Semen Analysis

DORIS J BAKER

ABBREVIATIONS: 2n = diploid number of chromosomes; ABP = androgen-binding protein; ART = assisted reproductive technology; CAP = College of American Pathologists; FDA = Food and Drug Administration; FSH = follicle stimulating hormone; GnRH = gonadotropin releasing hormone; ICSI = intracytoplasmic sperm injection; LH = leutinizing hormone; n = haploid number of chromosomes; QC = quality control; TZI = teratozoospermia index; VTS = viscosity treatment system; WHO = World Health Organization; Y = yellowish cast of eosin dye; ZP = zona pellucida.

INDEX TERMS: infertility; male factor infertility; semen analysis; seminal plasma; Strict Criteria sperm morphology.

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Doris J Baker PhD HCLD(ABB) MT(ASCP) CLS(NCA) *is* ***professor****, Center of Excellence in Reproductive Sciences, and* ***director****, Division of Clinical & Reproductive Sciences, University of Kentucky, Lexington KY.*

Address for correspondence: *Doris J Baker PhD HCLD(ABB) MT(ASCP) CLS(NCA) Professor, Center of Excellence in Reproductive Sciences, and Director, Division of Clinical & Reproductive Sciences, University of Kentucky, 126E CTW Building, 900 South Limestone, Lexington KY 40536-0200. (859) 323-1100 ext. 80854, (859) 323-8957 (fax). dbake0@uky.edu.*

Doris J Baker PhD *HCLD(ABB) MT(ASCP) CLS(NCA) is the* ***Focus: Seminal Plasma*** *editor.*

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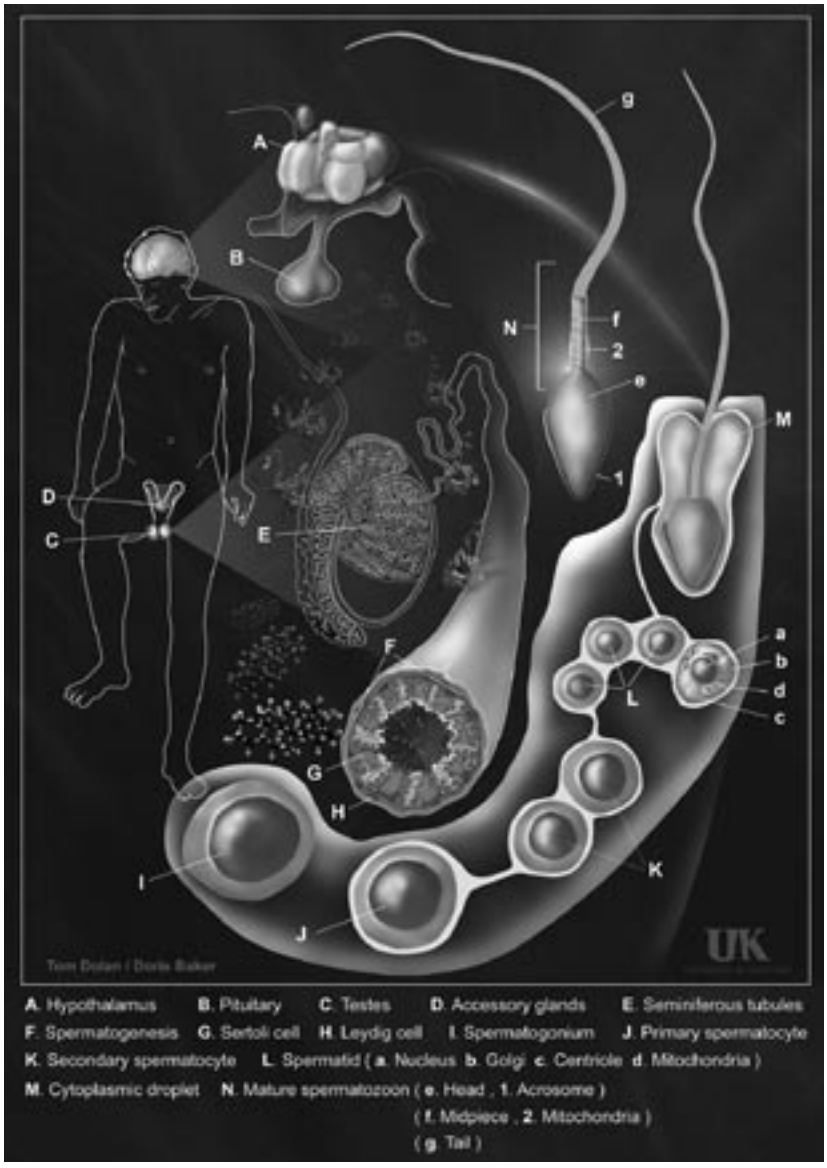
LEARNING OBJECTIVES

Upon completion of this article, the reader will be able to:

1. demonstrate a basic understanding of semen production.
2. describe the composition of seminal plasma, including key components included in the first and last portions of the ejaculate.
3. guide a patient through the proper steps for preparing for a semen analysis and for collecting and transporting the specimen to the laboratory.
4. perform a comprehensive semen analysis acceptable for fertility diagnosis that includes: (1) macroscopic evaluation; (2) assessment of the minimum number of spermatozoa to reduce statistical counting error for microscopic parameters; (3) correct calculations; (4) morphology assessment using Strict Criteria; and (5) appropriate quality control.
5. recognize abnormal semen findings and be able to troubleshoot.
6. use correct terminology associated with the production of semen and performance of a semen analysis.

Seminal plasma (semen) is composed of spermatozoa and glandular fluids and is produced by the primary organ of the male reproductive system, the testes and the accessory sex glands. This primary organ is a dual gland that produces exocrine products (spermatozoa and testicular fluid) and endocrine products (testosterone and inhibin). Testicular function is controlled by the pituitary hormones, leutinizing hormone (LH) and follicle stimulating hormone (FSH), under the influence of gonadotropin releasing hormone (GnRH) from the hypothalamus. FSH acts on Sertoli cells, located in the seminiferous tubules, to produce androgen-binding protein (ABP) and inhibin. Both ABP and testosterone produced by the testicular Leydig cells are required for spermatogenesis, the process in which spermatozoa are formed in the seminiferous tubules. Spermatogenic stem cells (2n) undergo transformation and a series of meiotic cell divisions to produce haploid (n) spermatids. The final step in the process, termed spermiogenesis, is the conversion of the haploid spermatid to a polarized, flagellated, motile spermatozoon, typically referred to as a sperm.[1] During spermatogenesis, which requires approximately ten weeks, the germinal cells

Figure 1. Male reproductive system



are supported and nourished by Sertoli cells (Figure 1).[2] The Sertoli cells have tight junctions that form a blood testis barrier providing the appropriate fluid environment for spermatogenesis. Sperm are released into the epididymis where, over an approximate two week period, they mature and gain the ability to become motile.

Upon sexual stimulation, the bulbourethral glands secrete a small amount of fluid that lubricates the penis for sexual intercourse and neutralizes the urethral contents that are toxic for spermatozoa. At ejaculation, spermatozoa stored in the epididymis are released along with a small amount of testicular fluid. Secretions from the accessory glands are then added: first, from the prostate gland followed by a second fluid from the seminal vesicles. Prostatic fluid is acidic, contributing to the pH of semen, and is rich in zinc and citric acid. This fluid also provides enzymes, including fibrinolysin, for liquefaction of the coagulum that forms at ejaculation. Seminal vesicle fluid is alkaline, rich in flavin that is responsible for semen color and contains fructose for nourishment as the male gamete travels through the female reproductive track to the site of fertilization in the oviduct. Seminal vesicle fluid contains enzymes responsible for semen coagulation and contributes approximately 70% of the total volume of seminal plasma (semen).

At ejaculation, the mixture of spermatozoa and glandular secretions (semen) empties through the penile urethra. The semen clot that is formed at ejaculation liquefies minutes later in response to enzymes from the prostate.[1] *In vivo* semen is ejaculated into the vagina and spermatozoa travel through the female reproductive track to the site of fertilization in the Fallopian tube. Sperm can only traverse the female reproductive track when the cervical mucus during mid-cycle of the menstrual cycle is receptive. As the sperm pass through the female reproductive track, they undergo capacitation, which is a series of biological and biochemical changes necessary for successful fertilization. Fertilization occurs at mid-cycle in the female when a mature oocyte is ovulated and competent sperm are present in the ampullar region of the Fallopian tube. The sperm must penetrate the cumulus cells that surround the oocyte in order to recognize receptors on the zona pellucida (ZP), the outer membrane of the oocyte. The sperm binds to the ZP and undergoes the acrosome reaction, releasing enzymes that allow penetration

of zona pellucida. The sperm plasma membrane then fuses with the vitelline membrane of the oocyte, the two gametes fuse, and the sperm enters the oocyte for fertilization.

A dysfunction or abnormality in the production of testicular products, or in the glands that contribute products to semen, may lead to pathology, including infertility. Inability of the sperm to traverse the female reproductive track and penetrate the oocyte also leads to infertility. Many of these abnormalities and/or deficiencies will be evident in the semen analysis. A comprehensive semen analysis, performed by a qualified laboratorian using appropriate quality control, on a specimen that has been properly collected, provides important diagnostic information.[3] Semen analysis is the first and most important test performed for the evaluation of male fertility. Infertility is often defined as not being able to become pregnant after one year of unprotected intercourse. The 2002 National Survey of Family Growth reported that seven percent of married couples in which the woman was of reproductive age (2.1 million couples) reported that they had not used contraception for 12 months and the woman had not become pregnant.[4] When a diagnosis of male factor infertility is made, knowledge of semen components and values of the associated parameters is essential for successful interpretation and treatment. For example, a specimen with a low sperm concentration and/or motility can be processed to select for the motile sperm fraction. The non-motile and dead sperm along with harmful debris are discarded and the enriched motile fraction is used for partner insemination, thus increasing the chances of fertilization.

PATIENT PREPARATION AND SPECIMEN COLLECTION

In order for the patient to properly prepare for the semen analysis, he must interact with laboratory personnel or the medical practitioner ordering the test. Patients must be provided with information that instructs them on: (1) preparation for the test; (2) specimen collection and transport; (3) documentation regarding medical history, preparation, specimen collection and transport; and (4) directions to the testing laboratory directions to the testing laboratory along with contact information.

a) Patients should be instructed to abstain from any type of ejaculation for three days (a two to five day range is acceptable) before collecting the sample for evaluation. A shorter abstinence period may result in a decreased sperm concentration and a longer abstinence may yield a higher concentration, but a decreased motility.

b) Patients should be directed to wash their genitals using only water (certain soaps are toxic to spermatozoa) before collecting the specimen by masturbation into a sterile container provided by the laboratory (seminal pouches are available at the request of the practitioner for patients who cannot collect by masturbation). The time of collection should be recorded on the collection container and/or a patient information form that is also provided by the laboratory. Ideally, the specimen is collected at the laboratory setting, but if this is not feasible, the patient should be instructed to maintain the specimen at or near body temperature (e.g., in shirt pocket) for delivery to the laboratory since both heat and cold adversely affect semen quality. If collected off-site, the specimen should reach the laboratory within 30 minutes of collection and must be delivered to the laboratory within 60 minutes of collection in order for the motility evaluation to be valid.

c) The patient should complete the patient information form and report days of abstinence, method of collection, and whether any part of the ejaculate was lost. If the first portion containing the spermatozoa and prostatic secretions is lost, the coagulum will fail to liquefy, the pH will be increased, and the sperm concentration will be decreased. If the last part of the ejaculate that consists of seminal vesicle fluid is missing, the coagulum will not formed, the pH will be decreased, the volume will be decreased, and the sperm concentration will be falsely elevated due to the loss of the diluting effect of the seminal vesicle fluid. Current medications should be recorded and illnesses or fevers within the past three months should be noted since increased temperature negatively impacts sperm production and quality.

ACCESSION AND PATIENT REPORTING

The specimen must be delivered directly to laboratory personnel who will denote time of specimen receipt and take responsibility for documenting chain of custody prior to specimen receipt and during testing and reporting of results. The technologist receiving the specimen should review the patient information form to determine if the specimen is acceptable for processing or if additional information is needed. Since two specimens that are collected one to three weeks apart should be evaluated, the patient should be reminded of any future appointments.

SPECIMEN HANDLING

The semen specimen should be maintained at 37°C during

Table 1. The semen examination

Test	Finding	Possible cause	Troubleshooting	Effect on other parameters
Coagulum	No coagulum	-Liquefied prior to lab examination -Lost second part of the ejaculate -Dysfunction of the seminal vesicles	Ask patient to self-report if practitioner needs this information Check patient information form to determine if second fraction of ejaculate was lost	If due to loss of the second part of the ejaculate, parameters, especially concentration, may be skewed; concentration will be increased; pH will be decreased
	Fails to liquefy	-Lost first part of the ejaculate -Dysfunction of the prostate	Check patient information form to determine if first fraction of ejaculate was lost Encourage liquefaction by going from the least aggressive to the most aggressive treatment: extend incubation time, followed by repeatedly pipetting specimen using a 10-mL pipette and, as last resort, enzymatically treat with 150 USP/mL chymotrypsin (Sigma Diagnostics, St. Louis MO) or Semen VTS proteolytic enzyme (Conception Technologies, San Diego CA.)	If due to loss of the first part of the ejaculate, parameters, especially concentration, may be skewed; concentration will be decreased; pH will be increased
Volume	Low volume	-Obstruction or blockage -Seminal vesicle aplasia -Congenital absence of the seminal vesicles -Retrograde ejaculation -Loss of first part of the ejaculate -Infection	Check patient information form to determine if second fraction was lost May need to dilute the specimen	If due to loss of the second part of the ejaculate, parameters, especially concentration, may be skewed; concentration will be increased; pH will be decreased If due to retrograde ejaculation, sperm will not be present but would be present in urine
Viscosity	Viscous specimen	-May indicate high mucus content -Associated with antibodies to sperm	Proceed with the least aggressive to the most aggressive treatment: extend incubation time, followed by repeatedly pipetting specimen using a 10-mL pipet; as last resort enzymatically treat with 150 USP/mL of chymotrypsin or Semen VTS proteolytic or Semen VTS proteolytic enzyme	May have lot of debris or abnormal sperm in the specimen High viscosity may be associated with decreased sperm concentration and decreased motility
Appearance	Specimen tinged with red indicates fresh blood and brownish color indicates the presence of old blood	-Infection -Trauma -Malignancy of the testes -Prostate cancer	Anticipate other abnormal parameters	If due to infection, may see increased bacteria and/or white blood cells; microbiology cultures may be positive

Table 1 continued on next page

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Test	Finding	Possible cause	Troubleshooting	Effect on other parameters
Appearance	Greenish specimens	-Infection -Use of certain medications -Bacterial contamination	Check time specimen received to ensure that processing was timely	If due to infection, may see increased bacteria and/or white blood cells microbiology cultures may be positive
			Check patient information form to determine use of medications	If due to contamination, bacteria, but not white blood cells, may be present
	White-yellow color	-Infection -Urine contamination -Prolonged abstinence	Check patient information form for abstinence period	If due to infection, may see increased bacteria and/or white blood cells; microbiology cultures may be positive
				If due to prolonged abstinence, sperm concentration may be increased and motility decreased
				If due to urine contamination, toxic effects will result in decreased motility and vitality; viscosity and sperm concentration will be decreased due to the diluting effects of urine
	Bright yellow color	-Presence of bilirubin -Use of certain medications	Check patient information form to determine use of medications	
	Other colors	-Use of certain medications	Check patient information form to determine use of medications	
			Be alert to other abnormal findings	
	Mucous threads	-Incomplete liquefaction	Be alert to other abnormal findings May need to treat to liquefy	Interference with assessment of motility and concentration
	Many clumps	-May indicate antibodies to sperm	Be alert to other abnormal findings	Interference with assessment of motility and concentration
	Watery	-Low sperm concentration -Absence of sperm	Be alert to other abnormal findings	Decreased sperm concentration
Odor not included in the analysis but may be noted by patient	Pungent or foul	-Collection in unacceptable container -Infection -Prolonged abstinence -Urine contamination	Check patient information form to determine abstinence period	If due to infection, may see increased bacteria and/or white blood cells; microbiology cultures may be positive
			Check patient information form to determine use of medications	If due to prolonged abstinence, sperm concentration may be increased and motility decreased
				If due to urine contamination, toxic effects will result in decreased motility and vitality; viscosity and sperm concentration will be decreased due to the diluting effects of urine
pH	< 7.2	-Disorder such as blockage of the seminal vesicles -Chronic prostatitis	Check patient information form to determine if the second fraction of the ejaculate was lost	If pH is acidic and the volume is decreased, this indicates blockage of the seminal vesticles

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Test	Finding	Possible cause	Troubleshooting	Effect on other parameters
pH	> 8.0	-Delayed reading -Microbial contamination -Infection -Disorder of the prostate (pH > 8.6 is indicative of acute prostatitis)	Check time to determine if processing was delayed Check patient information form to see if first part of specimen was lost	If due to infection, may see increased bacteria and/or white blood cells; microbiology cultures may be positive If due to microbial contamination, may see increased numbers of bacteria, but not white cells
Motility	Decreased	-Exposure to cold -Incomplete liquefaction -Improper collection container -Clumping -Exposure to toxins such as urine -Physiological causes -Kartagener syndrome (motility decreased due to ciliary immobility in some of the sperm population	Review transport, accession and time motility was performed	Immotile sperm due to Kartagener syndrome stain as live sperm on vitality stain
Vitality	Decreased	-Prolonged abstinence -Necrozoospermia -Kartagener syndrome -Exposure to urine	Check patient information form to confirm abstinence	If due to prolonged abstinence, sperm concentration may be increased, motility and vitality decreased, and pungent odor may be present Immotile sperm due to the Kartagener syndrome stain as live sperm on vitality stain If due to urine concentration, toxic effects will result in decreased motility and vitality; viscosity and sperm concentration will be decreased due to the diluting effects of urine
Agglutination	Present	-Anti-sperm antibodies -Infection	If more than 10% of sperm are agglutinated, motility should be assessed on the free spermatozoa only	May interfere with testing for motility and concentration
Concentration	Decreased	-Loss of the first part of the ejaculate -Short abstinence period -Anatomical defect -Physiological problem -Recent fever -Medications	Check patient information form to determine if the first part of the ejaculate was lost May need to concentrate the specimen to evaluate semen parameters	If due to the loss of the first part of the ejaculate, parameters, especially concentration, may be skewed; concentration will be decreased; pH will be increased
	Increased	-Loss of the second part of the ejaculate Prolonged abstinence	Check the patient information form to determine if second fraction of the ejaculate was lost	If due to the loss of the second part of the ejaculate, parameters, especially concentration, may be skewed; concentration will be increased; pH will be decreased If due to prolonged abstinence, motility may be decreased

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Test	Finding	Possible cause	Troubleshooting	Effect on other parameters
Morphology	Abnormal	-Anatomical defects -Physiological problems -Infection -Varicocele vein -Scrotal heating -Frequent ejaculations	Check patient information form to determine date of last ejaculate (may see immature sperm due to frequent emissions)	If due to infection, may see increased bacteria and/or white blood cells; microbiology cultures may be positive
Round cells	Increased white cells	Infection or inflammation	Check patient information form to see if patient had recent fever Check initial assessment to determine if bacteria were present	If due to infection/inflammation, may see increased bacteria and/or white blood cells; microbiology cultures may be positive
	Increased sperm precursors	Physiological or reproductive problem	If more than 5 X 10^6 round cells/mL recommend peroxidase staining to confirm leucocytes	

evaluation. The sample must be thoroughly mixed prior to each macroscopic and microscopic assessment.

Notes regarding reference ranges and quality control
When performing the comprehensive semen analysis, the technologist should be able to correlate abnormal parameter values with possible causes and be able to troubleshoot when necessary. Table 1 summarizes possible causes for semen parameters that fall outside reference ranges, ways the abnormal finding may influence other semen parameters, and associated troubleshooting. The values listed are based on the 4th edition of the WHO Laboratory Manual, published in 1999 for the evaluation of semen and sperm-cervical mucus interaction. The 5th edition, to be published in 2007, will report reference ranges for sperm concentration based on semen quality from men who impregnated their partners with a time to pregnancy of up to 12 months (personal correspondence with Trevor G Cooper PhD, chair, WHO Manual Editorial Committee, August, 2006).

MACROSCOPIC EXAMINATION[1]

The macroscopic portion of the test evaluates coagulum formation, liquefaction, volume, viscosity, appearance, and pH. If microbiology cultures are ordered, the specimen must be treated aseptically and plated immediately after volume is assessed.

Coagulum formation and liquefaction
Coagulum is the clot that forms in response to enzymes from the seminal vesicles. Liquefaction is due to enzymes in the prostatic fluid. The semen analysis can be performed once the clot liquefies.

Procedure: The semen specimen is swirled in the collection container to determine if the coagulum has liquefied. A liquefied sample will take the shape of the container.

Reference range: Coagulum liquefies within 15-30 minutes at 37°C or within one hour at room temperature.

Comments: In some patients the coagulum liquefies very soon after ejaculation and is therefore not detected in the laboratory. If formation is not detected and the practitioner needs this information, the patient can be asked to self-report on this parameter.

Volume
Procedure: Volume is assessed using a sterile 5-mL or 10-mL pipet and recorded to the nearest 0.1 mL.

Reference range: ≥ 2 mL; a volume of < 1 mL is referred to as oligospermia.

Comments: If volume is < 0.8 mL, the specimen may be diluted 1:1 using Sperm wash medium (Hepes based Human Tubal Fluid supplemented with protein or Dulbeccos' medium; Irvine Scientific, Irvine CA). If the specimen is diluted, the sperm concentration must be doubled prior to reporting results.

Viscosity
Viscosity refers to the fluid nature of semen.

Procedure: Viscosity is assessed after the volume has been measured by aspirating the sample into the pipet and allowing semen to drop back into the container by gravity. The length of any thread that forms is measured to the nearest 0.1 cm.

Reference range: Thread should be ≤ 2 cm. Grade as slightly viscous (thick), moderately viscous (difficult to pipet) or extremely viscous (cannot pipet).

Comments: Viscosity may indicate a high mucus content. Increased viscosity has been reported to be higher in men with oligoasthenospermia (decreased motility and decreased concentration) and has been associated with antibodies to spermatozoa.[6,7,8]

Appearance
Appearance refers to the color and consistency of semen.

Procedure: Note the exact appearance of the specimen.

Reference range: homogeneous, opalescent, and grayish-white in color

Comments: Colors other than grayish-white are associated with certain pathologies, contamination, and use of certain medications (Table 1).

pH
pH refers to the alkalinity of semen, which buffers against the acid pH of the vagina harmful to spermatozoa.

Procedure: Alkalinity is assed by placing a drop of semen on a pH paper with a range of 6.0-8.0 and reading against the calibrated strip after 30 seconds. The pH is recorded to the nearest 0.1.

Reference range: ≥ 7.2 (WHO Laboratory Manual, 1999); for clinical purposes, however, a pH below 7.6 or above 8.6 may be considered abnormal.

Comments: Have available pH paper in the range of 4.0-10.0 for specimens with values that fall outside the 6.0-8.0 range.

Quality control (QC): Standard commercial controls (Fisher Scientific International Inc., Pittsburgh, PA) should be used for pH, but quality control materials are not available for other macroscopic semen parameters. Photos or videos of normal and abnormal materials are recommended for training laboratorians and for comparison with specimens being evaluated (Semen Analysis Training Tool, Reproductive Educational Resources, Ltd., Lexington KY).

MICROSCOPIC EXAMINATION[1]

The microscopic analysis includes an assessment of semen quality, motility, vitality, sperm concentration, total sperm count, morphology, and round cells. Microscopic analyses for all unstained preparations of fresh semen (assessment of semen quality, motility, and vitality) should be made under high power (40X) using a phase contrast microscope or lowering the condenser on an ordinary light microscope.

Ideally all preparations for assessment of motility should be performed using a microscope with a stage warmed to 37°C. If this tool is not available, specimens should be placed in a 37°C incubator and the prepared slides incubated on 37°C warming tray prior to reading. Because motility is affected by temperature, slides should be read immediately following incubation on the warming tray.

Assessment of quality
Assessment of quality is performed before evaluating specific microscopic parameters to estimate motility, concentration, agglutination, clumping, bacteria, and cells. This will determine if additional parameters need to be assessed and proper dilutions to be made for counts.

Procedure: A 10 µl aliquot of semen is placed on a microscope slide, covered with a 22 X 22-mm coverslip, and incubated for one minute before viewing. The depth of preparation under the coverslip is 20.7 µm. The diameter of the microscopic field is calculated by dividing the diameter of the aperture ocular (e.g., 10) by the magnification power of the objective (e.g., 40). The field area is calculated using the formula area = πr^2 once the diameter has been calculated using a stage micrometer.[2] Using a standard 14-mm ocular and a 40X objective, the microscopic field is 0.0996 mm^2 and the number of spermatozoa in the field will approximate the sperm concentration in millions/mL. For example, if an average of 50 spermatozoa were counted, the concentration would be expected to be approximately 50 million spermatozoa/mL. The newer, wide-field oculars are 20 mm and the field area is 0.1963 mm^2 with the number of spermatozoa in the field being approximately one-half the sperm concentration. For example, using a 20 mm ocular, the 50 spermatozoa counted

Table 2. Sperm motility

Slide 1

Field	A: rapid progressive	B: sluggish progressive	C: non-progressive	D: immotile
1	22	8	0	20
2	25	10	0	22
3	19	9	0	26
4	20	10	0	20
5				
6				
7				
8				
9				
10				
Total	218	94	0	211

Total progressive motile sperm (A+B) = 312; A+B/A+B+C+D = 312/523 = 60% motile

Slide 2

Field	A: rapid progressive	B: sluggish progressive	C: non-progressive	D: immotile
1	29	8	1	25
2	17	11	0	28
3	22	12	0	19
4				
5				
6				
7				
8				
9				
10				
Total	240	88	1	198

Total progressive motile sperm (A+B) = 328 (A+B) = 328 A+B/A+B+C+D = 312/ 511 = 61.1% motile

$Y = 1.96 \times \sqrt{X}$
$328\text{-}312 = 1.96 \sqrt{328 + 312}$
$17 = 19.6 \times \sqrt{640}$
$17 = 19.6 \times 25.3$
$19.6X = 25.3\text{-}17$
$19.6X = 21.8$
The difference must be < 21.8; 17 is < 21.8, so the differences between duplicate slides is acceptable

in the microscopic field would result in a concentration estimate of 25 million spermatozoa/mL. Once the number of sperm is approximated, the estimate is used to set up proper dilutions for counting. Also estimated in the initial assessment are percent motility, clumping and agglutination, and cellular elements that are not spermatozoa, including leukocytes, erythrocytes, and microorganisms. Debris, bacteria, epithelial cells, and erythrocytes detected on the initial assessment are reported as none, slight, moderate, or excessive. If agglutination or round cells are noted on the initial assessment, additional testing is warranted and is described below.

Motility

Motility determines the number of sperm that are motile and rates their speed of progression. This assay should be determined within 30 minutes after collection and must be evaluated no later than one hour post-ejaculation.

Procedure: Motility is assessed by placing 10 uL of well-mixed semen on a microscope slide and adding a 22 X 22 mm coverslip. Preparations should be prepared in duplicate. Each slide is evaluated by counting sperm in ten random fields away from the edge of the coverslip. Because a minimum of 200 spermatozoa must be counted to reduce statistical counting error, it may be necessary to count additional fields for specimens with significantly decreased concentrations. Four categories of sperm motility are scored based on their speed of progression:

A = rapid progressive sperm have a speed of progression that is ≥ 25 µm/s at 37°C (approximately equal to half a sperm tail length or five sperm head lengths)

B = sluggish progressive sperm move at a rate 5-25 µ/s at 37°C

C = non-progressive sperm move at a rate of < 5 μm/s at 37°C, but do not make forward progress; the sperm may move in circles or "twitch" in place
D = immotile sperm do not exhibit movement

The motile sperm should be counted first, followed by the non-progressive population, and finally the immotile sperm. Sperm may enter and leave the field during the motility evaluation. Those sperm that enter after the count is initiated should not be counted and the evaluator should not be concerned about sperm that have left the field after having been counted. The percentage for each of the four categories is tallied and the evaluation is repeated on a duplicate preparation. Categories A and B are totaled to equal the percent of progressive motile sperm. The following formula is used to determine if the difference between duplicates is greater than that expected in 95% (confidence interval) of samples due to counting error alone:
$Y = \pm 1.96 \sqrt{X}$ when:

X = sum of the two progressive motile percentages (A+B) from the duplicate slides

1.96 = Z value for 95% confidence interval

In order to be accepted, the difference between the two counts must be less than the calculated value of Y (Table 2).[9]

If the difference between the counts is not greater than expected for counting error alone the values for the duplicate preparations are averaged and the percent progressive motile (A+B) is reported. If the difference occurring between the counts is greater than that due to counting error alone, the assessment must be repeated.

QC: Video depicting the types and rates of progression should be used to ensure consistent reading among laboratory personnel (Semen Analysis Training Tool, Reproductive Educational Resources, Ltd., Lexington KY). For ongoing QC, videos of sperm motility may be taped in-house or purchased commercially (Fertility Solutions, Cleveland OH). Aliquots of frozen semen with known post-thaw motility may be used for QC. These controls may be cryopreserved onsite and stored in liquid nitrogen. Motility controls also are available commercially. Conception Technologies (San Diego CA) markets frozen human semen with normal and low values for motility and kinematic assay and for sperm concentration.

Reference range: ≥ 50% (rapid progressive and sluggish progressive).

Comments: (1) Graphs and tables may be generated for determining acceptable counts between duplicates by determining points on a graph based on the formula $Y=1.96\sqrt{X}$; (2) Not all commercial quality control materials for semen analysis are FDA approved; (3) The 4th edition of the WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction reports the reference range for motility as ≥ 50% (rapid and sluggish progressive) or 25% rapid progressive.[5]

Vitality

Vitality staining is performed to determine if immotile sperm are alive or dead. Sperm that are alive and have an intact membrane will be able to extrude a vital dye whereas dead sperm will not be able to extrude the dye and will pick up the color of the stain. There are various methods for assessing vitality. The vital stain, eosin Y, may be added directly to semen and read immediately as described below or it may be mixed with nigrosin and air dried before reading.

Procedure-Eosin: Five microliters (5 μL) of 0.5% Eosin Y (Sigma-Aldrich, St. Louis MO) is placed on each of two glass slides and 25 μL of well-mixed semen is added to the stain and mixed using the tip of the pipet. A 22 X 22 mm coverslip is placed on the mixture and incubated at 37°C for one minute before reading. Live spermatozoa will be able to extrude the stain and appear colorless while the dead sperm will take in the eosin Y and stain red.

Procedure-Eosin-nigrosin: One drop (50 μL) of one percent is mixed with 50 μL of semen. Following a 30 second incubation, 150 μL of ten percent nigrosin solution is added, and mixed. A thin smear is made within 30 seconds of adding the nigrosin and allowed to air dry. Live sperm will appear white and the dead sperm will be stained red against a dark background.

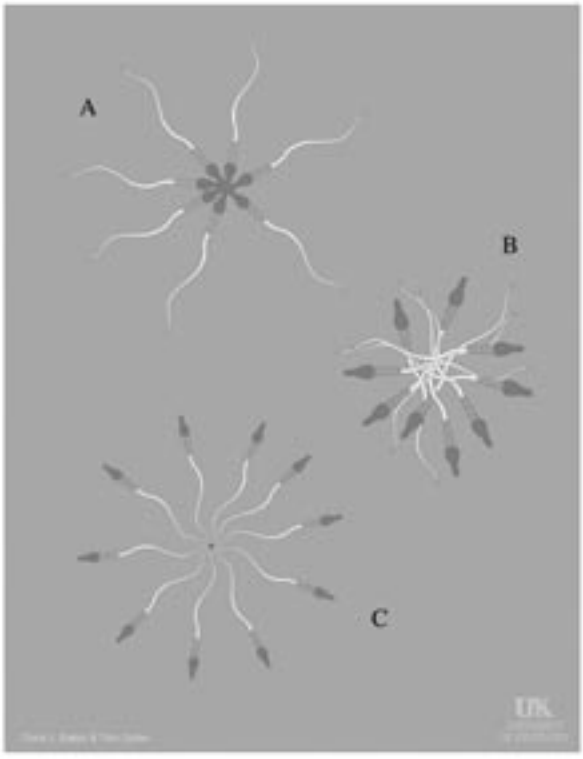
Reading: For both the eosin and the eosin-nigrosin preparations, slides are examined under oil immersion (1000X) with a light microscope. Using a laboratory tally counter, spermatozoa are counted in ten random fields away from the edge of the coverslip, counting at least 200 sperm. Each sperm is classified as either alive or dead. The percentage of live and dead sperm is calculated. The evaluation is repeated on a duplicate slide and the values are averaged and the average reported. As with the motility assessment, the counts must be repeated if not within 95% confidence limits.

QC: Frozen specimens with predetermined vitality values may be used for checking the quality of the stain and for assessments. Quality control slides are commercially available for the Eosin-nigrosin stain (Fertility Solutions, Inc., Cleveland OH).

Reference range: ≥ 75% alive.

Comments: (1) The vitality stain is a check on the motility because the total number of live sperm (% alive) should exceed the number of sperm that are motile (total motile) since there will be a population of sperm that are alive but immotile; (2) The term viability is often erroneously used to refer to the vital staining described; (3) Vitality refers to life or energy whereas viability means feasibility or capability; and (4) The 0.5% Eosin Y stain is prepared with a aqueous sodium chloride solution and the 1% Eosin Y stain for the Eosin-nigrosin assay is prepared by diluting with distilled water.[5]

Figure 2. Patterns of sperm agglutination: A – head-to-head agglutination; B – midpiece-to-midpiece; C – tail-to-tail agglutination



Agglutination

Agglutination refers to the specific attachment of motile sperm to each other. Agglutination is different from clumping which involves immotile sperm associated with debris and perhaps other cell types. Any agglutination may signify anti-sperm antibodies and is therefore significant. The assay is performed only when agglutination is noted on the initial assessment of quality.

Procedure: The preparation for counting is the same as that for motility: 10 µL of well-mixed semen is placed on a microscope slide and a 22 X 22 mm coverslip is added. Motile agglutinated sperm and motile non-agglutinated sperm are estimated in ten random fields away from the edge of the coverslip, counting at least 200 spermatozoa. Motile sperm agglutinate in specific patterns, including head-to-head, tail-to-tail, head-to-tail and midpeice-to-midpiece (Figure 2). Agglutination is estimated to the nearest five percent.

QC: Frozen semen samples that show agglutination or fresh semen exposed to antisperm antibody stock.

Reference range: Any agglutination is considered significant.

Comments: (1) Agglutination is associated with immunologic infertility and infection. *Escherichia coli* can colonize the prostate and induce production of IgA which may cause agglutination of sperm;[1,6] (2) Agglutination must not be confused with clumping of non-motile sperm associated with debris in a non-specific fashion;[1] (3) Antibody testing should be recommended to the physician when agglutination is present.

Concentration

Concentration refers to the number of spermatozoa per milliliter of semen. The term "sperm count" is frequently and erroneously used when referring to the concentration. Sperm count is NOT the same as sperm concentration; the total sperm count is the total number of spermatozoa in the entire ejaculate (concentration X semen volume).

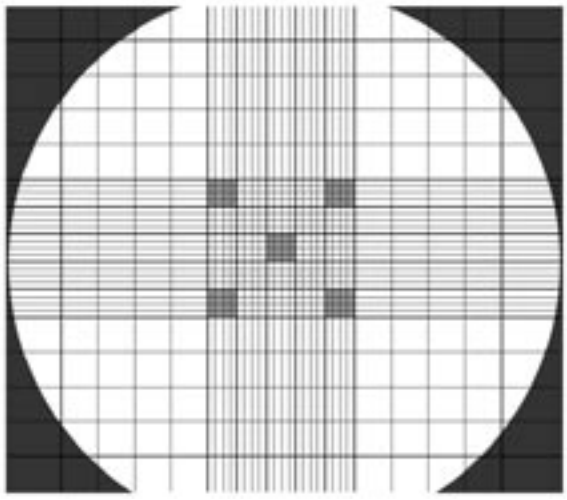
To determine the concentration of sperm, a minimum of 200 spermatozoa should be counted in the central grid on each side of a Neubauer hemacytometer, counting a total of five squares within the grid. The volume of five squares is calculated by multiplying the area (.2 mm^2) X chamber depth (0.1 mm) which is .02 mm^3 = .02 µL= 20 nL. Approximately 200 spermatozoa in five squares are equal to

10cells/nl (see Figure 3). The dilution is therefore made based on the formula:

$$\frac{\text{\# cells/nl in the initial assessment}}{10 \text{ cells/nL}}$$

Example: If a concentration of 50 X 10^6 was estimated based on counting 50 spermatozoa/field on the initial assessment, the dilution factor would be 50/10 or 5 and the dilution would be 1 in 5. The dilution can be made using a convenient volume (e.g., 10 µL semen + 40 µL sperm diluent).

Figure 3. Dilutions are made for counting approximately 200 spermatozoa in five central squares (four corners and middle)



Alternatively, the volume of semen can be calculated:

X µL @ 50 cells/nl = 50 µL (or any convenient volume) X 10 cells/nL

X = 10 µL semen (added to 40 µL diluent).

See Table 3.

Dilutions should be made in duplicate, mixed, and an aliquot of approximately 10 µL from each should be loaded on a side of a Neubauer hemacytometer. The hemacytometer is placed in a moist chamber for 10 to 15 minutes to allow the sperm to settle. The hemacytometer is viewed using 400X magnification, the central grid is located and sperm are counted in five squares within the central grid. Sperm on two of the four borders (left and top) should be counted and the established pattern followed by all laboratorians. The number of sperm on both sides of the chamber are counted and averaged. If the counts are not within 95% confidence levels the dilutions should be mixed and the counts repeated. If the rejected counts are still not acceptable, new dilutions should be made and counted. If the concentration is decreased, the undiluted specimen may be assessed or concentrated by centrifugation.[1] The sperm concentration is calculated using the formula:

$$\text{Sperm concentration} = \frac{\text{\# spermatozoa X dilution X (1000)}}{\text{depth X area}}$$

The number of spermatozoa is from the chamber count average (e.g., 50 X 10^6 was estimated and an average of 208 X 10^6 spermatozoa were counted). Dilution means, for example, one in five expressed as five. Depth of chamber is a constant of 0.1

Table 3. Objective: count approximately 200 spermatozoa in five squares, which is 10 cells/nl

Examples of estimates from initial microscopic assessment	Cells to be counted	Dilution calculation	Dilution volume*	Semen volume*	Diluent
10 X 10^6/mL	10/nL	10/10 = 1	1 *in* 2	20 µL	20 µl**
50 X 10^6/mL	10/nL	10/50 = 5	1 *in* 5	10 µL	40 µl
100 X 10^6/mL	10/nL	10/100 = 10	1 *in* 10	10 µL	90 µl

*Determine convenient volume
** A total of 40 µl is prepared since a total of 20 µl of the mixture is required to fill the two sides of the hemacytometer chamber
Sperm diluent described in Reference 1

mm. Area for five squares counted is 0.2 mm^2. Depth X area is expressed in mm^3, making it necessary to multiply by 1000 to express the concentration in millions per milliliter.

$$\text{Concentration} = \frac{208 \text{ X } 5}{0.1 \text{ X } 0.2 \text{ mm}^2} \text{ X } 1000 = 52 \text{ X } 10^6 \text{ spermatozoa/mL}$$

QC: Commercial beads that are similar in size to human sperm (Hamilton-Thorne Research, Beverly MA) may be used for quality control. Conception Technologies (San Diego CA) markets frozen human semen with normal and low values for kinematic assay for sperm concentration and concentrations of stabilized human sperm (Fertility Solutions, Inc., Cleveland OH) are available in different concentrations. Refrigerated or frozen semen with predetermined sperm concentration may be used, but the refrigerated aliquots should not be stored for more than one month.

Reference range: ≥ 20 X 10^6/mL (Comment 2).

Comments: (1) Recent reports suggest that semen concentrations are declining;[10-12] (2) Although alternate methods of evaluation for semen concentration are available including the Makler chamber (MidAtlantic Diagnostics, Mt. Laurel NJ) and the disposable Cell-Vu chambers (Fisher Scientific, Pittsburg PA), the hemacytometer remains the gold standard. Data from several studies that are being reanalyzed for semen quality for reference ranges for the 5th edition of the WHO included weighting factors to account for different techniques to establish semen quality. Samples that were not counted using hemacytometer chambers were not included (personal correspondence with Trevor G Cooper PhD, chair, WHO Manual Editorial Committee, August 2006).

Total sperm count

Total sperm count refers to the total number of spermatozoa in the ejaculate.

Procedure: Calculate the total sperm count using the formula:

Sperm concentration in millions/mL X the semen volume in mL

Reference range: ≥ 40 X 10^6 spermatozoa.

Total motile sperm count

Total motile sperm count refers to the total number of motile spermatozoa in the total ejaculate and is a value sometimes requested by fertility specialists ordering the semen analysis.

Procedure: Calculate the total motile sperm count using the formula:

Total sperm count X percent motile sperm

Reference range: ≥ 20 X 10^6 spermatozoa.

Morphology

Morphology is the assessment of the size and shape of spermatozoa. A considerable degree of morphological variability exists in human spermatozoa. A large number of defective sperm may be observed in a normal specimen. The morphology is important because it correlates with *in vivo* and *in vitro* fertilizing capability as well as *in vitro* sperm function tests. Morphology is assessed using the Strict Criteria as recommended in the 4th edition (1999) of the WHO manual[5] and will be recommended in the 5th edition WHO manual in progress for fertility evaluations. (personal correspondence with Trevor G Cooper PhD, chair, WHO Manual Editorial Committee, August 2006).

Procedure (staining, classification, and differential)

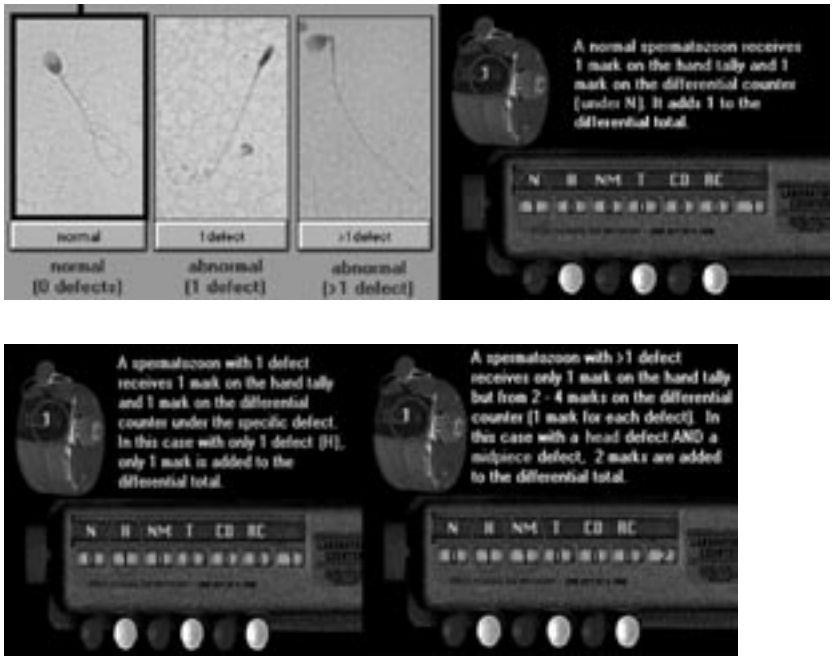
Staining: Slides for morphology assessment should be prepared using fresh, well-mixed semen, but slides can be batched for staining and, once stained, the reading can be delayed. Four slides should be prepared: two for duplicate staining and two for reserve. A drop of semen should be placed on each of two slides and a second slide placed over the slide containing the semen drop with edges overlapping the bottom slide. The semen is allowed to spread and the slides are pulled apart and allowed to air dry. Slides should be placed in an ether:ethanol fixative for 30 minutes prior to staining. The Papanicolaou stain is recommended for clarity of reading. Permount is added while the clearing agent, CitriSolv (Fisher Scientific International, Inc. Pittsburgh PA) is still wet on the stained slides and the slides are allowed to dry overnight in a 37° incubator. The morphology should be read using oil immersion X 1000, examining 200 spermatozoa in the area of the slide where sperm are evenly distributed. Stained sperm using the Papanicolaou method should exhibit a standard pattern with the acrosomal region of the head staining a light blue and the postacrosomal region staining a dark blue. The midpiece may stain blue or red; staining red is not considered abnormal unless the midpiece is distended or abnormal. The tail stains blue and the cytoplasmic droplets stain green.

Classifying spermatozoa: Sperm should be classified as normal or with defects (abnormal). Dimensions that define a normal spermatozoon are based on sperm that have been fixed and stained using the Papanicolaou method. To be considered normal, a spermatozoon must meet Strict Criteria guidelines for both shape and size, which is determined by measuring with a calibrated ocular micrometer. The sperm must be oval in shape with a length between 4.0 and 5.0 µm and a width between 2.5 and 3.5 µm. Spermatozoa having different dimensions are considered abnormal, regardless of shape. The length-to-width radio should be 1.50 to 1.75, the acrosome should cover the anterior head and should comprise 40% to 70% of the head area. The midpiece should be one µm in width and 1.5 times the length of the head. The tail should be thinner than the midpiece, approximately 45 µm long and should be uniform in shape.

There are four categories of defects within the abnormals: (1) head; (2) neck/midpiece; (3) tail; and (4) cytoplasmic droplets. An abnormal sperm may therefore have from one to four defects. Head defects may be attributed to abnormal size or abnormal shape. The acrosome may be absent or abnormal and the head may be amorphous, tapering, pyriform, double, or have a flat side/base. If the head is oval and meets all size requirements but tapers slightly, it is still considered normal. Vacuoles that occupy more than 20% of the head are considered a head defect. Heads that have surface irregularities or a flattened side or base may be difficult to assess and may be considered as either normal or abnormal depending on the severity of the defect. Some spermatozoa also exhibit surface irregularities. Neck/midpiece defects include thin or bent midpiece, noninserted tail, and abaxial implantation. Abnormal tails may be short, multiple, or have irregular widths. Tails may be broken, coiled, or have hairpin loops. Cytoplasmic droplets are usually located on the midpiece and are considered abnormal only if they are greater in size than one third of the normal head.. In order to perform the morphology differential, the reader must be able to distinguish normal and abnormal sperm and to identify the various defects associated with those that are abnormal.

Performing the differential: The morphology differential may be performed as a basic or complete evaluation. When performing either the basic or the complete differential, 200 spermatozoa are evaluated and each spermatozoon is identified as either normal or abnormal. When a basic morphology is requested, the sperm are evaluated as being either normal or abnormal and the specific defects are not recorded. When a complete morphology is performed, the type of defect (or defects) is recorded for each spermatozoon evaluated. A hand tally and a differential counter are used when performing the complete morphology evaluation because each sperm evaluated contributes to two separate but simultaneous counts. The hand tally is used to count the requisite 200 spermatozoa and a differential counter with a key labeled "normal" and keys marked for the four types of defects (head defect, neck/midpiece defect, tail defect and cytoplasmic droplet) are used to track the defects. A normal spermatozoon receives one mark on the hand tally and one mark

Figure 4. Morphology counting and scoring



From: Baker D, Witmyer J. Semen analysis training tool CD-ROM. Lexington KY: Reproductive Educational Resources, Ltd.; 2000

on the differential counter under "normal". A sperm with a single defect receives one mark on the hand tally and one mark on the differential counter under the type of defect (e.g., head). An abnormal sperm with more than one defect would receive one mark on the hand tally and two marks on the differential counter under the types of defects noted (e.g., head and neck/midpiece) (Figure 4).

Since abnormal sperm will receive one to four tallies on the differential counter for each count on the hand tally, this total will exceed 200. Sperm lying on the side are not included in the morphology differential nor are sperm without heads, with pinpoint heads, or without tails. Conjoined sperm are included in the count with each sperm counted separately and each defect tailed separately, including fused regions of the spermatozoa. An abundance of any particular defect such as more than 20% of the population with absent or abnormal acrosomes should be reported. The percent of normal sperm is reported for both the basic and complete evaluations and the teratozoospermia index (TZI) is also reported as part of the complete evaluation. TZI is the average number of defects per spermatozoon and is a predictor of sperm function both *in vivo* and *in vitro*. The TZI is calculated by dividing the total number of defects divided by the total number of abnormal sperm.

QC: Prestained morphology slides or illustrations (Reproductive Educational Resources, Ltd.) depicting various defects should be available for comparison; frozen semen with known morphology should be fixed and stained to check the quality of staining, and blank control slides should be passed through the staining process to ensure that there is no carry-over from previous specimens.

Reference range: >14% positively associated with *in vitro* fertilization rate; < 14% normal forms associated with decreased *in vitro* fertilization rate.

Strict Criteria morphology: Strict morphology: normal > 14% (threshold for assisted reproduction)

TZI: The TZI should be ≤ 1.6.

Comments: (1) Multicenter population-based studies, using Strict Criteria assessments have been performed and should be reported in the 5th edition of the WHO Manual; (2) A TZI that is ≥ 1.6 is associated with lower pregnancy rates in untreated couples.

Round cells

Round cells may be either leukocytes or sperm precursors. The total number of round cells should not exceed 5 X 10^6/mL of semen. If more than 5 X 10^6 round cells/mL are found, staining is indicated to determine whether the cells are leukocytes or sperm precursors.

Procedure: Round cells are tracked when performing either the basic or complete morphology. One key on the differential counter is designated for round cells. As the 200 spermatozoa are counted, each round cell encountered is noted. Only an occasional round cell may be seen. The number of round cells/mL of semen is calculated using the formula:

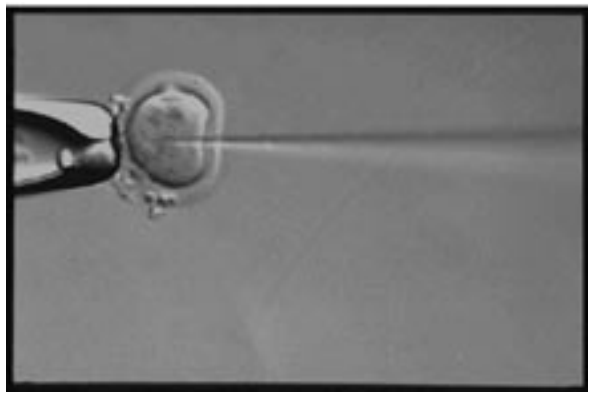
$$\frac{\text{Round cells/100 sperm X semen concentration}}{100}$$

Comments: If more than 5 X 10^6 round cells/mL are found, staining is indicated to determine if the cells are leucokytes or sperm precursors. Peroxidase staining (LeucoScreen, Conception Technologies, San Diego CA) or immunocytochemistry will detect leukocytes and the Bryan-Leishman stain is helpful for identifying sperm precursors. Semen should not contain more than one million white cells per mL of semen.

Proficiency testing

The College of American Pathologists (CAP, Chicago IL) has proficiency testing for sperm motility, vitality, concentration, and morphology. The American Association of Bioanalysts

Figure 5. Intracytoplasmic injection (ICSI)



A holding pipet stabilizes the oocyte in the dish; a single sperm is picked up and injected directly into the oocyte using an injection needle. Photo courtesy of Dr. Maria Bertero

has CAP-approved proficiency testing for sperm vitality, concentration and morphology.

Additional testing

Based on the results for repeated semen analyses, a complete physical, assessment of hormones and/or invasive testing (e.g., testicular biopsy) may be indicated. Additional testing on semen also may be warranted. Anti-sperm antibody testing is indicated when agglutination or a low motility (may indicate sperm immobilizing antibodies) are noted on semen analysis. Semen analysis results may suggest the need for assays for accessory gland function. Fructose serves as a marker for the seminal vesicles and enzymes are markers for the prostate gland (acid phosphatase) and the epididymis (α glucosidase). The hypo-osmotic swelling test is an alternative to the vitality test, and there are various assays to predict sperm function. Most sperm function assays are preformed in andrology reference laboratories and some are considered research assays and have not been approved for diagnostic testing.[3]

Treatments for male factor infertility

Treatment for male factor infertility is dependent on the final diagnosis. Hormone therapy (e.g., low FSH) or surgery (e.g., varicocele vein repair) may resolve the problem or laboratory interventions may be applicable. Intrauterine insemination may be performed to negate hostile cervical mucus or to allow for sperm concentration to select the motile fraction. Assisted reproductive technology (ART) procedures are indicated for semen with significantly abnormal parameters or for infertility of unknown etiology. Intracytoplasmic sperm injection (ICSI), the process of injecting a single sperm into the oocyte, circumvents many sperm problems, including very low sperm concentration and/or motility and morphology (Figure 5). ICSI has a high success rate for male infertility and is now a routine ART procedure. Use of donor sperm is the only option in cases of absolute azoospermia.

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REFERENCES

1. Baker DJ, Witmyer JF. Semen analysis training tool CD-Rom. Lexington, KY: Reproductive Educational Resources, Ltd.; 2000.
2. Mortimer D. Practical laboratory andrology. New York, NY: Oxford University Press; 1994
3. California study finds labs with trained personnel do more accurate testing. ASCP News. July 1997.
4. National Survey of Family Growth. Center for Health Statistics, Centers for Disease Prevention and Control; 2002. Available from http://www.cdc.gov/nchs/nsfg.htm. Accessed ???
5. WHO Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th. ed. New York, NY: Cambridge University Press; 1999.
6. Mendeluk FL, Flecha G, Castello PR, Bregni C. Factors involved in the biochemical etiology of human seminal plasma hyperviscosity. J Androl 2000;21:262-7.
7. Lin MC, Tsai TC, Yang YS. Measurement of viscosity of human semen with a rotational viscometer. J Formos Med Assoc 1992;4:419-23.
8. Moulik S, Kamala G, Hinduja I, Shahani S. Presence of sperm antibodies and association with viscosity of semen. Hum Reprod 1989;4: 290-1.
9. Daniel, WW. Biostatistics: a foundation of analysis in the health sciences, 4th ed. New York: John Wiley & Sons; 1987.
10. Crissman JW, Cooke PS, Hess, RA, Marty MS, Liberacki AB. Postulated human sperm count decline may involve historic elimination of juvenile iodine deficiency: a new hypothesis with experimental evidence in the rat. Toxicol Sci 2000;53:400-10.
11. Dindyal S, The sperm count has been decreasing steadily in Western industrialized countries: is there an endocrine basis for this decrease? Internet J Urol 2004:2-13.
12. Swan SH, Elkin EP, Fenster L. The question of declining sperm density revisited: An analysis of 101 studies published 1934-1996. Environ Health Perspect 2000;108:961-6.
13. Stephen EH, Chandra A. Declining estimates of infertility in the United States: 1982-2002. Fertil Steril 2006;96:516-23.