ABSTRACT

Approximately 15% of the couples trying to conceive are clinically infertile. Male factor is involved in half of these cases. Semen analysis remains the cornerstone of evaluating male infertility. It is one of the first tests done to evaluate a man’s fertility. It is inexpensive but can help determine if there is a problem in sperm production or it is the quality of the sperm that is causing infertility. In this chapter, the basics of semen analysis are explained. The tests are simple but can be meaningful if performed by highly trained and specialized professionals, in modern laboratories that are accredited to maintain the high standards and have strict quality controls.

INTRODUCTION

Approximately one in six couples is affected by infertility, a problem that can be caused by a number of factors, both male and female (Sigman and Jarow, 2007). The cause is attributed to the female in 30% of cases, to the male in 30%, to both in 30%, and is unknown in 10% of cases. The methods for evaluation of male infertility typically have been limited to a semen analysis that evaluates sperm count, motility, and morphology.

A semen analysis evaluates certain characteristics of a man’s semen and the sperm contained in the semen. It is an essential component of male infertility investigation, and interpretation of the results plays a vital role in the overall treatment of infertile couples. The basic aim of semen analysis is to evaluate descriptive parameters of the ejaculate (a mixture of spermatozoa suspended along with secretions from the testis, epididymis and other accessory glands).

For a clinician, semen analysis results are predictive of potential fertility and the possible causes of infertility; for an epidemiologist, the results are the basis for assessing hazards in the environment, occupational exposure, or effects of drugs and chemicals. The sample must be obtained and transported to the clinical laboratory according to World Health
Organization (WHO) guidelines (WHO, 1999), as semen analysis results can be significantly influenced by both the technique of semen collection and the methods of laboratory analysis.

The WHO manual recommends obtaining two samples for initial evaluation at an interval of not less than 7 days or more than 3 weeks. If the results from the two samples are distinctly different, additional samples have to be collected and examined.

A routine semen analysis includes the following important steps:

**PATIENT INSTRUCTIONS FOR SAMPLE COLLECTION, SAFE HANDLING AND DELIVERY**

1. Patient should be given clear and simple instructions explaining the need for semen analysis and what is required for specimen collection.
2. Patient should be informed about the importance of abstinence time. Ejaculate must be collected after 3-5 days (but not more than 7 days) of abstinence.
3. Samples should be obtained by masturbation and collected in a warm (20-40°C), sterile, nontoxic plastic or glass wide-mouth container. Prior to sample collection, the patient must void and wash hands and genitals to minimize the chances of contamination.
4. Use of lubricants and saliva should be avoided as their potential toxicity might influence the result. Semen samples should be protected from extremes of temperature (<20°C or >40°C) during transport to the laboratory.
5. All sample containers are labeled with adequate information to eliminate any chances of error.
6. Regular condoms should not be used because of their spermicidal effect. Ideally, the samples must be collected close to the laboratory. If the specimen cannot be produced close to the laboratory, it must be delivered to the laboratory as soon as possible, certainly within 1 hour of collection. During this period, the sample has to be kept warm by carrying it next to the body, and temperature extremes must be avoided.

**INFORMATION ON THE SAMPLE CONTAINER AND PATIENT SHEET**

Once the patient has collected the specimen, some preliminary information about the specimen should be obtained:

1. Label the specimen clearly, indicating patient’s complete name, clinic number, and collection date.
2. Record collection date and time.
3. Record abstinence time in days.
4. Record time the sample is received at the laboratory.
5. Record information about split ejaculate, noting when the sample was lost and whether the spill occurred at the beginning or after ejaculation. The samples always should be labeled as “Biohazard” and extreme precaution should be taken. Follow safety guidelines and protocols in handling the sample as the semen sample may contain infectious agents (e.g. hepatitis B, hepatitis C, HIV, herpes simplex).

**PHYSICAL EXAMINATION**

**Macroscopic Examination**

*Semen Age*

Record the time when the sample was received, to the liquefaction time.

*Liquefaction*

Incubation of the sample must be carried out at either ambient temperature or by placing the specimen in an incubator at 37°C. A normal sample usually liquefies within 60 minutes at room temperature, although usually this occurs within 15-20 minutes. It is determined by the time required for the gelatinous mass to liquefy. A normal sample might contain gel-like gelatinous corpuscles that do not liquefy. Exact liquefaction time is of no diagnostic importance unless >2 hours elapse without any change. This may indicate poor prostatic secretion since the liquefying enzymes are derived from the prostate gland. On the other hand, absence of coagulation may indicate ejaculatory duct obstruction or congenital absence of seminal vesicles.

For samples that do not liquefy, the sample can be mixed by rapidly mixing with a regular transfer pipette or, if the sample is viscous, viscosity can be broken by using a viscosity treatment system.

**Color and Odor**

It is important to note the color. Normal semen is homogeneously opaque, whitish grey or pearly white. The semen odor is unmistakable and pungent because of sperm oxidation. A yellowish tinge to the semen appears with an increase in the days of abstinence or probably due to carotene pigment. More pronounced yellow discoloration may indicate jaundice or contamination of semen with urine (e.g. bladder neck dysfunction). Drugs like methylene blue and pyridium may also color the semen. Fresh blood (hematospermia) will give semen a reddish tinge, while old blood gives it a brownish tinge. This could be due to the presence of inflammation. Prostatic secretions give semen a strong distinctive odor. Absence or uncharacteristic odor could be associated with an infection.
Volume

Volume of the ejaculate should be measured by transferring the liquefied sample into a graduated 15 mL conical centrifuge tube. The normal volume of ejaculate after 2-5 days of sexual abstinence is about 2-6 mL. Retrograde ejaculation, obstruction of lower urinary tract (urethra, congenital absence of vas deferens, seminal vesicles) may yield low volume. According to the WHO laboratory manual, the reference value for semen volume is ≥ 2.0 mL; however, for clinical purposes; semen volume is differentiated into three categories to facilitate interpretation and diagnosis:

Aspermia: No semen produced after orgasm (seen in certain clinical conditions).

Hypospermia: <0.5 mL of semen ejaculated (partial or complete retrograde flow of semen, accessory glands impairment).

Hyperspermia: > 6 mL of semen ejaculated (long period of sexual abstinence or overproduction of fluids from the accessory sex glands).

If the volume is <1 mL it is important to determine if the sample is complete. The highest sperm concentration is seen in the initial ejaculate.

Viscosity

Viscosity measures the seminal fluid’s resistance to flowing. It is measured by the length of the ‘thread-lines’ or ‘spinnbarkeit.’ It can be estimated by using a glass rod and observing the length of thread that forms on withdrawal of the rod. A normal sample leaves small, discrete drops; abnormal samples will form threads more than 2 cm long. High viscosity may interfere with determinations of sperm motility, concentration and antibody coating of spermatozoa. Viscosity can be categorized as ‘normal’, ‘moderate’ or ‘high.’ Viscous samples can be treated by a viscosity treatment system containing a premeasured vial of chymotrypsin (5 mg/vial (Conception Technologies, San Diego, CA). The sample can be swirled and left in the incubator for another 10-15 min till viscosity is completely broken down and the sample is suitable for analysis.

pH

The pH of liquefied semen is determined by using pH test strips; pH 6.5 to 10 has been found most suitable for this purpose. A drop of semen is spread evenly onto the pH paper. After 30 seconds, the color of the impregnated zone is compared with the calibrated strip.

Normal semen pH is in the range of 7.2 to 8.2, and it does tend to increase with time after ejaculation. Any change in the normal range of pH may be caused by inflammation of the prostate or seminal vesicles.
MICROSCOPIC EXAMINATION

Wet Preparation Examination

Load a 5 µL of well-mixed semen on a clean, warmed microscope slide with a cover slip on top (18 × 18 mm). If a 22 × 22 mm cover slip is used, the semen volume on the microscope slide should be 10 µL). This preparation has a depth of approximately ~20 µm. A depth less than 20 µm will hamper the rotational movements of the spermatozoa. Care should be taken to avoid formation of air bubbles that can be trapped between the cover slip and the slide. It is important to wait for the drifting to cease/stabilize before examination. In addition, a variety of other disposable two-well 20 µm counting chambers are also available.

A phase contrast microscope is recommended for all examinations of unstained preparations of fresh/washed semen. Initial examination is done under 100× total magnification (10× objective and 10× ocular), which provides an overview for determining mucus strands, sperm aggregation, and evenness of spread of spermatozoa on the slide. Subsequently, the sample should be examined for count and motility under 200× magnification.

Sperm Concentration

Determining accurate sperm concentration (million/mL of ejaculate) and total sperm count (million sperm per ejaculate) is important. The most accurate method of determining sperm concentration is volumetric dilution and hemocytometry. Gently mixing the semen sample using a positive displacement pipette before the volume is withdrawn is essential for an accurate determination of sperm concentration.

Hemocytometry

Principle
A fixed volume of a liquefied semen aliquot is used and fixed sperm are counted in a Neubauer hemocytometer chamber. Dilution of 1:19 is usually employed. Dilutions may be made in small, clean, glass or plastic vials. Extreme care must be taken while making dilutions and preparing the hemocytometer.

Reagents
1. The diluent consists of 50 g sodium bicarbonate
2. 10 ml of 35% of formaldehyde solution, and 0.25 g trypan blue dissolved in reagent water up to 1 liter.
Procedure
1. Filter the solution through Whatman No.1 papers into a clean bottle and store it at 4°C.
2. Add 50 µL liquefied semen to 950 µL diluent. Use a positive displacement pipette to ensure accurate handling of the viscous semen.
3. These dilutions can be stored for up to 4 weeks at 4°C.
4. Place the hemocytometer cover slip over the chamber.
5. Vortex the diluent for 10 s. Transfer 10 µL to each chamber.
6. Leave the hemocytometer in a humid chamber for 10 to 15 minutes for the spermatozoa to settle down onto the counting grid.
7. Count the spermatozoa using a 20× objective phase-contrast optics. The central square of the grid in an improved Neubauer chamber contains 25 large squares, each containing 16 small squares. The number of squares counted depends on the number of spermatozoa seen in the first large square as follows:
   i. < 10 spermatozoa in the first large square - count the whole grid of 25 large squares;
   ii. 10 - 40 spermatozoa per square - count only 10 large squares (two horizontal or vertical rows); and
   iii. >40 spermatozoa per square - count spermatozoa in the five large squares (the four corners plus the center).

Results
1. Counts of two hemocytometer chambers should be within 5% of their average. If not, discard, remix sample, and prepare another sample to be loaded on the hemocytometer, i.e. (higher value - lower value) must be < (sum of values/20) for the counts to be acceptable.
2. Sperm concentration (10⁶/mL) = total number of spermatozoa / appropriate correction factor (Table 11.1)
   Total sperm count = Sperm concentration × ejaculate volume.

| Table 11.1: Dilution and conversion factors for the improved Neubauer hemocytometer |
|-----------------------------------------------|-------------------|------------------|-------------|
| Spermatozoa per 400X field                   | Dilution (semen + diluent) | Conversion factors | Number of large squares counted |
| <15                                           | 1:5 (1 + 4)         | 20               | 8           | 4                   |
| 15-40                                         | 1:10 (1 + 9)        | 10               | 4           | 2                   |
| 40-200                                        | 1:20 (1 + 19)       | 5                | 2           | 1                   |
| >200                                          | 1:50 (1 + 49)       | 2                | 0.8         | 0.4                 |

Samples with low numbers of spermatozoa (<2/field, 400× should be centrifuged, a small aliquot of supernatant discarded, and the sample mixed and counted again after correcting for the volume of the supernatant that
was removed. Samples in which no spermatozoa are seen must be
centrifuged and examined for the presence of spermatozoa in the pellet.

*Specialized Counting Chamber*

*a. Makler chamber*

The Makler Counting Chamber (Sefi Medical Instruments, Heifa, Israel) is
widely used. It is only 10 microns deep, one-tenth the depth of an ordinary
hemocytometer, making it the shallowest of known chambers. It is
constructed from two pieces of optically flat glass; the upper layer serves
as a cover glass, with a 1 sq. mm fine grid in the center subdivided into 100
squares of 0.1 × 0.1 mm each. Spacing is firmly secured by four quartz
pins.

A small number of uncalibrated drops from a well-mixed, undiluted
specimen are placed in the center of the chamber by means of a simple rod
and immediately covered. A microscopic objective of × 20 is required.

*b. Disposable counting chambers*

Disposable counting chambers are available with multiple wells and
chamber depths. These are available as Micro Cell (Conception
Technologies, San Diego, CA) or CellVU (Advanced Meditech International,
Flushing, NY). These slides are easy to use. Each slide consists of two
separate chambers or wells, each 20 µ in depth.

*Loading the Chamber*

1. Load an appropriate amount (5 µL) of well-mixed sample to one of the
   loading zones. The amount of sample will depend on the chamber depth
   and design. Do not overfill the chamber.
2. The counting chamber fills by capillary action.
3. Wipe away any excess.
4. Counting can be done with an eyepiece reticule consisting of a 10 × 10
   box pattern.

*Sperm Motility Assessment*

Sperm motility is the ratio of the number of motile sperm to total number
of sperm in a given volume and is expressed as a percentage. Several scoring
systems exist for sperm motility assessments, but a simple grading system
is recommended. This provides an assessment of sperm motility without
requiring sophisticated equipments.

According to the WHO laboratory manual (WHO, 1999), five microscopic
fields are assessed in a systematic way to classify 200 spermatozoa. The
motility of each spermatozoon is graded into one of four groups:
a. Rapid progressive motility (i.e. > 25 µm/s at 37 ºC and > 20 µm/s at 20ºC; note that 25 µm is approximately equal to 5 head lengths or half a tail length).
b. Slow or sluggish progressive motility
c. Non-progressive motility (< 5 µm/s)
d. Immotility

A normal semen analysis must contain at least 50% progressively motile spermatozoa.

Computer-assisted Semen Analysis (CASA)

Manual semen analysis lacks the ability to measure the kinematics of sperm motion. Of the several systems in use for automated semen analysis, computer-aided sperm analysis (CASA) is given much attention because of its potential benefits for analyzing sperm motion (sperm head and flagellar kinematics). Some of these motion characteristics have been shown to be related to IVF outcome. Some of the important kinematic parameters are:

i. Curvilinear velocity: Curvilinear velocity (VCL) is the measure of the rate of travel of the centroid of the sperm head over a given time period. This is calculated from the sum of the straight lines joining the sequential positions of the sperm along the sperm’s track. Values are reported as µm/s.

ii. Average path velocity: Average path velocity (VAP) is the velocity along the average path of the spermatozoon. It is reported as µm/s.

iii. Straight-line velocity: Straight-line velocity (VSL) is the linear or progressive velocity of the cell. It is also the straight-line distance between the first and last centroid position for a given period of time. It is reported as µm/s.

iv. Linearity: Linearity of forward progression (LIN) is the ratio of VSL to VCL and is expressed as percentage. A value of 100% represents cells swimming in a perfectly straight line.

v. Amplitude of lateral head displacement: Amplitude of lateral head displacement (ALH) of the sperm head is calculated from the amplitude of its lateral deviation about the cells axis of progression or average path. It is reported as µm.

A man is considered to be asthenozoospermic if the spermatozoa in his ejaculate show less than 50% forward progressive movement within 60 minutes of ejaculation and necrozoospermic if all sperms are immotile.

Evaluation of Morphology Assessment

For a complete evaluation of a semen sample, the assessment of the morphological characteristics of the spermatozoa is important. The staining
of a seminal smear allows the quantitative evaluation of normal and abnormal sperm forms in an ejaculate.

**Smear Preparation**

Slides should be pre-cleaned with 95% ethanol to allow firm attachment of smears. A small drop of semen, approximately a 5µL aliquot, is placed on the slide. The fraction is then pulled out into a smear with a second slide; this is called the ‘feathering’ technique. This is done with minimum force to ensure that the spermatozoa tails do not fall apart, and care is taken to guarantee that the smear is not too thick. Two smears are made from each sample. If the sperm concentration is > 20 × 10⁶/ mL, then 5 µL of semen can be used; if the sperm concentration is < 20 × 10⁶/ mL, then 10-20 µL of semen is used. Smears are air-dried and fixed in 95% ethanol for 15 minutes. Air-dried smears can be batched prior to staining.

**Staining Methods**

The numerous staining techniques available include as the Papanicolaou, Giemsa, Shorr, modified Bryan-Leishman and Diff-Quik methods with Papanicolaou and Diff-Quik being the more common.

**Papanicolaou Stain**

Papanicolaou stain (WHO, 1999) is the most widely used. It is recommended by the WHO laboratory manual because it gives a good staining to spermatozoa and other cells as it distinguishes basophilic cell components and acidophilic cell components. It allows a comprehensive examination of nuclear chromatin pattern.

**Reagents**

i. **Fixative:** A freshly prepared solution of equal parts of analytical-grade absolute ethanol and diethyl ether.

ii. Graded ethanol (50%, 70%, 80%, 95%, and 99.5% (v/v)

**Staining solutions**

i. **Hematoxylin:** Orange G6 and EA-50 are commercially available.

ii. **Acid ethanol:** Prepared by mixing 300 mL of 095% (v/v) ethanol and 2.0 mL concentrated hydrochloric acid (36% HCL) in 100 mL reagent water.

iii. **Scott’s solution:** Prepare by dissolving 3.5 g NaHCO₃ and 20.0 g MgSO₄·7H₂O in reagent water to a total volume of 1000 mL.

**Procedure**

Prepare the air-dried smear and fix as explained above. Proceed with staining according to the sequence.
1. Graded ethanol (80%, 70%, 50%) 10 dips each
2. Running water 12-15 dips
3. Hematoxylin 3 minutes
4. Running water 3-5 dips
5. Acid ethanol 2 dips
6. Scott’s solution 4 min
7. Distilled water 1 dip
8. Graded ethanol 50%, 70%, 80% and 90% 10 dips
9. Orange G6 2 min
10. Ethanol 95% 10 dips
11. Ethanol 95% 10 dips
12. EA-50 5 min
13. Ethanol 95% (3 jars) 5 dips
14. Ethanol 99.5% 2 min
15. Xylene (3 staining jars) 1 min

With this stain, the head stains pale blue in the acrosomal region and dark blue in the post-acrosomal region. The midpiece may show some red staining. The tail is stained bluish or reddish and the cytoplasmic droplet stains green.

Diff-Quik Staining

Reagents

Diff-Quik stain (Baxter Healthcare, Deerfield, IL) comprises fixative and two solutions – Diff-Quik I and II.
1. Diff-Quik fixative: It contains 1.8 mg/L triarylmethane dye, 100% PDC (pure dye content) in methyl alcohol.
2. Diff-Quik solution I: It contains 1g/L xanthene dye 100% PDC, buffer and sodium azide (0.01%) as preservative
3. Diff-Quik solution II: It contains 1.25 g/L thiazine dye mixture, 100% PDC (0.625 g/L azure A, and 0.625 g/L methylene blue) and buffer.

Procedure

i. Slide is prepared as described above and labeled with the accession number, name, and date.
ii. Proceed with staining: Dip dry slide in Diff-Quik fixative solution containing methanol, 5 times for 1 sec each time and allowing 1 sec between dips.
iii. Allow slide to air dry for 15 min.
iv. Dip dried fixed slide in Diff-Quik solution I (xanthene dye) 3 times for 1 sec each dip and allowing 1 sec between dips. Allow excess stain to drip off. Do not dry slide.

v. Dip slide into Diff-Quik solution II (thiazine dye) 5 times for 1 sec each dip and allow 1 sec between dips. Allow excess stain to drip off. Do not dry slide.

vi. Rinse slide in deionized water gently and thoroughly to remove any excess stain.

vii. Allow stained slide to air dry in drying rack.

Mount cover slip using Accumount on the dried stained slide. For scoring, the slide can be viewed under oil immersion with magnification of 1000 × using a high quality 100 × nonphase-contrast objective and correctly adjusted bright-field optics. About 200 spermatozoa are scored for various abnormalities. The xanthine stain produces the red tones, and the thiazine increases the blue tones.

Scoring Sperm Morphology

Smears can be scored for morphology using the WHO classification (WHO, 1999). Spermatozoa abnormalities are categorized as head, neck and midpiece, and tail defects.

a. Head defects: Large, small, tapered, pyriform, round, amorphous, vacuolated (> 20% of the head area occupied by unstained vacuolar areas), heads with small acrosomal area (< 40% of head area), double heads, any combination of these.

b. Neck and midpiece defects: Bent neck; asymmetrical insertion of midpiece into head; thick, irregular midpiece; abnormally thin midpiece; any combination of these.

c. Tail defects: Short, multiple, hairpin, broken, bent, kinked, coiled tails, or any combination of these.

d. Cytoplasmic droplets: Greater than one-third of the area of a normal sperm head.

For a spermatozoon to be normal, the head, neck, midpiece, and tail must be normal. The head should be oval in shape. The length of the head should be 4.0-5.0 µm and the width 2.5-3.5 µm. The length-to-width ratio should be 1.5-0 to 1.75. Length and width can be measured with an ocular micrometer. The acrosome should be well defined and comprise 40-70% of the head area. The midpiece should be slender, less than 1 µm in width, about one-and-a-half times the length of the head, and attached axially to the head. Cytoplasmic droplets should be less than half the size of the normal head. The tail should be approximately 45 µm long. Reference range is considered as greater than 30% normal forms. In Kruger’s strict criteria classification, (Kruger et al. 1986), all ‘borderline’ forms are considered abnormal. Reference range includes spermatozoa with > 14% normal forms.
Leukocytospermia Test

This test is performed on suspended cells in a liquefied semen specimen and quantitated by counting stained cells in a Makler counting chamber. Peroxidase-positive granulocytes (neutrophils, polynuclear leukocytes, macrophages) are identified by histochemical staining using the Endtz test. This test is often referred to as the myeloperoxidase test.

Preparation of Stock Solution (stable 6 months)

Reagents
1. Ethanol
2. Benzidine 0.0625 g
3. Distilled water 25 mL

Procedure
1. Mix these chemicals in a clean 100 mL bottle. The solution should be clear and yellow.
2. Cover the bottle with aluminum foil and store in the dark.
3. Fresh stock solution should be prepared if it gets dark in color or forms a cloudy precipitate.

Benzidine is carcinogenic and should be handled carefully. Wear gloves and a face mask while weighing to avoid accidental contact or inhalation. The expired Endtz test solution should be discarded in concentrated bleach solution.

Preparation of Working Solution

i. Mix 4.0 mL of stock solution and 50 µL of 3% H₂O₂ in a 10 mL tube (dilute 30% stock H₂O₂ 10 times).

ii. Cover the bottle with aluminum foil and store in the dark.

iii. Prepare fresh working solution from stock every week and discard old solutions.

Equipment and Materials
1. Tyrode’s buffer
2. Makler counting chamber
3. Microcentrifuge tubes
4. Eppendorf pipette and tips (5 µL, 20 µL, 40 µL)

Procedure

i. Measure 20 µL of liquefied semen specimen into a micro-centrifuge tube; add 20 µL of Tyrode’s solution and 40 µL of working benzidene solution. Mix and let sit at room temperature for 5 minutes.

ii. Load a Makler counting chamber with 5 µL of the above solution and observe under 10 × magnification.
iii. All granulocytes will stain dark brown in color and retain their round shape.

iv. Count the cells in all the 100 squares of the Makler grid.

v. Number of white blood cells (WBC) can be calculated by multiplying total number of cells by 4 to correct for dilution factor. The total WBC number will be $10^5$/mL semen. This number should be corrected to million/ml by dividing by 10.

Report results as million/mL Endtz-positive cells. According to the WHO manual, the normal concentration of WBC in semen is $< 1 \times 10^6$/mL, leukocytospermia is defined as the presence of $>1$ million/ml WBCs.

Reference range: $0.0 - 0.9 \times 10^6$/mL (normal)

Panic value: Endtz test $> 1 \times 10^6$/mL (positive).

Quality Control

A weekly positive control should be run to check reagents. The results should be greater than $1.0 \times 10^6$/ml Endtz-positive cells.

Note: If semen specimen is not available, an EDTA anti-coagulated blood specimen may be used. Centrifuge the blood specimen to obtain the buffy coat. Remove the buffy layer containing WBC by using a transfer pipette. Dilute into 2 mL of Tyrode’s buffer and aliquot (0.1 mL). These aliquots may be used for approximately one month.

Sperm Viability

Sperm vitality is normally measured by testing cellular integrity, assessing the ability of the sperm plasma membrane to exclude extracellular substances. The cytologically intact ‘live’ cells can be determined using several vital staining techniques such as eosin Y and trypan blue. The hypo-osmotic swelling (HOS) test is also considered a test of sperm integrity.

Eosin-Nigrosin Stain

An eosin-nigrosin stain must be done on all specimens having a motility of 30% or less. The stain must be performed immediately following the initial motility examination (WHO, 1999).

Reagents

i. Eosin Y (1%): Weigh out 0.5 g of eosin Y and add it to 50 mL of deionized water. Dissolve this solution using gentle heat. Cool the liquid to room temperature and filter. This reagent is stable for 3 months at room temperature.
ii. **Nigrosin (10%)**: Weigh out 5 g of nigrosin and add it to 50 mL deionized water. Dissolve this solution using gentle heat. Cool the liquid to room temperature and filter. This reagent is stable for 3 months at room temperature.

**Procedure**

i. Place one drop of well-mixed semen on a Boerner slide.
ii. Add 2 drops of 1% aqueous eosin Y, stir with a wooden stirrer for 15 seconds.
iii. Add 3 drops of 10% aqueous nigrosin. Mix with a wooden stirrer.
iv. Immediately make two thin smears from this mixture by pipetting 10 µL onto each slide and air dry.
v. Place a cover slip with Accu-Mount mounting media (Baxter).
vi. Count 100 sperm on each slide in duplicate using high power (× 40).
vii. Calculate percentage of viable (unstained) and non-viable (stained) sperm.

Viability should be ≥ motility in samples with < 30% motility.

**Hypo-osmotic Swelling Test (HOS)**

The hypo-osmotic swelling (HOS) test was originally described as a test for sperm function (Jayendran, 1984, WHO, 1999). Now it is most appropriately considered as an additional test for sperm vitality. The HOS test is based on the principle that live spermatozoa withstand moderate hypo-osmotic stress. Dead spermatozoa in which the plasma membrane is no longer intact do not swell, whereas senescent cells show uncontrolled swelling that eventually results in rupture of the overdistended plasma membrane.

**Reagents**

i. Dissolve 0.735 g sodium citrate dihydrate
ii. 1.351g fructose
iii. Mix in 100 mL of distilled water.

**Procedure**

i. To 1 mL of HOS solution add 0.1 mL of liquefied semen and mix gently with the pipette.
ii. Incubate at 37°C for 30-60 minutes.
iii. Place a drop of semen mixture on a glass slide and place a cover slip.
iv. Examine under a phase contrast microscope.
v. Observe for tail swelling at × 40 magnification.
vi. Identify number of swollen cells in about 100 spermatozoa in duplicate.

Calculate the mean percentage of swollen cells:

\[
\text{Percent swelling} = \frac{\text{Number of sperm with swollen tails}}{\text{Number of spermatozoa with swollen + non-swollen tails}} \times 100
\]

Results

Normal values (fertile): > 60% spermatozoa with swollen tails
Abnormal values (infertile): < 50% spermatozoa with swollen tails.

HOS has a limited ability to predict male fertility, but it is useful in selecting non-motile but viable sperm for assisted reproductive technologies. An HOS result < 50% is associated with increased miscarriage rates.

Antisperm Antibodies

Immunological protection to sperm antigens is provided by the tight junctions of Sertoli cells forming the blood-testis barrier. The spermatozoon evokes an immune response when exposed to the systemic immune defense system. In conditions in which this barrier gets disrupted, formation of antisperm antibodies (ASA) may occur. Systemic and local immunoregulatory mechanisms control the development of antispermatic immunity, which may sometimes be overridden by genetic predispositions, non-physiological routes of inoculation, genital tract infections, etc., which may lead to ASA formation and sperm dysfunction.

Certain ASAs have a cytotoxic effect on the spermatozoa and can cause cell death and immobilization of sperm cells. Other effects of ASAs include creating agglutinated clumps of moving sperm in the semen sample, hampering passage of sperm through the cervical mucus, and zona binding and passage.

Immunobead Test

Principle

Antibodies bound to the human sperm surface can be detected by other antibodies that are against human IgG, IgA or IgM immunoglobulin molecules (Bronson et al 1984; WHO, 1999; Mortimer, 1994a).
Reagents

Immune beads: Anti-IgG, -IgA and -IgM beads (Irvine Scientific, Santa Ana, CA). For screening, beads for total B-cell labeling can be used. Reconstitute the immunobeads according to the manufacturer’s instructions. Beads can be kept for several months at 4°C in the original buffer, which contains a preservative (azide).

Stock buffer: Tyrode’s solution or Dulbecco’s phosphate-buffered saline (PBS) can be used.

Buffer I (0.3% BSA): Buffer for bead washing (10 mL) and sperm washing (2 x 10 mL for each semen sample). Add 0.6 g bovine serum albumin (BSA; Cohn fraction V) to 200 mL stock buffer. 200 mL buffer is sufficient to wash and run six unknown samples: one positive and one negative control and two sets of IgA and IgG beads.

Buffer II (5% BSA): Buffer for resuspension of beads and sperm pellets, 200 µL for each specimen. Add 250 mg BSA to 5 mL stock buffer. A total of 2 mL buffer II is needed for six samples, two controls and two sets of beads.

1. Filter all solutions through 0.22 or 0.45 µm filters and warm to 25-35°C before use.
2. At least 200 motile sperm should be assessed for each test. A positive (serum from a donor with high titers of antisperm antibodies) and negative control should be included in each run.

Direct Immunobead Test

1. Add 0.2 mL of stock bead suspension to 10 mL of buffer I in separate conical centrifuge tubes. Repeat this for each immunobead type.
2. Determine the amount of semen to be used, transfer that volume to a tube and add up to 10 mL with buffer I.
3. Centrifuge all tubes at 500 g for 6 min. at room temperature.
4. Tubes with sperm: Discard the supernatants. Resuspend the sperm pellets in 10 ml of fresh buffer I and centrifuge again as above.
5. Discard supernatants and resuspend sperm pellets in 200 µL of buffer II.
6. Tubes with beads: Discard the supernatants and resuspend the beads in 200 µL of buffer.
7. Add 5 µL droplets of each immunobead type on clean microscope slides.
8. Add 5 µL of washed sperm suspension to each droplet of beads and mix well using a yellow pipette tip.
9. Place a cover slip on each of the mixtures.
10. Leave the slides for 10 min at room temperature in a moist chamber and then assess under a 20 x phase contrast objective.
**Calculations and Results**

Only motile sperm should be assessed. Calculate the percentage of sperms that has two or more attached immunobeads. Those that have binding to the tip of the tail should be ignored. Count at least 200 motile sperm in duplicate for every preparation. Record the percentage of sperm carrying attached beads, the Ig class (IgG or IgA) and the site of binding (head, midpiece, and tail).

**Indirect Immunobead Test**

This is used to detect antisperm antibodies in heat-inactivated seminal plasma.

1. Wash normal donor sperm twice in buffer I as described above (Steps 2-4).
2. Add 0.2 mL of stock bead suspension to 10 mL of buffer I in separate conical centrifuge tubes. Repeat this for each immunobead type.
3. Determine the amount of semen to be used, transfer this volume to a tube and add up to 20 mL with buffer I.
4. Centrifuge all tubes at 500 g for 6 min. at room temperature.
5. **Tubes with sperm:** Discard the supernatants. Resuspend the sperm pellets in 10 mL of fresh buffer I and centrifuge again as above.
6. Discard supernatants and resuspend sperm pellets in 200 µL of buffer II or prepare them initially by swim-up procedure or density gradient centrifugation procedure followed by washing.
7. Adjust the washed sperm suspensions to a final motile sperm concentration of 50 × 10⁶/mL in buffer II.
8. Dilute 10 µL of the fluid to be tested with 40 µL of buffer II and mix with 50 µL of the washed donor sperm suspension. Incubate at 37°C for 60 min.
9. Wash the sperm twice as described above (Steps 2–4).
10. Place 5 µL droplets of each immunobead type on clean microscope slides.
11. Add 5 µL of washed sperm suspension to each droplet of beads and mix well using a yellow pipette tip.
12. Place a cover slip on each of the mixtures.

A positive and negative control should be included in each test run. A positive control can be prepared by using serum from a donor (e.g., from a vasectomized man) with high titers of serum sperm antibodies.

**Limitations**

Results are based on the analysis of motile sperm. Samples made using sperm with poor motility may give false negative results. A positive finding
of > 50% of motile sperm with attached beads is considered to be clinically significant.

**Normal Reference Values of Semen Variables**

Each laboratory must determine its own reference range for each variable. According to the World Health Organization guidelines (WHO, 1999) the following reference values for the semen sample are suggested:

<table>
<thead>
<tr>
<th>Reference Value</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>≥ 2.0 mL</td>
</tr>
<tr>
<td>pH</td>
<td>≥ 7.2</td>
</tr>
<tr>
<td>Sperm concentration</td>
<td>≥ 20 x 10^6 spermatozoa/mL</td>
</tr>
<tr>
<td>Motility</td>
<td>≥ 50% motile (grades a + b) or &gt; 25% with progressive motility (grade a) within 60 minutes of ejaculation</td>
</tr>
<tr>
<td>Vitality</td>
<td>&gt; 75% alive</td>
</tr>
<tr>
<td>White blood cells</td>
<td>&lt; 1 x 10^6/mL</td>
</tr>
<tr>
<td>Immunobead test</td>
<td>&lt; 50% motile spermatozoa with beads bound</td>
</tr>
</tbody>
</table>

**SEmen Microbiology and Virology**

Infection of the male reproductive tract can directly or indirectly cause infertility (Mortimer, 1994b). Inflammation caused by infection or various disorders can affect the secretory function of both the prostate and seminal vesicles. Asymptomatic infections of the prostate can cause partial or complete obstruction of the ejaculatory duct resulting in oligospermia and even azoospermia. Infection of the seminal vesicles often causes substantial reduction in ejaculate volume and a low seminal fructose concentration. Microbiological examination of the semen is required to differentiate a specific microbiological-induced pyospermia from other abnormalities that causes an increase in leukocytes.

**Pyospermia**

Pyospermia is a laboratory finding categorized as the abnormal presence of leukocytes in human ejaculate. Pyospermia is established when the concentration of seminal WBCs is in the range between 5 x 10^5/mL and 5 x 10^6/mL seminal fluid during semen analysis (Anderson DJ, 1995). Numerous studies have demonstrated that leukocytes in ejaculate have a physiological effect on sperm function, which may further impact male infertility (Wolff H, Anderson DJ, 1988).

The differential diagnosis of symptomatic pyospermia includes infection, autoimmune disease, and inflammation of the accessory sex glands and
Urogenital infections include acute and chronic prostatitis, seminal vesiculitis, epididymo-orchitis, cystitis, urethritis, urethral stricture, stone disease, foreign bodies, upper urinary tract infection, retrograde ejaculation, and localized sepsis of the adjacent lower gastrointestinal tract and asymptomatic bacteriuria. Chronic infections that may result in pyospermia include fungal, mycobacterial, and congenital lesions that cause urogenital tract infection.

Collection of Semen Specimens

1. Hands must be washed thoroughly with antiseptic soap.
2. Penis should be washed using antiseptic solution.
3. The semen specimen should be collected by masturbating directly into the sterile container.

Organisms Found in Semen

Many organisms found in semen are actually contaminants from the patient’s skin or from the air at the time of collection. Not all are associated with pyospermia, hence caution must be exercised in interpretation of positive cultures.

Ureaplasma and Mycoplasma

Ureaplasma and mycoplasma species are common commensal inhabitants of the lower genitourinary tract in adolescents and adult men and women who are sexually active. The organisms can be transmitted venereally and vertically from mother to offspring. Ureaplasma urealyticum and to a lesser extent Mycoplasma hominis are therefore often found in semen of infertile patients. U. urealyticum is part of the normal genital flora of both men and women and is found in about 70% of sexually active humans. Their presence is abnormal, and most authorities agree that the infection must be treated with antibiotics.

Chlamydia trachomatis

Chlamydia trachomatis is occasionally present in human semen. It is the cause of the most prevalent sexually transmitted bacterial disease worldwide and is responsible for an estimated 90 million infections. Considering the high worldwide prevalence of C. trachomatis infection, artificial insemination by donor (AID) is a potential route for the spread of C. trachomatis and has been reported as such. It cannot be cultured, since seminal plasma factor is toxic to the cell lines used for the culture. Treatment must extend to both the partners.
Neisseria gonorrhoeae

This organism causes severe symptoms in men. It can be identified in semen as gram-negative intracellular diplococci. Transmission is through sexual intercourse as well.

Trachomonas vaginalis and E. coli

In men with urinary tract infections, E. coli can colonize the prostate and cause production of IgA leading to sperm agglutination. Other organisms that form the normal flora of the reproductive tract may commonly be present; these include enterococci such as S. faecalis and staphylococci such as S. epidermidis. Mycobacterium tuberculosis maybe present in the absence of pyospermia.

Viruses

Many viruses can be isolated from semen, but the most important is human immunodeficiency virus (HIV). Its presence can be detected by either culture or polymerase chain reaction. Other viruses that could be present in semen are human T-cell lymphotrophic virus type I (HTLV-1), hepatitis B, hepatitis C, cytomegalovirus (CMV), and human papilloma virus (HPV). Genital herpes virus often is isolated from semen in infected patients.

BIOCHEMICAL ASSESSMENT OF SEMINAL PLASMA, PROSTATE, EPIDIDYMIS AND SEMINAL VESICLES

The prostate, seminal vesicles, and epididymis produce components such as zinc, citric acid and α-glucosidase that are uniquely specific to each accessory gland.

Measurement of Zinc

A colorimetric assay kit is used for determining the zinc content in the seminal plasma (Johnson and Eliasson, 1987, Mortimer, 1994c). It can be done in either a -well plate or spectrophotometer cuvettes. The assay is based on the principle that in the presence of zinc, 5-Br-PAPS (2-(5 bromo-2 - pyridylazo)-5-(N-propyl-N-sulphopropylamino)-phenol is converted to 5-Br-PAPS-Zn complex, which absorbs at 560 nm.

Reagents

1. Zinc kit: Chromogen solution is prepared by mixing color reagents A and B in proportions of 4:1. It is stable for 1 week at 4°C.
2. Zinc standard: (0.1 mM).
Specimens
1. Centrifuge semen at 1000 g for 15 minutes. 100 µL of semen is centrifuged to obtain 10 µL of neat seminal plasma.
2. A 10 µL aliquot of cell-free seminal plasma is diluted with 600 µL of water.

Procedure
1. Set absorbance readings on the spectrophotometer to 560 nm, and allow adequate time for stabilization.
2. Prepare a standard curve in duplicate (100 µM diluted to give 80, 60, 40, 20 and 10 µM).
3. Set the spectrophotometer to zero with a cuvette containing reagent water.
4. Add 2.5 mL working chromogen solution (mix 4 parts of color reagent A with 1 part of color reagent B). Add 20 µL of color reagent to 40 µL of diluted semen samples, standard, and blank.
5. Leave at room temperature for 5 minutes.
6. Measure absorbance at 560 nm and calculate results.
7. Calculation: Multiply by a dilution factor to obtain the concentration of zinc (mM) in undiluted seminal plasma. Multiply by ejaculate volume to obtain µmol/ejaculate.

Results
Zinc is a specific marker of prostatic function. Normal range of seminal zinc is 1.2-3.8 mmol/L or ≥ 2.4 µmol per ejaculate.

Measurement of Citric Acid in Seminal Plasma
Citric acid is an indicator of prostatic gland function. Decreased citric acid levels may indicate either prostate dysfunction or prostatic duct obstruction. It can be measured using the Boehringer enzymatic, NADH –linked kit (Mortimer, 1994c).

Reagents
1. Boehringer Kit No. 130976: Contains Solution 1: 3 x Bottle1 (mainly NADH), which is reconstituted by adding 12 mL reagent water and shaking well. 3 x Bottle 2 (citrate-lyase), reconstituted by adding 0.3 mL reagent water and shaking well.
2. Triethanolamine buffer (pH 7.7): Prepared by dissolving 14.9 g triethanolamine in 750 mL reagent water and adjusting the pH to 7.6 by adding 1 N HCl. Dissolve 0.027 g ZnCl₂ in 250 mL reagent water and
add it to the triethanolamine solution. Add 0.5 g of sodium azide and mix thoroughly.

3. *Trichloroacetic acid (TCA, 15%):* Dissolve 15 g trichloroacetic acid in 100 mL reagent water.

4. *NaOH (6 N):* Dissolve 24 g NaOH pellets dissolved in 100 mL reagent water.

5. *Citric acid standard:* 0.174 g citric acid in 10 mL reagent water. Make a 1 + 57 dilution.

**Specimens**

1. Centrifuge 250 µL liquefied semen (cell- and protein-free) in an Eppendorf tube at 1000 g for 15 minutes.

2. Add 100 µL of supernatant to 4.95 mL of 15% TCA in a small, capped vial and shake well.

3. Add 0.75 mL NaOH (6 N) and adjust the pH to 7.0.

4. Freeze three 0.5 mL aliquots of the extract in Eppendorf tubes at -20°C.

**Procedure**

1. Set the spectrophotometer to 340 nm and allow adequate time for stabilization.

2. Mix 0.5 mL of solution 1, 2.3 mL TRA buffer and 0.2 mL sample, standard, or blank in a disposable cuvette. Prepare each set in duplicate.

3. Adjust the spectrophotometer reading to zero with a cuvette containing reagent water.


5. Add 20 µL of solution 2. Shake it well, wait exactly 5 minutes, and measure the absorbance again (A2).

6. Calculate and analyze the results according to the formula:
   \[ \Delta A \times \left( \frac{V}{D} \right) \times DF \times MW \div E \div D + 100 = g/L \]
   where
   - \( \Delta A \) = Specimen dilution factor
   - \( V \) = final volume (3.02 mL)
   - \( MW \) = molecular weight of the substance analyzed (192.1)
   - \( E \) = absorption coefficient of NADH at 340 nm (6.3 cm³/µmol)
   - \( D \) = light path (1 cm)
   - \( V \) = sample volume (0.2 mL)
   - \( \Delta A \times 139.0 = \text{mmol citric acid/L} \)

**Results**

The normal range of seminal plasma citric acid concentration is 9.4-43.4 mmol/L or \( \geq 52 \mu \text{mol per ejaculate} \). Decreased levels of citric acid may indicate either prostatic dysfunction or duct obstruction. Further evaluation must be done by physician.
Measurement of Neutral $\alpha$-glucosidase in Seminal Plasma

Seminal plasma contains both neutral $\alpha$-glucosidase isoenzyme that originated from the epididymis and an acid isoenzyme contributed by the prostate. The latter can be selectively inhibited to allow measurement of neutral $\alpha$-glucosidase. P-nitrophenol $\alpha$-glucopyranoside in the presence of $\alpha$-glucosidase is converted to p-nitrophenol, and the absorbance can be read at 405 nm (Cooper et al 1990, Mortimer, 1994c).

**Reagents**

1. Phosphate buffer (0.2 M, pH 6.8). Prepare 1% SDS in phosphate buffer.
2. Color reagent 1 for stopping the reaction. 0.1 M sodium bicarbonate.
3. Color reagent 2 for diluting the product. Prepare color reagent 1 containing 0.1% SDS.
4. Substrate (p-nitrophenol glucopyranoside (PNPG, 5mg/mL in phosphate buffer, pH 6.8). Prepared fresh.
5. Glucosidase inhibitor for semen blanks Castanospermine (10 mM). Prepare 1mM working solution. Freeze in aliquots at -20°C.
6. 100 mM solution of sodium carbonate.
7. Standard: 5 mM p-nitrophenol. Make fresh every time.

**Specimen**

Use sperm-free seminal plasma prepared by centrifuging an aliquot of semen at 1000 g for 15 minutes.

**Procedure**

1. Set a water bath at exactly 37°C for the incubation step below.
2. Thaw specimens to be assayed and mix well.
3. Prepare 100 µL of PNPG substrate solution in Eppendorf tube.
4. Using a positive displacement pipette, add 10 µL specimen aliquots in duplicate into the Eppendorf tube.
5. Mix each tube and incubate at 37°C for 2 h.
6. Include internal quality control samples consisting of high, medium, and low activities of neutral $\alpha$-glucosidase.
7. To two high activity quality control semen pools. Add 8 mL of 1 mM castanospermine to provide semen blank value.
8. Prepare PNP standard curve (160, 120, 80, 40 µm) with color reagent 2 (within an hour of incubation).
9. Stop the reaction by adding 1.0 mL of color reagent 1 and mix.
10. Read absorbance of each sample at 405 nm against the blank (water).
Results

1 unit of glucosidase activity is equal to the production of 1 µmole product (PNP) per minute at 37°C. In this assay, the activity is derived from 15 µL of semen in a total volume of 1.115 mL over 120 minutes. Therefore the correction factor is 1115/15/120 or 0.6194.

1. Read the concentration of PNP produced by the sample from the standard curve (µM).
2. Multiply by the correction factor (0.6194) to obtain the activity of neutral glucosidase in undiluted seminal plasma (U/l).
3. Subtract the activity of the castanospermine semen blank from each sample to obtain the corrected (glucosidase-related) activity.
4. Multiply the corrected activity by the ejaculate volume to obtain glucosidase activity (mU) per ejaculate. α-glucosidase is a specific indicator for epipdymis function. Normal values are ≥ 20 mU/ ejaculate.

Quantitative Seminal Fructose

Sperm in semen sample are lysed, and addition of resorcinol and subsequent heating at 70°C results in a salmon-pink color, which is read at 420 nm (Davis and Gander, 1967, Moon and Bunge, 1968).

Reagents used

1. Concentrated HCl
2. Deionized water.
3. Fructose (0.32 mmole/L). Add 14.4 g of fructose to make 250 mL of deionized water (5.56 mg/dL).
4. Resorcinol 0.05%. Add 25 mg of resorcinol to 50 mL of ethanol (95%).

Freeze three aliquots (150 µL) aliquots of supernatant in 1.5 mL Eppendorf tubes at –20°C.

Procedure

1. Turn on the 77°C water bath.
2. Set spectrophotometer absorbance at 420 nm.
3. Adjust the reading to zero with cuvette containing reagent water.
4. Label three beakers for patient, positive control (pooled seminal plasma from normal donors), and negative controls (no semen added).
5. Treat with acid: To a clean beaker add 7.5 mL of deionized water + 2.5 mL HCl and 50 µL of semen or seminal plasma. Mix each patient and control sample carefully (200-fold dilution).
6. Label beaker for each patient and control. Using a Whatman #1 filter paper, filter each patient and control mixture into appropriately labeled beaker.
7. Label in duplicate 13 × 100 mm glass tubes for each standard, control, and patient sample. Add the following to the appropriate tube (Table 11.2).

8. Mix the tubes carefully and add 3.0 mL of concentrated HCL to each tube under the fume hood. Mix carefully.

9. Add 1.90 mL of 0.05% resorcinol to each tube, cap and vortex carefully.

   Incubate all tubes at 77°C for 8 min.

10. Place the tubes in an ice bath. Cool to room temperature. Transfer to disposable cuvettes and read the tubes at 420 nm.

11. Calculate the average OD from the standard. Multiply by the dilution (200) to obtain the final results in mg/dL.

### Results

Fructose is a marker for seminal vesicle function. Normal range for seminal fructose is >150 mg/dl.

### SAFETY AND QUALITY CONTROL IN THE ANDROLOGY LABORATORY

All general safety precautions related to electrical or chemical hazards, compressed gases, fire, and physical injury must be observed in the laboratory. Safety precautions must be exercised when handling biological specimens in the laboratory. All semen samples must be treated as a biological hazard, and extreme caution should be exercised. This includes use of protective clothing, eye protection, use of safety gloves, and any other protective measures as necessary.

Strict quality control and appropriate training of technical staff performing semen analysis and other laboratory procedures are important. All andrology technicians must be periodically trained to ensure uniformity, accuracy and

<table>
<thead>
<tr>
<th>Table 11.2: Preparing a sample for fructose measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fructose standard (mg/dL)</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.36</td>
</tr>
<tr>
<td>0.72</td>
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<tr>
<td>1.44</td>
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<tr>
<td>2.88</td>
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<tr>
<td>5.76</td>
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<td>0</td>
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</tbody>
</table>
reproducibility, especially in sperm count, motility and sperm morphology (Mortimer, 1994d). Internal quality controls, proficiency testing of slides for viability and morphology both by WHO and Kruger’s strict criteria are available, and laboratories can enroll in a proficiency testing program. Technicians score the slides in a blind fashion, and the results are mailed to the proficiency testing program. Adequate remedial measures should be in place to minimize inter- and intraobserver variations. In addition, strict quality control for monitoring all equipment in use is critical, and all solutions and reagents must be subjected to strict quality control measures to optimize performance and accuracy of results.

CONCLUSION

Semen analysis is fairly simple and easy to perform and remains the cornerstone of the andrology laboratory even as newer tests are being introduced. Maintaining strict interlaboratory, inter-observer, and intraobserver quality control measures and making quality control materials available is important for optimizing results.

BIBLIOGRAPHY