low sensitivity strips, the following Pearson's correlation coefficient was produced. The following plot is as the result of the combination of both vortex and stir techniques as the focus of this analysis is on the strips performance and not the mixing technique.

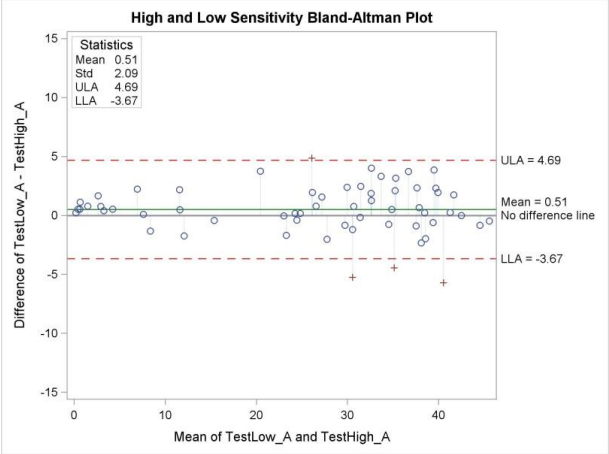
Figure 51: Pearson's correlation between high and low sensitivity strips



Graph shows high levels of agreement between the high and low sensitivity strips. Pearson's correlation of 0.989 (95%CI (0.981, 0.993)).

The following Bland-Altman graph shows that there is no clear trend for the average difference between high and low sensitivity but the variation increases as %A increases. The upper and lower limits of agreement are not greater than 5%A from 0.

Figure 52: Bland-Altman plot for high and low sensitivity strips



On average the low sensitivity strip gives a higher result by 0.51%A and 0 is contained within the limits of agreement therefore demonstrating a high level of agreement.

There are 4 possible outliers in the graph above. These outlying samples are demonstrated in the following table:

Table 38: Outlying semen samples for sensitivity strips comparison

Sample	Produced	Method	Conc (mil/ml)	Vol (ml)	TestLow%A	TestHigh%A	Difference	Comment
4	Home	Vortex	48.16	2.7	37.66	43.37	-5.71	-
10	onsite	Stir	30.30	5.5	32.90	37.36	-4.46	Moderate agglutinations
20	onsite	Stir	45.66	2.5	27.93	33.18	-5.25	Large aggregations
21	onsite	Vortex	6.51	2.5	28.52	23.65	4.87	Many epithelial cells

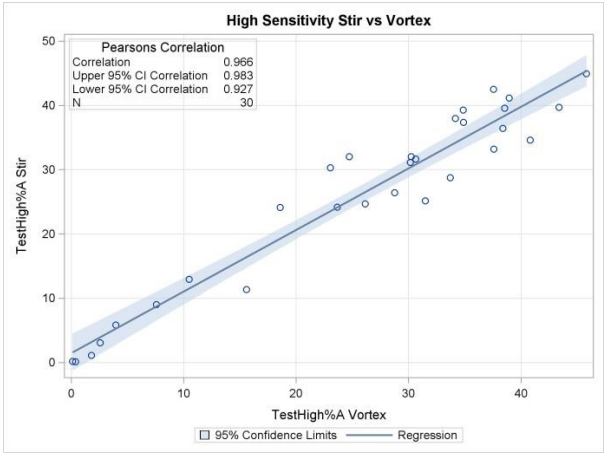
3 out of 4 outlying samples have expressed biological restrictions (the comments section) which may have an impact on the microscopic sperm count.

3.9 Predicate vs. controlled sampling: device results

This section investigated the agreement of the sample processing methods stirring (predicate) and vortex (controlled) for high and low sensitivity strips. Once the agreement for the high and low sensitivity strips was established, the data was combined to assess the overall agreement

between stir and vortex methods. The following graph demonstrates the findings of the analysis.

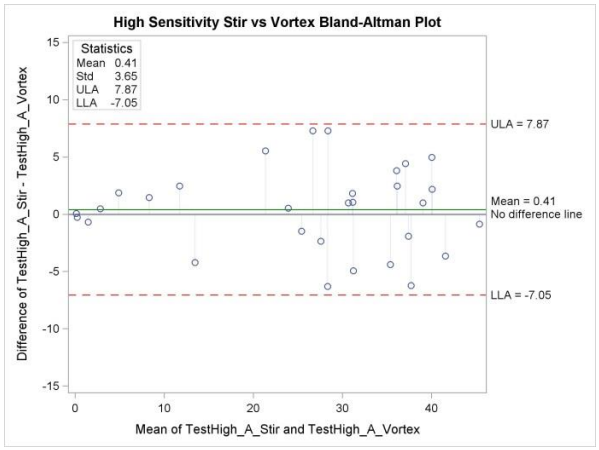
Figure 53: Pearson's correlation between mixing methods on the high sensitivity strip



Strong level of agreement between the sample processing methods. High sensitivity strip with a correlation of 0.966 (95%CI (0.927, 0.983)).

The following Bland-Altman plot also illustrates the agreement between the mixing methods. The average agreement is that the stir method has a higher %A value compared to vortex by only 0.41%A.

Figure 54: Bland-Altman plot: mixing methods on high sensitivity strip



There is no clear trend for average differences against %A value. However, the variation increases as %A increases. The upper and lower limits of agreement are not greater than 8%A from 0.

3.9.1 Sensitivity, specificity and the overall accuracy

The cut-off points of 5 mil/ml and 15 mil/ml were chosen to detect the sensitivity, specificity and the overall accuracy of the device. The analysis focused on each strip as well as both strips combined data for both thresholds using both stir and vortex methods. For the 15mil/ml threshold using the vortex method, the overall accuracy of the dataset is demonstrated in the table below:

Table 39: Overall accuracy of the device performance at 15 mil/ml threshold using vortexing

Statistic	Result	95% Confidence Interval	Number of Samples
Accuracy	85.0%	(73.4% - 92.9%)	60
Sensitivity	71.9%	(53.3% - 86.3%)	32
Specificity	100.0%	(87.7% - 100.0%)	28
PPV	100.0%	(85.2% - 100.0%)	23
NPV	75.7%	(58.8% - 88.2%)	37

Sensitivity, specificity and the overall accuracy of the device for the 15 mil/ml threshold in comparison with the analysed CASA data post vortex.

The 15 mil/ml threshold was also analysed for the stir method.

Table 40: Overall accuracy of the device performance at 15 mil/ml threshold using stirring

Statistic	Result	95% Confidence Interval	Number of Samples
Accuracy	78.3%	(65.8% - 87.9%)	60
Sensitivity	59.4%	(40.6% - 76.3%)	32
Specificity	100.0%	(87.7% - 100.0%)	28
PPV	100.0%	(82.4% - 100.0%)	19
NPV	68.3%	(51.9% - 81.9%)	41

Sensitivity, specificity and the overall accuracy of the device for the 15 mil/ml threshold in comparison with the analysed CASA data after stirring the sample.

The following table summarise the results of the analysis for stirring and vortexing for the 5 mil/ml threshold combined.

Table 41: The accuracy of the device performance at 5 mil/ml using vortexing and stirring combined

Statistic	Result	95% Confidence Interval	Number of Samples
Accuracy	100.0%	(94.0% - 100.0%)	60
Sensitivity	100.0%	(92.0% - 100.0%)	44
Specificity	100.0%	(79.4% - 100.0%)	16
PPV	100.0%	(92.0% - 100.0%)	44
NPV	100.0%	(79.4% - 100.0%)	16

Sensitivity, specificity and the overall accuracy of the device at 5 mil/ml threshold in comparison with the analysed CASA after vortexing and stirring.

The accuracy of the device was then analysed at 15 mil/ml for the vortex method. The following tables illustrate the accuracy of the high sensitivity strip at 33%A and low sensitivity at 36%A for the threshold of 15 mil/ml.

Table 42: Accuracy of the high sensitivity strip at the chosen percentage absorbance cut-off point for 15 mil/ml threshold

Statistic	Result	95% Confidence Interval	Number of Samples
Accuracy	86.7%	(69.3% - 96.2%)	30
Sensitivity	75.0%	(47.6% - 92.7%)	16
Specificity	100.0%	(76.8% - 100.0%)	14
PPV	100.0%	(73.5% - 100.0%)	12
NPV	77.8%	(52.4% - 93.6%)	18

The accuracy of the high sensitivity at 33%A for the 15 mil/ml threshold using the vortex method was determined as 86.7%.

Table 43: Accuracy of the low sensitivity strip at the chosen percentage absorbance cut-off point for 15 mil/ml threshold

Statistic	Result	95% Confidence Interval	Number of Samples
Accuracy	83.3%	(65.3% - 94.4%)	30
Sensitivity	68.8%	(41.3% - 89.0%)	16
Specificity	100.0%	(76.8% - 100.0%)	14
PPV	100.0%	(71.5% - 100.0%)	11
NPV	73.7%	(48.8% - 90.9%)	19

The accuracy of the low sensitivity strip at 36%A for the 15 mil/ml threshold using the vortex method was determined as 83.3%

Subsequently, the same statistical analysis was carried out for the stirring method (high sensitivity at 33%A and low sensitivity at 36%A). The following tables show the result of the analysis.

Table 44: Accuracy of the high sensitivity strip at the chosen percentage absorbance cut-off point for 15 mil/ml threshold using stirring

Statistic	Result	95% Confidence Interval	Number of Samples
Accuracy	83.3%	(65.3% - 94.4%)	30
Sensitivity	68.8%	(41.3% - 89.0%)	16
Specificity	100.0%	(76.8% - 100.0%)	14
PPV	100.0%	(71.5% - 100.0%)	11
NPV	73.7%	(48.8% - 90.9%)	19

The accuracy of the high sensitivity strip at 33%A for the 15 mil/ml threshold using the stir method was determined as 83.3%.

Table 45: Accuracy of the low sensitivity strip at the chosen percentage absorbance cut-off point for 15 mil/ml threshold using stirring

Statistic	Result	95% Confidence Interval	Number of Samples
Accuracy	73.3%	(54.1% - 87.7%)	30
Sensitivity	50.0%	(24.7% - 75.3%)	16
Specificity	100.0%	(76.8% - 100.0%)	14
PPV	100.0%	(63.1% - 100.0%)	8
NPV	63.6%	(40.7% - 82.8%)	22

The accuracy of the low sensitivity strip at 36%A for the 15 mil/ml threshold using the stir method was determined as 73.3%.

The readings of the high sensitivity and low sensitivity strips are demonstrated in the following table for both cut-off points using stirring and vortexing the sample.

Table 46: Accuracy of both sensitivity strips at the chosen percentage absorbance cut-off point for 5 and 15 mil/ml thresholds using both mixing methods

Statistic	Result	95% Confidence Interval	Number of Samples
Accuracy	100.0%	(88.4% - 100.0%)	30
Sensitivity	100.0%	(84.6% - 100.0%)	22
Specificity	100.0%	(63.1% - 100.0%)	8
PPV	100.0%	(84.6% - 100.0%)	22
NPV	100.0%	(63.1% - 100.0%)	8

The accuracy of the high and low sensitivity strip at 18%A for the 5 mil/ml and 15 mil/ml thresholds using the vortex and stirring methods was determined as 100%.

4.0 Discussion

The primary objective of this project was to examine the feasibility of a novel diagnostic device for the purpose of indirect sperm counting. In order to lay the foundation for validating the novel device, the effect of semen viscosity and homogeneity on accurate sperm count was determined. In addition to device validation, the gathered data on this part of the thesis also has service improvement application in the andrology laboratory. The data in turn can assist the manufacturer in optimising device functionality i.e. whether a 1 or 2 step analysis is preferable. Furthermore, different methods of sample production and preparation were closely examined to determine their potential impact on seminal qualities and quantities.

It was hypothesised that homogeneity is an important factor and lack thereof can result in inaccurate seminal analysis. Therefore a set of experiments on trypsin were carried out to determine whether it is essential in homogenisation and viscosity reduction of semen. Although the results of the analysis demonstrated that trypsin significantly reduces semen viscosity and contributes towards homogenisation of semen, it was decided that use of a proteolytic enzyme is not essential for the current prototype. Moreover, the use of trypsin may be more applicable for homogenisation of hyper-viscous samples in the laboratory where a lysis buffer is not used for routine analysis.

The impact of the lysis buffer on semen viscosity and homogeneity was very similar to trypsin. The lysis buffer is essential for purpose of the current prototype, since it successfully punctured the membrane of all sperm acrosomal membrane and effectively released SP-10 into the solution. Additionally, 0.25% trypsin doesn't effectively impair the acrosome of all sperm cells in an ejaculate which makes it ineffective for indirect sperm concentration analysis using the novel device. Also since sperm motility is partially impaired after addition of trypsin, it may not be suitable for use as a medium in an indirect motility assessment device.

Vitality analysis (section 3.4.3) on the lysis buffer showed that at its current concentration, the lysis buffer instantly impairs the membrane of the entire sperm population. The limitation of using the lysis buffer is the damage it causes to the sperm membrane, making it impossible to add sperm motility measurement as an additional assay function. For future advancements of the device where motility measurement may be implemented into the kit, use of such strong buffer will not be suitable. Addition of motility as another diagnostic test to the current prototype would benefit from using a semipermeable membrane through which motile sperm can migrate.

Comparison of sperm concentration analysis using both manual (haemocytometer) and CASA showed a strong correlation. The average concentration of 34.17 mil/ml was recorded for CASA method and 36.43 mil/ml for manual analysis. The results showed a high degree of parity between methods for a wide range of samples with various levels of

viscosity. In addition, the results of this experiment were in line with the report of Tomlinson *et al* (2008), suggesting that the concentration count for CASA is slightly lower than manual analysis but within the acceptable difference.

Tjioe and Oentoeng (1968) and Dunn and Picologlou (1977) were in agreement that the viscosity of liquefied semen was on average less than 10 mPa.s at 37°C. As previously mentioned, the findings of this experiment produced the average viscosity of neat semen as 11.35 mPa.s at room temperature. This figure is higher than the previous data suggest and the elevated quantitative viscosity reading could be due to measuring viscosity at room temperature for the current study.

Overall, the viscometer wasn't an ideal tool for routine seminal viscosity measurement as it often struggled to determine the viscosity measure for neat semen and it showed 'error' and 'out of range' messages. An upper limit of 40 mPa.s was set for viscous samples for which the viscometer was unable to determine viscosity and classified them as 'out of range'. The viscosity of some of the analysed samples was so high, they were almost in a solid state.

Even after regularly washing the flow tube inside the viscometer using the manufacturer recommended solution, at times the machine didn't seem to function efficiently. An interfering factor in the measure of viscosity using the viscometer may have been the presence of seminal débris and

proteinous gels. In 11% of the analysed samples, large seminal plasma constituents were visibly detected in the sample by macroscopic analysis. These constituents may appear as droplets, globules or lipid molecules. As suggested by Mann (1981), these seminal bodies can interfere with liquefaction and viscosity measurement of semen. It is understood that these sticky mucinous objects are by-products of the accessory glands as they have also been observed in vasectomised males (Metz *et al*, 1968). It is therefore probable that the presence of these bodies in semen has interfered with the performance of the viscometer. As previously mentioned, even regularly cleaning the flow tube inside the viscometer didn't seem to effectively remove the mucinous bodies which is a limiting factor for regular usage.

Measuring viscosity in mPa.s uses a predetermined measuring scale and has no limitations imposed by user subjectivity. In theory, a viscometer provides a more accurate viscosity reading compared with manual methods. In practice however, it proved to have disadvantages that outweigh its advantages. This is a high maintenance device that requires constant cleaning of the flow tube and the waste container for accurate function and minimising the potential risk of spreading infections. The operation of this device is also costly as it requires specialised disposable syringes for each analysis. Furthermore, the viscometer proved inconsistent in time it took to produce results; it took up to 4 minutes for completing the analysis cycle which is slower than measuring viscosity using a pasture pipette or a velocity chamber. The viscometer would not be a suitable method of viscosity measurement in applied andrology but the data gathered from the

viscometer helped to produce the normative viscosity scale (table 18) and support the hypothesis that chamber filling time is a reproducible method of viscosity measurement.

Viscosity measurement by injecting the sample in to a leja slide and measuring the time it takes for 5 µl of the sample to fill the tube appeared suitable and accurate. This method appeared to be more consistent than the viscometer even though it shared some of its limitations. The mucinous bodies that potentially interfered with the performance of the viscometer appeared to reduce the velocity of semen advancement in the chamber. For a few samples that were clearly liquefied and had normal viscosity throughout, the rate of chamber filling time was not as rapid as expected. An advantage of this method was that similarly to the viscometer, it had no user subjectivity therefore measure of viscosity was more universally acceptable than dripping the sample from a pipette and measuring the formed threads or droplets. In addition, viscosity measurement using the chamber filling time can be done when a wet-prep slide is prepared for motility analysis, making it a fitting step in the current andrology semen analysis routine. Using a leja slide is cheaper than using a viscometer and it is more accurate and less subjective than dripping the sample from a pasture pipette therefore it is the most suitable method for viscosity analysis. The analysis showed that measuring the thread length of dripping semen (as recommended by WHO) can actually represent the viscosity of semen given that it is performed consistently and using a traceable scale which is calibrated.

Further analysis on impact of different dilutions of lysis solution on semen viscosity led to making a series on dilutions. A serial dilution of 1:2, 1:5, 1:10, 1:15 and 1:20 were prepared for 63 samples. The purpose of this analysis was to determine the viscosity of the semen-lysis solutions at different semen concentrations. If the viscosity is not reduced significantly as the result of dilution, seminal viscosity may interfere with the flow of semen in the immunoassay. The mean viscosity for every dilution apart from 1:2 (2.75 mPa.s) were lower than 2 mPa.s. This experiment showed that all of the dilutions, reduce viscosity significantly enough to flow laterally. Table 18 classifies the viscosity of 2.75 mPa.s as normal viscosity which indicates that even at a 1:2 dilution, the resistance of semen to flow is insignificant and will not interfere with the function of the device. The next stage of validation, turned the focus of the analysis to the 1:20 dilution. In order to determine the sensitivity and overall accuracy of the device's lower limit, a more diluted solution of semen is preferable. Another advantage of 1:20 dilution is that a smaller amount of semen is required for dilution and therefore patients who produce ejaculates with low volumes can still use the device without restrictions. Eliminating any excluding criteria in terms of semen volume is an important feature of any device for general patient use. For this purpose the 3rd phase of the project focused on the 1:20 dilution which only requires 50µl of semen for the 1ml device required solution.

Conventional seminal fluid analysis relies heavily on the homogeneity of semen as the analysis is based on a small volume of the sample to represent the qualities of the entire ejaculate. It was hypothesised that lack of homogenisation may have a significant impact on the accuracy of laboratory sperm concentration and motility analysis. Mixing semen contributes to homogeneity of semen which may be delayed as a result of hyper-viscosity. Homogeneity is an independent seminal characteristic and shouldn't be synonymised with viscosity. Methods of semen mixing such as using a vortex contribute towards achieving seminal homogeneity by mixing the sample well. Following liquefaction semen becomes more homogeneous, however both liquefaction and viscosity can vary enormously across an ejaculate with likely consequences on the ability to carry out reliable analysis.

WHO (2010) advises on mixing the samples thoroughly but avoid using a high speed-vortex. It has also been shown that vortexing a sample inserts mechanical stress on sperm and may result in reduction of percentage motile sperm (WHO 2010), therefore using a vortex on samples undergoing IVF and ICSI is generally avoided. This part of the experiment aimed to determine the effect of different mixing methods on sample homogenisation and whether this characteristic affects providing reliable and reproducible results. First, this experiment aimed to highlight the effects of different mixing techniques on sperm motility which is off significance in the conventional andrology laboratory; and second the determination of reproducibility of analysing undisturbed, stirred, swirled and pipetted samples in comparison with using a high-speed vortex. Determining the

most reproducible non-vortex mixing technique is an important stage of developing a streamlined protocol for using the lateral flow immunoassay.

Mixing data showed that the most reproducible method is vortexing. Using a vortex has a significant impact on motility of the sample. Therefore for diagnostic andrology, the best method of sample preparation is gently swirling the sample pot for wet-prep motility analysis followed by 10 seconds of vortexing prior to removing an aliquot of semen for concentration analysis. A mixture of swirling and vortex is therefore the most suitable mixing combination to manual semen analysis. The deteriorating impact of a high-speed vortex on sperm motility, suggests that vortexing should be avoided for semen samples that are being used for ART.

One possible explanation for reduction of percentage motile sperm post vortexing was that a vortex mixes the sample so well that some sperm that had sedimented during the liquefaction of semen will be mixed back with the rest of the sperm. This theory however was rejected as the sperm concentration analysis of samples taken from undisturbed samples and post vortex didn't differ significantly (sections 3.5.1.1, 3.5.2.1. and 3.5.3.1). Elimination of this theory can only prove that using a high speed-vortex reduces sperm motility at various degrees amongst samples.

For the purpose of the immunoassay concentration analysis, stirring the sample appears to be a suitable mixing method, as this analysis is intended

to take place at patient's private homes where there's no access to a vortex. This method yielded in reproducible data for sperm concentration and it appears to be user friendly from the manufacturers point of view.

It was hypothesised that mixing semen is an essential stage of sample preparation before analysis. The result of the analysis support this claim as analysis of undisturbed samples resulted in a wide range of sperm concentration between multiple counts of the same sample whereas multiple counts of the sample post stirring, swirling and vortexing yielded in more reproducible results. Although vortexing produced the best results, other methods of semen mixing were acceptable compromises. Mixing semen indeed contributes towards homogenisation of semen which results in a more accurate count. As previously mentioned, andrology laboratories across the UK and the world do not follow a particular protocol for sperm homogenisation and therefore may be reducing the number of motile sperm before analysis which may alter the correct diagnosis on the report particularly for patients with borderline motility or concentration.

For the purpose of the immunoassay, pipetting the sample is not a suitable method of semen mixing, even though section 3.5.3 of the results showed it is a reproducible method of semen mixing. Pipetting the sample increases the chance of spillage and therefore it may result in loss of the sample and potential spread of infections. Additionally, patients are likely to create air bubbles in the sample by pipetting semen in and out of the pipette which can affect the way the device functions.

In order for the device to get validated for the purpose of indirect sperm concentration count, it is crucial that the quantitative measure of the test line signal intensity for both strips directly correlate with one another and the laboratory sperm concentration counts. 3.8 and 3.9 stages of the project therefore aimed to determine preliminary thresholds for strip sensitivity. This part of the analysis used the improved prototype for the analysis of 30 patient samples. According to the WHO (2010) guidelines, samples with concentration of less than 15 mil/ml are considered to be oligozoospermic. The thresholds for the device function were set at 15 mil/ml and 5 mil/ml to identify low and very low sperm concentration in samples. The main driver for these cut-off choices was that no sample that has low sperm concentration according to the CASA analysis, would be classified as normal by the device. For the high sensitivity strip the test line signal intensity for said cut-off points were 18% and 33% respectively. The percentage threshold for the low sensitivity strip was set at 18% for 5 mil/ml and 36% for 15 mil/ml.

The accuracy of the set preliminary thresholds were then determined against sperm concentration. The combined results from both high and low sensitivity strips showed a high level of agreement between the two strips. For the 15 mil/ml threshold using the vortex method, the device specificity was 100% therefore every sample that was diagnosed with oligozoospermia using CASA, was classified as having low sperm concentration by the device. However, 9 samples that were shown to have normal sperm count

using CASA, were classified as having low sperm count by the device resulting in sensitivity of 72%.

The 15 mil/ml threshold was also analysed against the stir method. The analysis showed the specificity of the device as 100% for this cut-off point. Similarly to the vortex method, all participants that were classified as oligozoospermic by CASA, were classified by the device as having low sperm count. However, 13 samples that had normal concentration were classified as having low sperm count by the device resulting in the sensitivity of 59%. The sensitivity of 59% may have been as a result of wicking effect, device malfunction or simply the absorbance percentage thresholds are yet not optimal.

Comparison of the stirring and vortexing techniques showed that the stirring method was less accurate at the 15 mil/ml threshold by about 7%. The stirring method also showed lower sensitivity than vortexing at the 15 mil/ml cut-off point by 13%. It was hypothesised that stirring semen can result in reproducible readings competing with vortexing the sample. Although the sensitivity of stirring method at 15 mil/ml was lower than vortex, streamlining the strips and additional testing will yield in a more accurate test line signal intensity that will have the optimal sensitivity and specificity compared with the result of the analysis in the laboratory.

The combined readings from high and low sensitivity strips for the 5 mil/ml threshold showed a perfect level of accuracy for both vortex and stir

techniques. The specificity, sensitivity, positive predictive value and negative predictive value all matched the CASA classifications on the training dataset. The accuracy of the device was then analysed at 15 mil/ml for the vortex method. The overall accuracy of the high sensitivity appeared superior to the low sensitivity strip by 3.4% due to its higher sensitivity. Subsequently, the same statistical analysis was carried out for the stirring method (high sensitivity at 33%A and low sensitivity at 36%A). The accuracy of the high sensitivity strip was higher by 10% in comparison to the low sensitivity strip due to higher sensitivity.

At the 5 mil/ml threshold, both strips recorded a 100% accuracy. Both the device and CASA categorised 22 samples as having normal sperm and 8 patient samples as low. This level of accuracy was due to perfect specificity and sensitivity at 5 mil/ml threshold for both strips using both methods.

Out of the 30 analysed samples, 1 outlying result did not agree with the trend for both sensitivity strips and CASA concentration analysis at the chosen test line signal intensity. Sample viscosity was very high for this sample, as it took over 60s to fill the leja chamber and formed a continues thread as measured by a pasture pipette expressing the upper limit of viscosity measurement of the used methods. In fact this sample was so viscous that it was almost in a solid state. Such samples are more likely to be anomalies regardless of the used assay. In addition, at 1ml, this sample had the lowest volume out of the 30 sample pool.

The analysis on sample processing methods of vortex and stir for the low sensitivity strip expressed a strong level of agreement however, there were 3 potential outliers for this analysis. Interestingly, the same 3 outliers were observed in the sample processing methods of vortex and stir for the high sensitivity strip. Microscopic investigation of these 3 samples, determined gross aggregations and agglutinations of sperm in semen. Aggregation and agglutination of sperm have been known to have an impact on the accuracy of sperm count in samples as they create random concentrated clumps of sperm in parts of the sample, while other parts contain much lower sperm concentration.

Biological restrictions such as aggregation and agglutination impose limiting effects on both manual and CASA counts. Additionally, expression of aggregation is a common concern amongst the participants of external quality assurance schemes and it has been credited as one of the leading causes of inaccurate sperm counts in quarterly NEQAS distributions. Indirect concentration analysis such as the current prototype will not share this limitation as regardless of clumps, all present sperm will release SP-10 upon dilution in the lysis buffer. In cases where semen contains large agglutinations or aggregations of sperm, concentration analysis using the lateral flow assay may have a significant advantage over manual or CASA analysis.

It is worth pointing out that the results obtained from the current set thresholds may have yielded better results in the present laboratory

experiment than they would being used by patients at home. This is due to the fact that the thresholds for percentage absorbance of the sensitivity strips were set on the very data that were analysed and in order to implement a more accurate and reliable threshold, testing is required on a larger pool of data. Future analysis on the determination of the percentage absorbance thresholds requires testing the device more rigorously by analysing a training set of samples where the thresholds are set against a test set of samples where the thresholds are tested. The perfect accuracy of the device at the lowest cut off point of 5 mil/ml is encouraging and shows that the device development has moved towards the right direction, however, given there were only 30 samples analysed for this part of the experiment, it cannot be taken as absolute.

A 31st sample was also analysed using the improved prototype of the device. This sample was a great negative control as the patient suffered from globozoospermia. Globozoospermic samples lack acrosomes altogether and therefore in theory should not express any SP-10. The device reacted accordingly with this condition as the verdict was low or no SP-10 even though CASA analysis of the sample resulted in 44 mil/ml sperm concentration. Sperm from men with total globozoospermia are unable to fertilise an egg as they won't be able to complete the acrosome reaction which is essential prior to fertilisation. This is a very rare condition and has an occurrence rate of <0.1% (Dam *et al* 2007) in the human population. The aim of the final product is suggesting the patients seek further fertility advise and book an appointment with a GP if they the device indicates low

sperm count and globozoospermia can be diagnosed during further andrology investigation.

Upon commercial availability of this device, it is important to inform the patients that producing semen with more than 15mil/ml of sperm does not guarantee natural conception but rather it means that they have enough sperm to do so. This test only rules out oligozoospermia as a contributing factor to infertility and additional analysis of quality and quantity of semen still require laboratory assessment.

For the purpose of this experiment, only ejaculated semen were used. In order to broaden the device application and increasing the data pool for device validation, future testing could include retrograde and epididymal sperm counts as well. Analysing retrograde and epididymal samples will pose many challenges for any future trial but they will be able to provide valuable information regarding the potential back ground noise that may interfere with the function of the strips.

It is important to determine the possible factors interfering with the percentage absorbance of the device strips. Semen contains round looking cells that could be immature germ cells or leukocytes. At X400 magnification microscopy it's impossible to correctly determine whether they are germ cells. Immature germ (or immature sperm) cells may contain SP-10 and therefore could result in a false positive reading in large numbers. The current trial included one sample which had a low sperm count but a high concentration of round cells. This sample was not an outlier

and was diagnosed with low sperm count by the device. Future analysis could also spike the sample with immature germ cells, bacteria or viruses to establish whether their presence has an impact on the way the strips function.

Determining sperm concentration using an immunoassay has the potential to count sperm more accurately than any other method of sperm count. Viscosity and lack of homogenisation have an impact on accuracy of sperm count both manually and using CASA but these factors are eliminated after diluting the semen in lysis buffer. In addition, lack of standardisation and human error can account for further inaccuracies for counting sperm in the andrology laboratory and again this device is exempt from such limitations. Perhaps as the technology evolves in the future, using a lateral flow immunoassay would become the gold standard method of sperm count for andrology semen analysis.

A potential advantage of analysis techniques that have a higher level of accuracy is drawing a clearer line between patients that may achieve natural conception by trying for a longer period of time and patients that need ART for fertilisation. Current andrology protocols are in need of an update and standardisation, therefore research that challenge the current methods can improve the quality of the semen analysis report and minimise the effect of factors that reduce reproducibility of the analysis.

Different methods of sample production were compared to determine whether samples produced as a result of masturbation and *coitus* differ in quality. A total of 7 patients were analysed for this trial and significant differences were isolated. In order to minimise the effect of abstinence period on seminal qualities, patients were given clear instructions about keeping a similar period of abstinence for each sample they produced. The abstinence period ranged between 2-7 days between patients. The masturbation and condom production abstinence periods for each patient were all within the acceptable difference. The mean volume of samples produced in a condom at home was 0.64 ml higher than samples produced as a result of masturbation onsite. In addition, the data showed that the total sperm concentration in samples was higher for samples that were produced at home. For the purpose of this experiment, sperm was counted both manually and using CASA. The results of both counting methods were in harmony and indicated that samples that were produced in condoms were superior in total sperm number compared to the samples of the same patients producing a sample onsite. CASA count showed an average of 43.13 million increase and haemocytometer 46.92 million. No significant difference was identified for the percentage motility of samples due to the method of sample production as the samples expressed very similar percentage of motile sperm.

Searching for previous experiments that compared the quality of onsite and at home samples resulted in only a handful of studies. Sofikitis and Miyagawa (1993), reported that seminal volume, total concentration, motility and percentage of morphologically normal sperm were higher for

coital samples. Findings of Sofikitis and Miyagawa (1993) matched the claims of Purvis *et al* (1986) regarding the difference between condom and masturbation sample qualities. The results of the current study after analysing 7 patients, generally agree with the previous trials. So far the only difference between this study and previous ones is in percentage of motile sperm where no significant difference was outlined between the 2 methods of production.

Similar studies were also done in this field. Elzanaty and Malm (2008) reported that samples that were produced at home had higher concentration in comparison with onsite samples (Elzanaty and Malm 2008). Elzanaty and Malm's experiment was designed differently to the current study as they compared the quality of onsite masturbation and home masturbation of the same patients. Additional findings of Elzanaty and Malm (2008) reported an increase in motility when the samples were produced at home and found no significant difference in the volume of semen produced which were not in line with the results of the current study.

A study by Hotchkiss *et al* (1938) analysed semen of 200 fertile men whose wives were in the first period of gestation in order to establish a better basis for semen assessment. This study focused on a single specimen from the participants where 33 produced a sample in a condom during sex and 167 men provided a specimen by masturbation. They reported that the mean ejaculate volume of masturbation samples was 3 ml, whilst the mean value was 2.3 ml for condom samples. Hence they concluded that masturbation

averages in a higher sample volume compared to using a condom. They also claimed that using a condom will impair sperm motility therefore wouldn't provide the optimal sample. The claim of Hotchkiss *et al* (1938) on the effect of non-spermicidal condoms on sperm motility is no longer valid since the development of toxicity tested condoms. Toxicity testing of non-spermicidal condoms has advanced in recent decades and they are shown to have minimal effects on sperm motility. Pasante unique condoms which are commercially available and male factor pack (MFP) that are used for patients undergoing IVF and ICSI treatment have shown the same sperm survival rate after various time points as sterile specimen containers (Tomlinson *et al* 2012).

Another study carried out by Freund (1969) analysed samples of volunteers that produced regular samples via masturbation with a group of married men who provided samples in condoms during sex. The main focus of this study was on the correlation of increased frequency of ejaculation and depletion of human sperm reservoir. A section of results compared the concentration from specimens collected by masturbation and at intercourse for 5 patients that had produced up to 5 samples by the same method. It's important to point out that the 2 groups had different patients and at no point a patient attempted sample production by both methods. The mean value for sperm concentration of condom group was 7.86 mil/ml higher than the masturbation group. Although the experimental design for Freund's (1969) experiment is so different that the superiority of the condom group cannot be used to support the findings of the current study on sperm

concentration, it is interesting that the report found a higher sperm concentration in coital samples.

The studies of Elzanaty and Malm (2008), Hotchkiss *et al* (1938) and Freund (1969) have a major limitation in not having the same patient to produce samples using different production methods. This limitation makes the findings in terms of comparing the quality and quantity of semen samples by masturbation and condom off little clinical significance.

Published literature on effects of anxiety and psychological stress have all agreed on the negative impact it can have on semen quality (Clarke *et al.* 1999; Gollenberg *et al* 2010. Vellani *et al.* 2013). Producing a sample in a clinical environment no doubt is not as comfortable as producing a sample at home. The lower volume and concentration of samples produced onsite may have therefore been affected by psychological stress. Even though the evidence suggests that coital samples have significantly better quality, it is not common practice to ask patients to produce a sample by sex before fertility treatment as it is not convenient and onsite samples are not exposed to environmental temperature. Furthermore, the probability of contamination in samples may be elevated for coital samples which could have an impact on the outcome of ART. This theory may reinforce the need for such devices that can assess the semen quality that exclude stressful factors such as attending a hospital appointment and producing the sample in a clinical cubicle. Patients can produce the sample when they feel up to

the task and they don’t need to take some time out from their day to day life for a hospital analysis.

The investigation on the impact of semen production on the quality of semen is an ongoing investigation and it is aimed to recruit a total of 20 patients to take part in this trial in the near future. Early signs of this experiment seem promising and there seem to be a difference in sample quality depending on how it was produced. Although the current 7 patient set is too small to be able to draw a definitive conclusion, the addition of the remaining 13 samples will give a clearer indication of difference in sample quality due to production. Significant differences in semen quality due to method of sample production could potentially result in misrepresentation of the fertility status of the patients.

On the quest to outline key sample quality and quantity differences due to production method, a future trial could compare the presence of microorganisms in onsite and intercourse samples as it is important to outline whether different methods of sample production increase the possibility of contaminations. It would also be interesting to find out which semen production method is more suitable for the patients to collect the entire sample. Missing a small part of the ejaculate may not have a detrimental impact for diagnostic analysis, however it is essential for the samples undergoing treatment to contain the entire ejaculate.

5.0 Conclusion

In conclusion, it is evident that indirect measurement of sperm concentration using the device has the ability to result in accurate sperm count. SP-10 is present in the acrosome of the entire sperm population and it is a suitable protein marker for this purpose. Further analysis is required to determine the optimal threshold for the absorbance percentage of the device sensitivity strips to achieve higher accuracy at the 15mil/ml cut-off point.

The lysis buffer was a suitable solution in achieving homogeneity, semen viscosity reduction and SP-10 release from sperm. Based on that, no additional solution with proteolytic characteristics is required for the current purpose of the device.

Future experiments can focus on epididymal and retrograde semen samples in addition to ejaculated semen. Detecting SP-10 in urine and epididymal fluid has the capability of broadening the application of the device as well as providing valuable information regarding potential interfering factors with the function of the device. Should the developer aim to increase the diagnostic ability of the device and measure the percentage of motile sperm, a semi permeable membrane through which motile sperm can migrate would be a suitable future trial. The addition of lysis buffer to semen results in complete lack of sperm motility therefore lysis buffer cannot be part of any indirect motility analysis of the device in a single step test.

6.0 References

ABOU-HAILA, A. TULSIANI, D. (2000) Mammalian sperm acrosome: formation, contents and function. Archives of biochemistry and biophysics. Vol. 379, No. 2, pp. 173-182

AFZELIUS, B.A. (2004) Cilia-related diseases. The journal of pathology vol: 204(4) pp: 470-477

AGARWAL, A. SAID, T. (2011) Interpretation of basic semen analysis and advanced semen testing. Male infertility. 6(2) pp.15-21

ALIZADEH, R. NADID, S. ABBASI, N. YARI, A. MAZAHERI, Z. DANESHI, E. AGARWAL, A. ABBASI, M. (2016) The effect of aminoguanidine on sperm motility and mitochondria membrane potential in varococelized rats. Iranian journal of basic medical science. Vol:19 pp: 1279-1284

ALVAREZ, C. CASTILLA, JA. RAMIREZ, JP. VERGARA, F. YOLDI, A. FERNANDEZ, A. GAFORIO, JJ. External quality control program for semen analysis:Spanish experience (2005) Journal of assisted reproduction and genetics. Vol: 22 (11-12) pp: 379-387)

ALWAAD, A. BREYER, B. LUE, T. (2015) Normal male sexual function: emphasis on orgasm and ejaculation. Fertility and sterility. Vol: 104 No. 5 pp: 1051-1060

AMARAL, A. LOURENCO, B. MARQUES, M. RAMALHO-SANTOS, J (2013) Mitochondria functionality and sperm quality. Reproduction (Cambridge, England) 146(5) pp.163-174

ARIKAN, I.I. DEMIR, B. BOZDAG, G. ESINLER, I. SOKMENSUER, L.K. GUNALP, S. (2012) ICSI cycle outcomes in oligozoospermia. Clinical and experimental obstetrics & gynecology. 39 (3) pp: 280-282

ASHRAFZADEH, A. KARSANI, S.A. NATHAN, S. (2013) Mammalian sperm fertility related proteins) International journal of medical sciences. Vol.10 No.12 pp:1649-1657

BAKER, R. R. BELLIS, M. A. (1995). Human sperm competition: Copulation, Masturbation, and Infidelity. Chapman and Hall, London

BAILEY, E. FENNING, N.R. CHAMBERLAIN, S. DEVLIN, L. HOPKISSON, J.H. TOMLINSON, M,J. (2007) Validation of sperm counting methods using limits of agreement. Journal of andrology. Vol: 28 pp: 364-373

BALHORN, R. (2007) The protamine family of sperm nuclear proteins. Genome Biology. Vol. 8 Issue: 9 Article 227

BERKOVITS A, ELTES, F. SOFFER, Y. ZABLUDOVSKY, N. BEYTH, Y. FARHI, J. LEVRAN, D. BARTOOV (1999) ART success and in vivo sperm cell selection depend on the ultramorphological status of spermatozoa. Andrologia 31 pp. 1-8

BJÖRNDAHL, L. MORTIMER, D. BARRATT, C. CASTILLA, J. MENKVELD, R. KVIST, U. ALVAREZ, J. HAUGEN, T. (2010) A practical guide to basic laboratory andrology. Cambridge university press pp. 24

BJÖRNDAHL, L. SÖDERLUND, I. KVIST, U. (2003) Evaluation of one-step eosin-nigrosin staining technique for human sperm vitality assessment. Human reproduction. Vol:18 No:4 pp.813-816

BURGOS, M.H. DI PAOLA, G. (1951) Eosin test for the evaluation of sperm vitality. Fertility and sterility. Vol: 2 p: 445

CALVEL, P. ROLLAND, A. JEGOU, B. PINEAU, C. (2010) Testicular postgenomics: targeting the regulation of spermatogenesis. Philosophical transactions of the royal society B. Vol: 365 pp: 1481-1500

CARPINO, A. SICILIANO, L. (1998) Unaltered protein pattern/genital tract secretion marker levels in seminal plasma of highly viscous human ejaculates. Archives of Andrology, 41(1) pp: 31-35

CASTIGLIONE, R. SALEMI, M. VICARI, L. O. VICARI, E. (2014) Relationship of semen hyperviscosity with IL-6, TNF-α, IL-10 and ROS production in seminal plasma of infertile patients with prostatitis and prostato-vesiculitis. Andrologia. Vol 46, Issue 10. Pp: 1148-1155

CHEMES, H.E. RAWE, V.Y. (2003) Sperm pathology: a step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperm phenotypes in infertile men. Human Reproduction Vol.9 No.5 pp. 405-428

CHENG, Y. MRUK, D. (2010) The biology of spermatogenesis: the past, present and future. Philosophical transactions of the royal society. No. 365 pp. 1459-1463

CHENOWETH, P. (2005) Genetic sperm defects. Theriogenology (64) pp. 457-468

CLARKE, R.N. KLOCK, S.C. GEOGEHAN, A. TRAVASSOS, D.E. (1999) Relationship between psychological stress and semen quality among in-vitro fertilization patients. Human reproduction. Vol.14 No.3 pp.753-758

CLERMONT, Y. (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiological reviews. Vol: 52, No.1, pp. 198-236

COMHAIRE, F.H. (1996) [edited by] Male infertility: clinical investigations, cause evaluation and treatment. 1st ed. London: Chapman & Hall. Chapter 6 pp: 3, 123, 146

DAM, A.H. FEENSTRA, I. WESTPHAL, J.R. RAMOS, L. VAN GOLDE, R.J.T. KREMER, J.A.M. Globozoospermia revisited. Human reproduction update. Vol.13, No.1 pp:63-75

DE JONGE, C.J. (2014) Biological basis for human capacitation. Human reproduction update, Vol: 11, No, 3 pp: 205-214

DE JONGE, C J. BARRATT, C. (2006) The sperm cell: production,

maturation, fertilisation, regeneration. Cambridge university press, UK. Vol. 1 pp. 8-9, 15-16, 108,

DE KRETSER, D.M. LOVELAND, K.L. MEINHARDT, A. SIMORANGKIR, D. WREFORD, N. (1998) Spermatogenesis. . Human Reproduction. Vol: 13 pp: 1-8

DU PLESSIS, S. S. GOKUL, S. AGARWAL, A. (2013) Semen hyperviscosity: causes, consequenses, and cures. Frontiers in bioscience (Elite Ed) 1;5 pp. 224-231

DUBE, J. Y. GAUDREAULT, D. TREMBLY, R. R. (1989) The concentration of immunoreactive prostate specific antigen is not decreased in viscous samples. Andrologia, 21 pp: 136-139

DUNN, P. PICOLOGLOU, B.F. (1977) Variation in human viscoelastic properties with respect to time post ejaculation and frequency of ejaculation. International journal of infertility. Vol: 22 No.4 pp: 217- 224.

ELZANATY, S. MALM, J. (2008) Comparison of semen parameters in samples collected by masturbation at a clinic ad at home. Fertility and sterility. Vol.89 No.6 pp: 1718-1722s

ELZANATY, S. MALM, J. GIWERCMAN, A. (2004) Visco-elasticisy of seminal fluid in relation to the epididymal and accessory sex gland function and its impact on sperm motility. International journal of andrology, 27 pp: 94-100

EMAMI, N. SCORILAS, A. SOOSAIPILLAI, A. EARLE, T. MULLEN, B. DIAMANDIS, E. P. (2009) Association between kallikrein-related peptidases (KLKs) and macroscopic indicators of semen analysis: Their relation to sperm motility. Biological Chemistry. Vol: 390, pp: 921-929

ESFANDIARI, N. BURJAQ, H. GOTLIEB, L. CASPER, R. (2008) Seminal hyperviscosity is associated with poor outcome of *in vitro* fertilisation and embryo transfer: a prospective study. Fertility and sterility Vol:90 No.9 pp:1739- 1743

FREDRICSSON, B. BJORK, G. (1977) Morphology of postcoital spermatozoa in the cervical secretion and its clinical significance. Fertility and sterility 28 pp. 839-841

FERRAMOSCA, A. ZARA, V. (2014) Bioenergetics of mammalian sperm capacitation. BioMed Research International. Volume 2014, Article ID 902953 pp: 1-8

FERRAMOSCA, A. PROVENZANO, S.P. COLLOLA, L. ZARA, V. (2012) Mitochondrial respiratory efficiency is positively correlated with human sperm motility. Urology. Vol:79(4) pp: 809-814

FREUND, M. (1962) Interrelationships among the characteristics of human semen and factors affecting semen-specimen quality. Journal of reproduction & fertility. Vol. 4 pp. 143-159

GAFFNEY, E.A. GADELHA, H. SMITH, D.J. BLAKE, J.R. KIRKMAN-BROWN, J.C. (2011) Mammalian sperm motility: observation and theory. The annual review of fluid mechanics. Vol: 43 pp: 501-528

GIULIANO, F. CLEMENT, P. (2005) Review-Erectile dysfunction/andrology. Physiology of ejaculation:Emphasis on serotonergic control. European urology Vol: 48 pp: 408-417

GOLLENBERG, A.L. LIU, F. BRAZIL, C. DROBNIS, E.Z. GUZICK, D. OVERSTREET, J.W. REDMON, J.B. (2010) Semen quality in fertile men in relation to psychosocial stress. Fertility and sterility Vol.93 No.4 pp:1104-1111

GONZALES, G. F. KORTEBANI, G. MAZZOLLI, A. B. (1993) Hyperviscosity and hypo-function of the seminal vesicles. Archives of Andrology. 30 pp: 63-68

GUYTON, ARTHUR, C. (1981) textbook of medical physiology 6th Ed. USA: W.B. Saunders Company pp. 994-995

GUZICK, D.S. OVERSTREET, J.W. FACTOR-LIVAK, P. BRAZIL, C.K. NAKAJIMA, S.T. COUTIFARIS, C. *et al* (2001) Sperm morphology, motility and concentration in fertile and infertile men. The new England journal of medicine. Vol: 345, No.19 pp: 1388-1393

HAMADA, A. ESTEVES, S.C. AGARWAL, A. (2011) Unexplained male infertility: potential causes and management. Review article. Human andrology. Vol:1 pp:2-16

HAMADA, A. ESTEVES, S.C. NIZZA, M. AGARWAL, A. (2012) Unexplained male infertility: diagnosis and management. Review article. International Brazil journal of urology. Vol: 38 (5) pp: 576-594

HASHIMOTO, H. ISHIKAWA, T. GOTO, S. KOKEGUCHI, S. FUJISAWA, M. SHIOTANI, M. (2010) The effects of severity of oligozoospermia on ICSI cycle outcome. Systems biology in reproductive medicine.

HERR, J.C. FLICKINGER, C.J. HOMYK, M. KLOTZ, K. JOHN, E. (1990a) Biochemical and morphological characterization of the intra-acrosomal antigen SP-10 from human sperm. Biology of reproduction. Vol: 42. Pp: 181-193

HERR, J.C. WRIGHT, R.M. JOHN, E. FOSTER, J. KAYES, T. FLICKINGER, C. (1990b) Identification of human acrosomal antigen SP-10 in primates and pigs. Biology of reproduction vol.42 pp: 377-382

HFEA.com Fertility facts and figures 2008. London, UK (2010); Cited on 02/08/17 [pdf] URL: http://hfeaarchive.uksouth.cloudapp.azure.com/www.hfea.gov.uk/docs/2010-12-08_Fertility_Facts_and_Figures_2008_Publication_PDF.pdf

HOTCHKISS, R.S. BRUNNER, E.K. GRENLEY, P.K. (1938) Semen analyses of two hundred fertile men. The Americal journal of the medical sciences. Vol.196 No.3 pp: 362-383

IAMMARRONE, E., BALET, R., LOWER, A. M., GILLOTT, C. & GRUDZINSKAS, J. G. 2003. Male infertility. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 17, pp.211-229.

INABA, K (2003) Molecular architecture of the sperm flagella: Molecules for motility and signalling. Zoological Science, 20(9) pp. 1043-1056

JENSEN, M.B. BJERRUM, P.J. JESSEN, T.E. NIELSEN, J.E. JOENSEN, U.N. OLESEN, I.A. PETERSEN, J.H. JUUL, A. DISSING, S. JORGENSEN, N. (2011) Vitamin D is positively associated with sperm motility and increases intracellular calcium in human spermatozoa. Human reproduction. Vol: 26 No.6 pp: 1307-3017

JEQUIER, M. J. (2011) *Male Infertility (Cambridge Clinical Guides)*. 2nd Edition. Cambridge University Press pp: 30-34, 53

KEIL, M. WETTERAUER, U. HEITE, H.J. (1979) Glutamic acid concentration in human semen—its origin and significance. Andrologia vol.11(5) pp: 385-391

KIRICHOK, Y. LISHKO, P.V. (2011) Rediscovering sperm ion channels with the patch-clamp technique. Molecular human reproduction. Vol: 17, No.8 pp:478-499

KIRKMAN-BROWN, J. SMITH, D. (2011) Sperm motility: is viscosity fundamental to progress? Molecular Human Reproduction 17(8) pp. 539-544

KLOTZ, K. COPPOLA, M. LABRECQUE, M. BRUGH, V. RAMSEY, K. KIM, K. CONAWAY, M. HOWARDS, S. FLICKERINGER, C. HERR, J. (2008) Clinical and consumer trial performance of a sensitive immunodiagnostic home test that qualitatively detects low concentrations of sperm following vasectomy. The journal of urology. Vol: 180, pp: 2569-2576

KURTH, B.E. KLOTZ, K. FLICKINGER, C.J. HERR, J.C. (1991) Localization of sperm antigen SP-10 during the six stages of the cycle of the seminiferous epithelium in men. Vol.44 pp: 814-821

LA VIGNERA, S. CONDORELLI, R. A. VICARI, E. D'AAGATA, R. SALEMI, M. CALOGERO, A E. (2011) Hyperviscosity of semen in patients with male accessory gland infection: direct measurement with quantitative viscometer. Andrologia. 44 pp: 556-559

LAMIRANDE, E. LAMOTHE, G. 2010. Levels of semenogelin in human spermatozoa decrease during capacitation: involvement of reactive oxygen species and zinc. Human Reproduction, vol.25, No.7 pp.1619-1630

LEE, C. KEEFER, M. WEN ZHAO, Z. KROES, R. BERG, L. LIU, X. SENSIBAR, J. 1989. Demonstration of the role of prostate-specific antigen in semen liquefaction by two-dimensional electrophoresis. Journal of andrology, vol.10, No, 6. Pp.432-438

LEVITAS, E. LUNENFELD, E. WEISS, N. FRIGER, M. HAR-VARDI, I. KOIFMAN, A. POTASHNIK, G. (2005) Relationship between the duration of sexual abstinence and semen quality: analysis of 9,489 semen samples. Fertility and sterility vol.83(6) pp. 1680-1686

LILJA, H. ABRAHAMSSON, P. LUNDWALL, A. (1989) Semenogelin, the Predominant protein in Human Semen. The journal of Biological Chemistry. Vol: 264, No. 3. Pp: 1894-1900

LILJA, H. OLDBRING, J. RANNEVIK, G. LAURELL, C. (1987) Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. Journal of Clinical Investigation. Vol.80 pp. 281-285

LIU, D.Y. BAKER, H.W.G. (2000) Defective sperm-zona pellucida interaction: a major cause of failure of fertilization in clinical in-vitro fertilization. Human Reproduction. Vol. 15. No. 3 pp. 702-708

LIPSHULTZ, L.I. HOWARDS, S.S. NIEDERBERGER, C.S. (2009) Infertility in the male. 4th ed. Cambridge university press: UK [chapters 11, 33]

LUNDWALL, A. BJARTELL, A. OLSSON, A. MALM, J. (2002) Semenogelin I and II, the predominant seminal plasma proteins, are also expressed in non-genital tissues. Molecular Human Reproduction. Vol.8, No.9. pp.805-810

MANDAL, A. BHATTACHARYYA, A.K. (1986) Grouping of human ejaculates according to the degree of coagulation and the relationship to the levels of choline and cholinesterase. International journal of andrology. Vol:9 Iss:6 pp: 407-415

MANN, T. LUTWAK-MANN, C. (1981) Male reproductive function and semen: themes and trends in physiology, biochemistry and investigative andrology. 1st ed. Springer-Verlag Berlin Heidelborg: New York. Pp: 29-30, 58-59.

MANN, T. LUTWAK-MANN, C. (1948) Studies on the metabolism of semen. 4. Aerobic and anaerobic utilization of fructose by spermatozoa and seminal vesicles. Biochemical Journal. 43(2) pp: 266-270

MCLACHLAN, R.I. (2013) Approach to the patients with oligozoospermia. Journal of clinical endocrinology and metabolism. Vol: 98(3) pp: 873-880

MENKVELD, R. FRANKEN, D. KRUGER, T. OEHNINGER, S. HODGSON, G (1991) Sperm selection capacity of the human zona pellucida. Molecular reproduction and development. Vol. 30 Issue:4 pp. 346-352

METZ, C.B. HINSCH, G.W. ANIKA, J.L. (1968) Ultrastructure and antigen of particles from rabbit semen. Journal of reproduction and fertility. Vol.17 pp. 195-198

MING-CHUNG, L. TSONG-CHANG, T. YU-SHIH, Y. (1992) Measurement of viscosity of the human semen with a rotational viscometer. Journal of the Formosan Medical association. 4; pp: 419-423

MORTIMER, S.T. (1997) Evaluation of new smoothing-independent kinematic measures of capacitating human sperm movement. Human Reproduction. Vol.12 suppl. 1 p.182

MORTIMER, S.T. SWAN, M.A (1999) The development of smoothing-independent kinematic measures of capacitating human sperm movement. Human reproduction vol.14 no.4 pp: 986-996

MOULIK, S. GOPALKRISHNAN, K, HINDUJA, SHAHANI, S. K. (1989) Presence of sperm antibodies and association with viscosity of semen. Human Reproduction. 4 pp: 290-291

MULHALL, J.P. APPLEGARTH, L.D. OATES, R.D. SCHLEGEL, P.N. (2013) Fertility preservation in male cancer patients. 1st ed. Cambridge university press: New York, US. pp: 19, 52-53

MUNDY, A.J. RYDER, T.A. EDMONDS, D.K. (1995) Asthenozoospermia and the human sperm mid-piece. Human reproduction. Vol. 10 no.1 pp. 116-119.

MUNUCE, M. J. BREGNI, C. CARIZZA, C. MENDULEK, G. (1999) Semen culture, leukocytospermia, and the presence of sperm antibodies in seminal hyperviscosity. Archives of Andrology. 42 pp: 21-28

NEHRA, A. WERNER, M. BASTUBA, M. TITLE, C. OATES, R. (1996) Vibratory stimulation and rectal probe electroejaculation as therapy for patients with spinal cord injury: Semen parameters and pregnancy rates. The journal of urology. Vol: 155 pp: 554-559

Nidacon.com Sperm VitalStain™ | NidaCon International AB. Mölndal, Sweden. pdf [2015-03-30] Cited on 01/08/17 URL: http://nidacon.com/inserts/spermvitalstain.pdf

NIESCHLAG, E. HABENICHT, U.F. (1992) Spermatogenesis, fertilization, contraception: molecular, cellular and endocrine events in male reproduction. Vol 4. Berlin. Springer-Verlag. Pp: 470

OKO, R. MARAVEI, D. (1995) Distribution and possible role of perinuclear theca proteins during bovine spermatogenesis. Microscopy research and technique. Vol.32 No.6 pp: 520-532.

OWEN, DEREK H. KATS, DAVID F. (2005) A review of the physical and chemical properties of human semen and the formulation of a semen simulant. Journal of Andrology. Vol.26 No.4 pp. 459-469

PERRIN, A. COAT, C. NGUYEN, M.H. TALAGAS, M. MOREL, F. AMICEC, J. DE BRAEKELEER, M. (2013) Molecular cytogenetic and genetic aspects of Globozoospermia: A review. Andrologia **45** pp: 1-9

PIOMBONI, P. FOCARELLI, R. STENDARDI, A. FERRAMOSCA, A. ZARA, V. (2012) The role of mitochondria in energy production for human sperm motility. International journal of andrology. Vol.35 Issue:2 pp. 109-124

PETER, A. LILJA, H. LUNDWALL, A. MALM, J. (1998) Semenogelin I and semenogelin II, the major gel-forming proteins in human semen, are substrates for transglutaminase. European journal of biochemistry. 252 pp. 216-221

PUBLICOVER, S.J. BARRATT, C.L.R. (2011) Sperm motility: things are moving in the lab! Molecular human reproduction, Vol.17 No.8 pp: 453-456

RHEOSENSE.com *Portable, Small Sample Viscometer | RheoSence Inc*. California, United States (2016); Cited on 01/03/17 URL: http://www.rheosense.com/products/viscometers/microvisc/how-it-works

RIJNDERS, S. BOLSCHER, J. MCDONNELL, J. VERMEIDEN, J. (2007) Filling time of a Lamellar capillary-filling semen analysis chamber is a rapid, precise, and accurate method to assess viscosity of seminal plasma. Journal of Andrology. Vol: 28, No. 4 pp: 461-465

SANDERS, D. FENSOME-RIMMER, S. WOODWARD, B. (2017) Uncertainty of measurement in andrology: UK best practice guideline from the association of biomedical andrologists. British journal of biomedical science. Pp:1-6

SEAMAN, E.K. GOLUBOFF, E. BARCHAMA, N. FISCH, H. (1996) Accuracy of semen counting chambers as determined by the use of latex beads. Fertility and sterility. Vol: 66, No. 4 pp: 662-665

SIGMAALDRICH.COM(a), Sigma-aldrich (2017) Date accessed: 24/05/17.URL:http://www.sigmaaldrich.com/catalog/product/sigma/59428c?lang=en

SIGMAALDRICH.COM(b), Sigma-aldrich (2017) Date accessed: 25/05/17.URL: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Roche/Bulletin/1/11332481001bul.pdf

SLAMA, R. EUSTACHE, F. DUCOT, B. JENSEN, T.K. JORGENSEN, N. HORTE, A. IRVINE, S. SUOMINEN, J. ANDERSEN, A.G., AUGER, J. VIERULA, M. TOPPARI, J. ANDERSEN, A.N. KEIDING, N. SKAKKEBAEK, N.E. SPIRA, A. JOUANNET, P. (2002) Time to pregnancy and semen parameters: a cross-sectional study among fertile couples from four European cities. Human Reproduction. vol.17 No.2 pp.503-515

SHARLIP I, JAROW J, BELKER A, LIPSHULTZ L, SIGMAN M, THOMAS, A, et al. (2002) Best practice policies for male infertility. Fertility and Sterility; 77 pp:873–82.

SOBRERO, A.J. MACLEOD, J. (1962). The immediate postcoital test. Fertility and Sterility, 13: pp.184-189.

SUAREZ, S.S. (2008) Control of hyperactivation in sperm. Human reproduction update. Vol.14 No.6 pp: 647-657

SUAREZ, S.S PACEY, A.A. (2006) Sperm transport in the female reproductive tract. Human reproduction update. Vol: 12 No.1 pp: 23-37

TALWAR, P. HAYATNAGARKAR, S. (2015) Sperm function test. Review article. Journal of human reproductive sciences. Vol: 8 Issue: 2 pp: 61-69

TELKKA, ANTTI. FAWCETT, DON, D. CHRISTENSEN, KENT (1961) Further observations on the structure of the Mammalian sperm tail. The Anatomical Record. Vol.141, Issue.3, pp. 231-245

The WHO laboratory manual for the examination and processing of human semen. 5th ed. 2010. pp: 21, 26-31, 33, 70, 168.

TJIOE, D.Y. OENTOENG, S. (1968) The viscosity of human semen and the percentage of motile spermatozoa. Sterility and fertility. Vol: 19(4) pp: 562-565

THONNEAU, P. MARCHAND, S. TALLEC, A. FERIAL, M. L. DUCOT, B. LANSAC, J. LOPES, P. TABASTE, J. M. SPIRA, A. (1991) Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988-1989) Human reproduction. Vol:6 pp. 811-816

TOMLINSON, M.J. (2016) Uncertainty of measurement and clinical value of semen analysis: has standardisation through professional guidelines helped or hindered progress? Andrology. Vol: 4 pp: 763-770

TOMLINSON, M.J. NAEEM, A.HOPKISSON, J.F. CAMPBELL, B. (2012) Brief communication: assessment and validation of nonspermicidal condoms as specimen collection sheaths for semen analysis and assisted conception.

TOMLINSON, M.J. (2010) Is your andrology service up to scratch? Human fertility. Vol:13 pp: 194-200

TOMLINSON, M. POOLEY, K. SIMPSON, T. NEWTON, T. HOPKISSON, J. JAYAPRAKASAN, K. JAYAPRAKASAN, R. NAEEM, A. PRIDMORE, T. (2010) Validation of a novel computer-assisted sperm analysis (CASA) system using multitarget-tracking algorithms. Fertility and Sterility. Vol: 93, Issue: 6, pp: 1911-1920

TORRES-FLORES, V. PICAZO-JUAREZ, G. HERNANDEZ-RUEDA, Y. DARSZON, A. GONZALEZ-MARTINEZ, T. (2011) Sodium influx induced by external calcium chelation decreases human sperm motility. Human reproduction, Vol: 26, No.100 pp: 2626-2635.

TULSIANI, D.R.P. ABOU-HAILA, A. LOESER, C. R. PERREIRA, B. (1998) The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. Experimental cell research 1;240 (2) pp. 151-164

VASAN, S.S. (2011) Semen analysis and sperm function tests: how much to test? Indian journal of urology. Vol: 27(1) pp: 41-48

VELLANI, E. COLASANTE, A. MAMAZZA, L. MINASI, M.G. GRECO, E. BEVILACQUA, A. (2013) Association of state and trait anxiety to semen quality of in vitro fertilization patients: a controlled study. Fertility and sterility. Vol.99 No.6 pp: 1565-1572

VILLANUEVA-DIAZ, C. VEGA-HERNANDEZ, E. DIAZ-PEREZ, M. ECHAVARRIA-SANCHEZ, M. KARCHMER-KRIVITSKY, S. (1999) Sperm dysfunction in subfertile patients with varicocele and marginal semen analysis. Andrologia. Vol: 31 pp: 263-267

WILTON, L.J TEMPLE-SMITH, P.D. BAKER, H.W.G. DE KRETSER, D.M. (1988) Human male infertility caued by degeneration and death of sperm in the sipidymis. Fertility and sterility. Vol: 49, No: 6 pp: 1052-1058

WRIGHT, R.M. JOHN, E. KLOTZ, K. FLICKINGER, C.J. HERR, J.C. (1990) Cloning and sequencing of cDNAs coding for the human intra-acrosomal antigen SP-10. Biology of reproduction. Vol.42 pp: 693-701