Semen Analysis
Examination & Processing of Human Semen
Based on WHO Guidelines 2010

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What is Semen?

- Semen is concentrated suspension of spermatozoa in seminal plasma.

- During ejaculation, it is produced from a concentrated suspension of spermatozoa, stored in the paired epididymides, mixed with, and diluted by, fluid secretions from the accessory sex organs. It is emitted in several boluses. Comparison of pre- and post-vasectomy semen volumes reveals that about 90% of semen volume is made up of secretions from the accessory organs, mainly the prostate and seminal vesicles, with minor contributions from the bulbourethral (Cowper’s) glands and epididymides.

- Semen has two major quantifiable attributes:
  - The total number of spermatozoa: this reflects sperm production by the testes and the patency of the post-testicular duct system
  - The total fluid volume contributed by the various accessory glands: this reflects the secretory activity of the glands.
Contents of Semen

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<th>Produces</th>
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<td>1. Testes</td>
<td>Spermatozoa</td>
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</table>
| 2. Epididymis (sperms mature here, storage site) | 1. choline (energy source for sperms)  
                                      | 2. Alpha glucosidase  
                                      | 3. Carnitine                                                               |
| 3. Vas deferens (also storage site) | Ergothioneine                                                            |
| 4. Seminal Vesicle (nutritive fluid secreted during ejaculation) | Fructose                                                                  |
| 5. Prostate                          | 1. citric acid  
                                      | 2. acid phosphatase  
                                      | 3. proteolytic enzymes  
                                      | 4. zinc                                                                    |
| 6. Bulbourethral glands of Cooper    | Mucous                                                                   |
Fractions of Ejaculation

- 4 Fractions-
  - PRE-EJACULATORY FRACTION: by COWPER’S or LITTER’S GLANDS
  - PRELIMINARY FRACTION: by Prostate gland
    - Odour
    - Enzymes which liquefies the spermatozoa coagulum
  - MAIN FRACTION: by Seminal vesicles, testes, epididymis and by Prostate gland partially
  - TERMINAL FRACTION: by Seminal vesicles
    - Gelatinous
    - Immotile spermatozoa.
Fraction of Semen contributed by various glands

- Urethral glands: 2 - 4%
- Prostate: 20-30%
  - Acidic fluid
  - citrate, zinc, acid phosphatase and proteolytic enzymes
- Seminal vesicles: 46-80 %
  - alkaline viscous
  - yellowish secretion - rich in fructose, vitamin C, prostaglandin, protein kinase
- Testis & Epididymis: 5-10%
  - Epididymis -α-glucosidase isoenzyme
Sperm

- Human sperm cell is about 65 µm long.

- The head size: 4-5 µm
  - Nucleus - contains the 23 chromosomes
  - Acrosome

- Mid-piece: 4-5 µm
  - The energy for motility is generated

- Tail: 55 µm
  - Motility-Propagated along the tail.
Indications for semen analysis

- Investigation of infertility
- Post-vasectomy by confirming absence of sperm.
- To support or disprove a denial of paternity on the grounds of sterility.
- To examine vaginal secretions or clothing stains for the presence of semen in medicolegal cases.
- For selection of donors for artificial insemination.
- For selection of assisted reproductive technology, e.g. in vitro fertilization, gamete intrafallopian transfer technique.
- After reversal of vasectomy to confirm the success of procedure.
Steps of Semen analysis

❖ **First 5 minutes:**
  Placing the specimen container on the bench or in an incubator (37°C) for liquefaction.

❖ **Between 30 and 60 minutes:**
  • Assessing Liquefaction and appearance of the semen
  • Measuring Semen volume
  • Measuring Semen pH
  • Preparing a wet preparation for assessing microscopic appearance, sperm motility and the dilution required for assessing sperm number.
  • Assessing sperm vitality (if the percentage of motile cells is low).
  • Making semen smears for assessing sperm morphology.
  • Making semen dilutions for assessing sperm concentration.
  • Assessing sperm number.
  • Performing the mixed antiglobulin reaction (MAR) test (if required).
Steps of Semen Analysis

- **Between 30 and 60 minutes:**
  - Assessing peroxidase-positive cells (if round cells are present).
  - Preparing spermatozoa for the immunobead test (if required).
  - Centrifuging semen (if biochemical markers are to be assayed).
- **Within 3 hours:**
  - Sending samples to the microbiology laboratory (if required).
- **After 4 hours:**
  - Fixing, staining and assessing smears for sperm morphology.
  - **Later on the same day (or on a subsequent day if samples are frozen):**
    - Assaying accessory gland markers (if required).
    - Performing the indirect immunobead test (if required).
Sample collection- Preparation

- Sample collection in Private room - The sample should be collected in a private room near the laboratory, in order to limit the exposure of the semen to fluctuations in temperature and to control the time between collection and analysis.
- Sexual abstinence - **2-7 days** - The sample should be collected after a minimum of 2 days and a maximum of 7 days of sexual abstinence. If additional samples are required, the number of days of sexual abstinence should be as constant as possible at each visit.
- Two separate samples at least 7 days apart should be analysed.
- Recommended procedure - **Masturbation**
- Pre-warmed (21°C), sterile, non-toxic, wide-mouth container made of glass or plastic
- The man should be given clear written and spoken instructions concerning the collection of the semen sample. These should emphasize that the semen sample needs to be complete and that the man should report any loss of any fraction of the sample.
- Other methods of collection: *Coitus interruptus, Condom collection*
Sample Collection Preparation
Labelling of sample

- Patient name
- Age
- Clinic or Doctor name
- Laboratory analysis form:
  - The period of abstinence (in days)
  - Date & Time of collection
  - Place of collection
  - Method of collection
  - Complete or incomplete
  - Any difficulty in producing the sample
  - The time interval from collection to analysis.
Sample Collection

1- Collection of semen for diagnostic or research purposes

- The sample should be obtained by masturbation and ejaculated into a clean, sterile, nontoxic wide-mouthed container made of glass or plastic.

- The specimen container should be kept at ambient temperature, between 20 °C and 37 °C, to avoid large changes in temperature that may affect the spermatozoa after they are ejaculated into it. It must be labelled with the man’s name and identification number, and the date and time of collection.

- The specimen container is placed on the bench or in an incubator (37 °C) while the semen liquefies.

- Note: in the report if the sample is incomplete, especially if the first, sperm-rich fraction may be missing. If the sample is incomplete, a second sample should be collected, again after an abstinence period of 2–7 days.
Sample Collection

2-Sterile collection of semen for assisted reproduction

- This is performed as for diagnostic collection but the specimen containers, pipette tips and pipettes for mixing must be sterile.
Sample collection
3-Sterile collection of semen for microbiological analysis

- In this situation, microbiological contamination from non-semen sources (e.g. commensal organisms from the skin) must be avoided. The specimen containers, pipette tips and pipettes for mixing must be sterile.

  The man should:
  - Pass urine.
  - Wash hands and penis with soap, to reduce the risk of contamination of the specimen with commensal organisms from the skin.
  - Rinse away the soap.
  - Dry hands and penis with a fresh disposable towel.
  - Ejaculate into a sterile container.

- Precautions:
  - Lubricants should be avoided - interfere with motility
  - Collect the entire sample - 70% of sperm is in the first part of the ejaculate.

Note: The time between collection of the semen sample and the start of the investigation by the microbiological laboratory should not exceed 3 hours.
Sample collection
4-Collections of Semen at home

- A sample may be collected at home in exceptional circumstances, such as a demonstrated inability to produce a sample by masturbation in the clinic or the lack of adequate facilities near the laboratory.
- The man should be given clear written and spoken instructions concerning the collection and transport of the semen sample. These should emphasize that the semen sample needs to be complete, i.e. all the ejaculate is collected, including the first, sperm-rich portion, and that the man should report any loss of any fraction of the sample. It should be noted in the report if the sample is incomplete.
- The man should be given a pre-weighed container, labelled with his name and identification number.
- The man should record the time of semen production and deliver the sample to the laboratory within 1 hour of collection.
- During transport to the laboratory, the sample should be kept between 20 °C and 37°C.
- The report should note that the sample was collected at home or another location outside the laboratory.
Sample Collection
5- Collection of Semen by Condom

❖ A sample may be collected in a condom during sexual intercourse only in exceptional circumstances, such as a demonstrated inability to produce a sample by masturbation.
❖ Only special non-toxic condoms designed for semen collection should be used; such condoms are available commercially.
❖ The man should be given information from the manufacturer on how to use the condom, close it, and send or transport it to the laboratory.
❖ The man should record the time of semen production and deliver the sample to the laboratory within 1 hour of collection.
❖ During transport to the laboratory, the sample should be kept between 20 °C and 37 °C.
❖ The report should note that the sample was collected by means of a special condom during sexual intercourse at home or another location outside the laboratory.
❖ Note: Ordinary latex condoms must not be used for semen collection because they contain agents that interfere with the motility of spermatozoa (Jones et al., 1986).
Coitus interrupts

Coitus interruptus is not a reliable means of semen collection, because

- the first portion of the ejaculate, which contains the highest number of spermatozoa, may be lost.
- Moreover, there may be cellular and bacteriological contamination of the sample, the low pH of the vaginal fluid could adversely affect sperm motility.

*If a man cannot provide a semen sample, the postcoital test may provide some information about his spermatozoa.*
Safe handling of specimen

- Semen samples may contain dangerous infectious agents (e.g. human immunodeficiency virus (HIV), hepatitis viruses or herpes simplex virus) and should therefore be handled as a biohazard.
- If the sample is to be processed for bioassay, intra-uterine insemination (IUI), in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) or if semen culture is to be performed, sterile materials and techniques must be used.
- Safety guidelines should be strictly followed.
Initial Macroscopic Examination or Physical Examination

1- Liquefaction
2- Semen Viscosity
3- Appearance of Ejaculate
4- Semen Volume
5- Semen pH

- Semen analysis should begin with a simple inspection soon after liquefaction, preferably at 30 minutes, but no longer than 1 hour after ejaculation, to prevent dehydration or changes in temperature from affecting semen quality.
1- Liquefaction

- Immediately after ejaculation into the collection vessel, semen is typically a semisolid coagulated mass.
- Within a few minutes at room temperature, the semen usually begins to liquefy (become thinner), at which time a heterogeneous mixture of lumps will be seen in the fluid.
- During liquefaction, continuous gentle mixing or rotation of the sample container on a two-dimensional shaker, either at room temperature or in an incubator set at 37 °C, can help to produce a homogeneous sample.
- As liquefaction continues, the semen becomes more homogeneous and quite watery, and in the final stages only small areas of coagulation remain.
- The complete sample usually liquefies within 15 minutes at room temperature, although rarely it may take up to 60 minutes or more.
- If complete liquefaction does not occur within 60 minutes, this should be recorded.
Semen samples collected at home or by condom will normally have liquefied by the time they arrive in the laboratory.

Normal liquefied semen samples may contain jelly-like granules (gelatinous bodies) which do not liquefy; these do not appear to have any clinical significance.

The presence of mucus strands, however, may interfere with semen analysis.

Liquefaction can be recognized both macroscopically, and microscopically. Immobilized spermatozoa gain the ability to move as the semen liquefies. If immobilized spermatozoa are observed on microscopic examination, more time must be allowed for the liquefaction process to be completed.

If the semen does not liquefy within 30 minutes, do not proceed with semen analysis but wait for another 30 minutes. If liquefaction has not occurred within 60 minutes, proceed for delayed liquefaction.
**Delayed Liquefaction**

- Occasionally samples may not liquefy, making semen evaluation difficult. In these cases, additional treatment, mechanical mixing or enzymatic digestion may be necessary.

1. Some samples can be induced to liquefy by the addition of an equal volume of physiological medium (e.g. Dulbecco’s phosphate-buffered saline), followed by repeated pipetting.

2. Inhomogeneity can be reduced by repeated (6–10 times) gentle passage through a blunt gauge 18 (internal diameter 0.84 mm) or gauge 19 (internal diameter 0.69 mm) needle attached to a syringe.

3. Digestion by bromelain, a broad-specificity proteolytic enzyme, may help to promote liquefaction.

- These treatments may affect seminal plasma biochemistry, sperm motility and sperm morphology, and their use must be recorded. The 1 + 1 (1:2) dilution of semen with bromelain must be accounted for when calculating sperm concentration.
Semen viscosity

- After liquefaction, the viscosity of the sample can be estimated by gently aspirating it into a wide-bore (approximately 1.5 mm diameter) plastic disposable pipette, allowing the semen to drop by gravity and observing the length of any thread.
- A normal sample leaves the pipette in small discrete drops. If viscosity is abnormal, the drop will form a thread more than 2 cm long.
- Alternatively, the viscosity can be evaluated by introducing a glass rod into the sample and observing the length of the thread that forms upon withdrawal of the rod. The viscosity should be recorded as abnormal when the thread exceeds 2 cm.
- In contrast to a partially unliquefied sample, a viscous semen specimen exhibits homogeneous stickiness and its consistency will not change with time.
- High viscosity can be recognized by the elastic properties of the sample, which adheres strongly to itself when attempts are made to pipette it.
- The methods to reduce viscosity are the same as those for delayed liquefaction.
- High viscosity can interfere with determination of sperm motility, sperm concentration, detection of antibody-coated spermatozoa and measurement of biochemical markers.
Appearance of Ejaculate

- A normal liquefied semen sample has a homogeneous, grey-opalescent appearance.
- It may appear less opaque if the sperm concentration is very low; the colour may also be different, i.e. red-brown when red blood cells are present (haemospermia), or yellow in a man with jaundice or taking certain vitamins or drugs.
Semen Volume

- The volume of the ejaculate is contributed mainly by the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymides.
- Precise measurement of volume is essential in any evaluation of semen, because it allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated.
- The volume is best measured by weighing the sample in the vessel in which it is collected.
- Collect the sample in a pre-weighed, clean, disposable container. Weigh the vessel with semen in it. Subtract the weight of the container.
- Calculate the volume from the sample weight, assuming the density of semen to be 1 g/ml (Auger et al., 1995). Volume = mass/density
- Semen density varies between 1.043 and 1.102 g/ml (Huggins et al., 1942; Brazil et al., 2004a; Cooper et al., 2007).
Semen Volume

- Empty specimen containers may have different weights, so each container should be individually pre-weighed. The weight may be recorded on the container before it is given to the client. Use a permanent marker pen on the vessel itself or on a label. If a label is used for recording the weight, it should be attached before the empty container is weighed.

- Alternatively, the volume can be measured directly.

- Collect the sample directly into a modified graduated glass measuring cylinder with a wide mouth. These can be obtained commercially. Read the volume directly from the graduations (0.1 ml accuracy).

- Note:
  - Measuring volume by aspirating the sample from the specimen container into a pipette or syringe, or decanting it into a measuring cylinder, is not recommended, because not all the sample will be retrieved and the volume will therefore be underestimated. The volume lost can be between 0.3 and 0.9 ml (Brazil et al., 2004a; Iwamoto et al., 2006; Cooper et al., 2007).
Semen Volume

Lower reference limit:

The lower reference limit for semen volume is 1.5 ml (5th centile, 95% confidence interval (CI)).

❖ Low semen volume is characteristic of obstruction of the ejaculatory duct or congenital bilateral absence of the vas deferens (CBAVD) (de la Taille et al., 1998; Daudin et al., 2000; von Eckardstein et al., 2000; Weiske et al., 2000), a condition in which the seminal vesicles are also poorly developed.

❖ Low semen volume can also be the result of collection problems (loss of a fraction of the ejaculate), partial retrograde ejaculation or androgen deficiency.

❖ High semen volume may reflect active exudation in cases of active inflammation of the accessory organs.
Semen pH

- The pH of semen reflects the balance between the pH values of the different accessory gland secretions, mainly the alkaline seminal vesicular secretion and the acidic prostatic secretion. The pH should be measured after liquefaction at a uniform time, preferably after 30 minutes, but in any case within 1 hour of ejaculation since it is influenced by the loss of CO2 that occurs after production.

- For normal samples, pH paper in the range 6.0 to 10.0 should be used.

- Mix the semen sample well.

- Spread a drop of semen evenly onto the pH paper.

- Wait for the colour of the impregnated zone to become uniform (<30 seconds).

- Compare the colour with the calibration strip to read the pH.

- **Note:** The accuracy of the pH paper should be checked against known standards.
Semen pH

- For viscous samples, the pH of a small aliquot of the semen can be measured using a pH meter designed for measurement of viscous solutions (Haugen & Grotmol, 1998).
- Reference value: 7.2 as a lower threshold value.
- If the pH is less than 7.0 in a semen sample with low volume and low sperm numbers, there may be ejaculatory duct obstruction or congenital bilateral absence of the vas deferens (de la Taille et al., 1998; Daudin et al., 2000; von Eckardstein et al., 2000; Weiske et al., 2000), a condition in which seminal vesicles are also poorly developed.
- Semen pH increases with time, as natural buffering decreases, so high pH values may provide little clinically useful information.
Initial Microscopic Examination

- Motility
- Viability
- Sperm count
- Morphology
Initial Microscopic Examination

- A phase-contrast microscope is recommended for all examinations of unstained preparations of fresh semen. An initial microscopic examination of the sample involves scanning the preparation at a total magnification of ×100 (i.e. a combination of a ×10 objective lens with a ×10 ocular lens).

- This provides an overview of the sample, to reveal:
  - mucus strand formation;
  - sperm aggregation or agglutination;
  - the presence of cells other than spermatozoa, e.g. epithelial cells, “round cells” (leukocytes and immature germ cells) and isolated sperm heads or tails.

- The preparation should then be observed at ×200 or ×400 total magnification (i.e. a combination of a ×20 or a ×40 objective with a ×10 ocular). This permits:
  - assessment of sperm motility
  - determination of the dilution required for accurate assessment of sperm number
Initial Microscopic Examination
Thorough mixing and representative sampling of semen

- The nature of the liquefied ejaculate makes taking a representative sample of semen for analysis problematical. If the sample is not well mixed, analysis of two separate aliquots may show marked differences in sperm motility, vitality, concentration and morphology.

- To be certain of obtaining reproducible data, the sample should be thoroughly mixed before aliquots are taken for assessment, and results for replicate aliquots should agree before the values are accepted. Agreement between replicates is determined for sperm numbers by the Poisson distribution and for percentages by the binomial distribution.

Thorough mixing of semen:
Before removing an aliquot of semen for assessment, mix the sample well in the original container, but not so vigorously that air bubbles are created. This can be achieved by aspirating the sample 10 times into a wide-bore (approximately 1.5 mm diameter) disposable plastic pipette (sterile when necessary). Do not mix with a vortex mixer at high speed as this will damage spermatozoa.
Initial Microscopic Examination
Making a wet preparation

- Mix the semen sample well.
- Remove an aliquot of semen immediately after mixing, allowing no time for the spermatozoa to settle out of suspension.
- Remix the semen sample before removing replicate aliquots.
- The volume of semen and the dimensions of the coverslip must be standardized, so that the analyses are carried out on a preparation of fixed depth of about 20um, which allows the spermatozoa to swim freely: Place a standard volume of semen, e.g. 10ul, onto a clean glass slide. Cover it with a coverslip, e.g. 22 mm × 22 mm for 10ul, to provide a chamber approximately 20 um deep. The weight of the coverslip spreads the sample.
- Take care to avoid the formation and trapping of air bubbles between the coverslip and the slide.
- Assess the freshly made wet preparation as soon as the contents are no longer drifting.
Initial Microscopic Examination
Making a wet preparation

- The depth of a preparation ($D, \text{ um}$) is obtained by dividing the volume of the sample ($V, \text{ul} = \text{mm}^3$) by the area over which it is spread ($A, \text{mm}^2$): $D = \frac{V}{A}$. Thus, a volume of 10 ul of semen delivered onto a clean glass slide and covered with a 22 mm × 22 mm coverslip (area 484 mm$^2$) provides a chamber of depth of 20.7 um; a 6.5 ul sample covered with an 18 mm × 18 mm coverslip (area 324 mm$^2$) provides a depth of 20.1 um; an 11 ul sample covered by a 21 mm × 26 mm coverslip (area 546 mm$^2$) provides a depth of 20.1um. Occasionally, a deeper chamber may be required: a 40 ul sample covered by a 24 mm × 50 mm coverslip (area 1200 mm$^2$) provides a depth of 33.3 um.

- A chamber depth of less than 20 um constrains the rotational movement of spermatozoa (Le Lannou et al., 1992; Kraemer et al., 1998).

- If the chamber is too deep, it will be difficult to assess spermatozoa as they move in and out of focus.

- If the number of spermatozoa per visual field varies considerably, the sample is not homogeneous. In such cases, the semen sample should be mixed again thoroughly and a new slide prepared. Lack of homogeneity may also result from abnormal consistency, abnormal liquefaction, aggregation of spermatozoa or sperm agglutination.
Aggregation of Spermatozoa

- The adherence either of immotile spermatozoa to each other or of motile spermatozoa to mucus strands, non-sperm cells or debris is considered to be nonspecific aggregation (Fig. 2.2) and should be recorded as such.

Fig. 2.2 Non-specific aggregation of spermatozoa in semen

Views of spermatozoa aggregated with an epithelial cell (a), debris (b) or spermatozoa (c, d).

Micrographs courtesy of C Brazil.
Agglutination of spermatozoa

- Agglutination specifically refers to motile spermatozoa sticking to each other, head-to-head, tail-to-tail or in a mixed way.
- The motility is often vigorous with a frantic shaking motion, but sometimes the spermatozoa are so agglutinated that their motion is limited. Any motile spermatozoa that stick to each other by their heads, tails or mid pieces should be noted.
- The major type of agglutination (reflecting the degree (grades 1–4) and the site of attachment (grades A–E) should be recorded (Rose et al., 1976):
  - grade 1: isolated <10 spermatozoa per agglutinate, many free spermatozoa
  - grade 2: moderate 10–50 spermatozoa per agglutinate, free spermatozoa
  - grade 3: large agglutinates of >50 spermatozoa, some spermatozoa still free
  - grade 4: gross all spermatozoa agglutinated and agglutinates interconnected
- Motile spermatozoa stuck to cells or debris or immotile spermatozoa stuck to each other (aggregation) should not be scored as agglutination.
- The presence of agglutination is not sufficient evidence to deduce an immunological cause of infertility, but is suggestive of the presence of anti-sperm antibodies; further testing is required.
- Severe agglutination can affect the assessment of sperm motility and concentration.
**Fig. 2.3** Schematic diagram of different extents of sperm agglutination

<table>
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<tr>
<th>Degree of agglutination</th>
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<tbody>
<tr>
<td>1. Isolated (&lt;10 sperm/agglutinate, many free sperm)</td>
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<tr>
<td>2. Moderate (10–50 sperm/agglutinate, free sperm)</td>
</tr>
<tr>
<td>3. Large (agglutinates &gt;50 sperm, some sperm still free)</td>
</tr>
<tr>
<td>4. Gross (all sperm agglutinated, and agglutinates interconnected)</td>
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</tbody>
</table>

A. Head-to-head

B. Tail-to-tail (heads are seen to be free and move clear of agglutinates)

C. Tail-tip-to-tail-tip

D. Mixed (clear head-to-head and tail-to-tail agglutinations)

E. Tangle (heads and tails enmeshed. Heads are not clear of agglutinates as they are in tail-to-tail agglutination)

Reproduced from Rose et al. (1976) by permission of Wiley-Blackwell.
Sperm Motility

❖ Motility: Ability of the sperms to move.
❖ The extent of progressive sperm motility is related to pregnancy rates (Jouannet et al., 1988; Larsen et al., 2000; Zinaman et al., 2000).
❖ Sperm motility within semen should be assessed as soon as possible after liquefaction of the sample, preferably at 30 minutes, but in any case within 1 hour, following ejaculation, to limit the deleterious effects of dehydration, pH or changes in temperature on motility.
❖ Categories of sperm movement
  • Progressive motility (PR): spermatozoa moving actively, either linearly or in a large circle, regardless of speed.
  • Non-progressive motility (NP): all other patterns of motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed.
  • Immotility (IM): no movement.
❖ When discussing sperm motility, it is important to specify total motility (PR + NP) or progressive motility (PR).
❖ Lower reference limit
  The lower reference limit for total motility (PR + NP) is 40% (5th centile, 95% CI 38–42).
  The lower reference limit for progressive motility (PR) is 32% (5th centile, 95% CI 31–34).
Preparing and assessing a sample for Motility

- Mix the semen sample well.
- Remove an aliquot of semen immediately after mixing, allowing no time for the spermatozoa to settle out of suspension.
- Remix the semen sample before removing a replicate aliquot.
- For each replicate, prepare a wet preparation approximately 20 um deep.
- Wait for the sample to stop drifting (within 60 seconds).
- Examine the slide with phase-contrast optics at ×200 or ×400 magnification.
- Look for spermatozoa in an area at least 5 mm from the edge of the coverslip, to prevent observation of effects of drying on motility.
Preparing and assessing a sample for Motility

- The procedure may be performed at room temperature or at 37 °C with a heated microscope stage, but should be standardized for each laboratory. If sperm motility is to be assessed at 37 °C, the sample should be incubated at this temperature and the preparation made with prewarmed slides and coverslips.

- Assess the motility of all spermatozoa within a defined area of the field. This is most easily achieved by using an eyepiece reticle. Select the portion of the field or grid to be scored from the sperm concentration, i.e. score only the top row of the grid if the sperm concentration is high; score the entire grid if the sperm concentration is low.

- Assess progressive motility first, then non-progressive motility and immotility. Limiting the area, and thus the number of spermatozoa assessed, ensures that several areas of the preparation are examined for motility.

- Evaluate at least 200 spermatozoa in a total of at least five fields in each replicate, in order to achieve an acceptably low sampling error.
Preparing and assessing a sample for Motility

❖ Assess only intact spermatozoa (defined as having a head and a tail), since only intact spermatozoa are counted for sperm concentration.
❖ Do not count motile pinheads.

Note-
❖ If spermatozoa are being scored in two stages (i.e. PR first, followed by NP and IM from the same area) and a count of 200 spermatozoa is achieved before all motility categories from that area have been scored, counting must continue beyond 200 spermatozoa until all categories have been counted, in order to avoid bias towards the motility category scored first.
❖ It is common to overestimate sperm motility, but this can often be avoided by reversing the order of analysis (NP and IM first), using an eyepiece reticle, and being aware of, and avoiding, to the extent possible, potential sources of bias. On rare occasions, with inhomogeneous samples, even a third set of replicates may provide unacceptable differences. In this case, calculate the mean of all replicates and note this in the report.
❖ The total number of progressively motile spermatozoa in the ejaculate is of biological significance. This is obtained by multiplying the total number of spermatozoa in the ejaculate (see Section 2.8.7) by the percentage of progressively motile cells.
Fig. 2.4 Aids to assessing sperm motility

(a) An eyepiece reticle makes it easier to count motile and immotile spermatozoa. (b) Systematic selection of fields for assessment of sperm motility, at least 5 mm from the edges of the coverslip.
Habitual factors affecting sperm density / motility

- High intake of soya – decrease sperm density
- High consumption of tobacco – decrease sperm density / motility
- Consumption of cocaine / Marijuana – decrease sperm motility
- Vaginal lubricants – decrease sperm motility
- Alcoholism – affects all semen parameters.
Semen Vitality

- Sperm vitality is estimated by assessing the membrane integrity of the cells
- It may be determined routinely on all samples, but is especially important for samples with less than about 40% progressively motile spermatozoa.
- The percentage of live spermatozoa is assessed by identifying those with an intact cell membrane, from dye exclusion or by hypotonic swelling.
- Sperm vitality should be assessed as soon as possible after liquefaction of the semen sample, preferably at 30 minutes, but in any case within 1 hour of ejaculation, to prevent observation of deleterious effects of dehydration or of changes in temperature on vitality.
- *Lower reference limit:* The lower reference limit for vitality (membrane-intact spermatozoa) is 58% (5th centile, 95% CI 55–63).
Semen Vitality

– It is clinically important to know whether immotile spermatozoa are alive or dead. Vitality results should be assessed in conjunction with motility results from the same semen sample.

– The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum (Chemes & Rawe, 2003); a high percentage of immotile and non-viable cells (necrozoospermia) may indicate epididymal pathology (Wilton et al., 1988; Correa-Perez et al., 2004).
Methods of Vitality testing
1- Dye exclusion 2- Hypo osmotic Swelling

1- Dye exclusion- using eosin- nigosine

Most commonly used

Principle- damaged plasma membranes, such as those found in non-vital (dead) cells, allow entry of membrane-impermeant stains.

This one-step staining technique uses nigrosin to increase the contrast between the background and the sperm heads, which makes them easier to discern. It also permits slides to be stored for re-evaluation and quality-control purposes.

2. The hypo-osmotic swelling test (HOS)

- Alternative method
- Principle: only cells with intact membranes (live cells) will swell in hypotonic solutions
Vitality test using eosin–nigrosin

This one-step staining technique uses nigrosin to increase the contrast between the background and the sperm heads, which makes them easier to discern. It also permits slides to be stored for re-evaluation and quality-control purposes.

Procedure

1. Mix the semen sample well.
2. Remove a 50-l aliquot of semen and mix with an equal volume of eosin–nigrosin suspension, e.g. in a porcelain spot plate well or test-tube, and wait for 30 seconds.
3. Remix the semen sample before removing a replicate aliquot and mixing with eosin–nigrosin and treating as in step 2 above.
4. For each suspension make a smear on a glass slide and allow it to dry in air.
5. Examine immediately after drying, or later after mounting with a permanent non-aqueous mounting medium.
6. Examine each slide with brightfield optics at ×1000 magnification and oil immersion.
7. Tally the number of stained (dead) or unstained (vital) cells with the aid of a laboratory counter.
Vitality test using eosin–nigrosin

- 8. Evaluate 200 spermatozoa in each replicate, in order to achieve an acceptably low sampling error.
- 9. Calculate the average and difference of the two percentages of vital cells from the replicate slides.
- **Scoring**
  - 1. The nigrosin provides a dark background that makes it easier to discern faintly stained spermatozoa.
  - 2. With brightfield optics, live spermatozoa have white heads and dead spermatozoa have heads that are stained red or dark pink. Spermatozoa with a faint pink head are assessed as alive.
  - 3. If the stain is limited to only a part of the neck region, and the rest of the head area is unstained, this is considered a “leaky neck membrane”, not a sign of cell death and total membrane disintegration. These cells should be assessed as alive.
Fig. 2.5 Eosin–nigrosin smear observed in brightfield optics

Spermatozoa with red (D1) or dark pink (D2) heads are considered dead (membrane-damaged), whereas spermatozoa with white heads (L) or light pink heads are considered alive (membrane-intact).
Vitality test using hypo-osmotic swelling

- As an alternative to dye exclusion, the hypo-osmotic swelling (HOS) test may be used to assess vitality.
- This is useful when staining of spermatozoa must be avoided, e.g. when choosing spermatozoa for ICSI. Spermatozoa with intact membranes swell within 5 minutes in hypo-osmotic medium and all flagellar shapes are stabilized by 30 minutes.

Procedure
- 1. Thaw the frozen swelling solution and mix well before use.
- 2. Warm 1 ml of swelling solution or 1 ml of 1 + 1 (1:2) diluted medium in a closed microcentrifuge tube at 37 °C for 5 minutes.
- 3. Mix the semen sample well.
- 4. Remove a 100-l aliquot of semen and add to the swelling solution. Mix gently by drawing it in and out of the pipette.
- 5. Incubate at 37 °C for exactly 5 minutes or 30 minutes, then transfer a 10-l aliquot to a clean slide and cover with a 22 mm × 22 mm coverslip.
Vitality test using eosin–nigrosin

6. Remix the semen sample, remove a replicate aliquot, mix with swelling solution, incubate and prepare a replicate slide, as above.
7. Examine each slide with phase-contrast optics at ×200 or ×400 magnification.
8. Tally the number of unswollen (dead) and swollen (vital) cells with the aid of a laboratory counter.
9. Evaluate 200 spermatozoa in each replicate, in order to achieve an acceptably low sampling error.
Calculate the average and difference of the two percentages of vital cells from the replicate preparations.

**Scoring**
1. Swollen spermatozoa are identified by changes in the shape of the cell, as indicated by coiling of the tail.
2. Live cells are distinguished by evidence of swelling of the sperm tail; score all forms of swollen tails as live spermatozoa.
Schematic representation of typical morphological changes in human spermatozoa subjected to hypo-osmotic stress

(a) No change. (b)–(g) Various types of tail changes. Swelling in tail is indicated by the grey area.
Semen sample → Hypo-osmotic test

- Live sperms (curled tail)
- Dead sperms (straight tail)
Sperm Numbers

❖ The terms “total sperm number” and “sperm concentration” are not synonymous.
❖ Sperm concentration refers to the number of spermatozoa per unit volume of semen and is a function of the number of spermatozoa emitted and the volume of fluid diluting them.
❖ Total sperm number refers to the total number of spermatozoa in the entire ejaculate and is obtained by multiplying the sperm concentration by the semen volume.
❖ Normal Sperm Concentration ≥ 15 x 10⁶/ml
❖ Normal Sperm numbers ≥ 39 x 10⁶
Sperm Numbers

Determination of sperm number comprises the following steps:

- Examining a well-mixed, undiluted preparation of liquefied semen on a glass slide under a coverslip, to determine the appropriate dilution and appropriate chambers to use. This is usually the wet preparation used for evaluation of motility.
- Mixing semen and preparing dilutions with fixative.
- Loading the haemocytometer chamber and allowing spermatozoa to settle in a humid chamber.
- Assessing the samples within 10–15 minutes (after which evaporation has noticeable effects on sperm position within the chamber).
- Counting at least 200 spermatozoa per replicate.
- Comparing replicate counts to see if they are acceptably close. If so, proceeding with calculations; if not, preparing new dilutions.
- Calculating the concentration in spermatozoa per ml.
- Calculating the total number of spermatozoa per ejaculate.
Counting chamber

- 100 µm deep haemocytometer chamber recommended
- Most commonly used – Improved Neubauer chamber
- Disposable chambers also available
- Shallow chambers not recommended
- Improved Neubauer chamber – consists 9 grids
- Each grid hold 100 nL
- Cover slip to be used – thickness no 4, 0.44 mm

- After cleaning, soak reusable chambers and coverslips overnight in disinfectant to avoid contamination with potentially infectious agents in semen.
1. Wet mount examination

<table>
<thead>
<tr>
<th>Spermatozoa per ×400 field</th>
<th>Spermatozoa per ×200 field</th>
<th>Dilution required</th>
<th>Semen (µl)</th>
<th>Fixative (µl)</th>
<th>Chamber</th>
<th>Area to be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;101</td>
<td>&gt;404</td>
<td>1:20 (1 + 19)</td>
<td>50</td>
<td>950</td>
<td>Improved Neubauer</td>
<td>Grids 5, 4, 6</td>
</tr>
<tr>
<td>16–100</td>
<td>64–400</td>
<td>1:5 (1 + 4)</td>
<td>50</td>
<td>200</td>
<td>Improved Neubauer</td>
<td>Grids 5, 4, 6</td>
</tr>
<tr>
<td>2–15</td>
<td>8–60</td>
<td>1:2 (1 + 1)</td>
<td>50</td>
<td>50</td>
<td>Improved Neubauer</td>
<td>Grids 5, 4, 6</td>
</tr>
<tr>
<td>&lt;2</td>
<td>&lt;8</td>
<td>1:2 (1 + 1)</td>
<td>50</td>
<td>50</td>
<td>Improved Neubauer or large-volume</td>
<td>All 9 grids or Entire slide</td>
</tr>
</tbody>
</table>

- If a 1 + 19 (1:20) dilution is inadequate, use 1 + 49 (1:50)
2 & 3. Preparing dilutions & Loading the haemocytometer chamber

❖ Fixative for diluting semen—**SODIUM BICARBONATE FORMALIN DILUTING FLUID**
  • Dissolve 50 g of sodium bicarbonate (NaHCO₃) and 10 ml of 35% (v/v) formalin in 1000 ml of purified water.
  • Store at 4°C
❖ Appropriate dilutions made in 2 test tubes
❖ Neubauer chamber loaded with both dilutions
❖ Kept for 3-4 minutes
4&5. Assessing sperm numbers in the counting chambers and counting at least 200 spermatozoa

- Count at least 200 spermatozoa in each replicate, for low sampling error
- First assess the central grid (number 5) of one side of the improved Neubauer chamber, row by row
- Continue counting until at least 200 spermatozoa have been observed and a complete row (of five large squares) has been examined.
- Counting must be done by complete rows; do not stop in the middle of a row.
- If 200 spermatozoa are not observed in the five rows of the central grid, continue counting in the rows (of 4 large squares) of the two adjacent grids
Make a note of the number of rows assessed to reach at least 200 spermatozoa

Switch to the second chamber of the haemocytometer
The same number of rows will be counted from the other chamber of the haemocytometer (the same volume) as the first replicate, even if this yields fewer than 200 spermatozoa.

If fewer than 200 spermatozoa are found in grids 4, 5 and 6, do not continue to count in grids 1, 2, 3, 7, 8 or 9, since the volume of each row in these grids differs from that of the rows in grids 4, 5 and 6

Calculate the sum and difference of the two numbers.

Determine the acceptability of the difference
If the difference is acceptable, calculate the concentration
6. Comparing replicate counts

<table>
<thead>
<tr>
<th>Sum</th>
<th>Acceptable Difference*</th>
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</thead>
<tbody>
<tr>
<td>144–156</td>
<td>24</td>
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<tr>
<td>157–169</td>
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<td>170–182</td>
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<td>539–562</td>
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<tr>
<td>563–587</td>
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</table>
Calculation of the concentration of spermatozoa in semen & total number of spermatozoa per ejaculate

\[
\text{Spermatozoa concentration} = \frac{\text{total number of sperms counted in both replicates}}{\text{volume in which they are found}} \times \text{dilution factor}
\]

- Total number of spermatozoa per ejaculate = sperm concentration \times volume of whole ejaculate
- Each grid = 100nL
- Grid 4,5 & 6 contain 5 rows. So, each row contains = 100/5 = 20 nL
- Grid 1,2,3,7,8 & 9 contain 4 rows each, so each row = 25 nL
- In 1:20 and 1:5 dilution, only grids 4,5, & 6 counted, that too row wise because each row have same volume
- In 1:2 dilution, all grids are assessed.
Calculation of sperm concentration for grid 4, 5, 6:

- Spermatozoa concentration (per mL) = \( \frac{\text{total number of sperms counted in both replicates}}{\text{volume in which they are found}} \) x dilution factor

  - i.e., \( SC = \frac{\text{total number of sperms counted in both replicates} \times (N)}{\text{number of rows} \times (n) \times 20} \times \text{dilution factor} \)

- i.e., \( C = \frac{(N/n) \times (1/20)}{\times \text{dilution factor}} \)

Example 1.: With a 1:20 dilution, replicate 1 is found to contain 201 spermatozoa in seven rows, while replicate 2 contains 245 spermatozoa in seven rows.

- \( \text{SUM} = (201 + 245) \text{ is 446 in 14 rows} \)
- \( \text{difference} (245–201) \text{ is 44} \).
- \( \text{See the acceptable difference in table = 41} \)
- \( \text{so new replicate dilutions are made} \)
Example 2: With a 1:20 dilution, replicate 1 is found to contain 220 spermatozoa in four rows, while replicate 2 contains 218 spermatozoa in four rows. The volume of whole ejaculate is 3ml.

- Sum = (220 + 218) = 438 in eight rows
- Difference (220 – 218) is 2.
- See table
- Values are accepted.
- \( C(\text{per nL}) = \frac{N}{n} \times \frac{1}{20} \times \text{dilution factor} \)
- \( C = \left(\frac{438}{8}\right) \times \left(\frac{1}{20}\right) \times 20 \)
  = 54.75 spermatozoa/nl
  = 55 \times 10^6 \text{spermatozoa per ml of semen}
- Total sperm count = 55 \times 10^6 \times 3
  = 165 \times 10^6 \text{spermatozoa /ejaculate}
Example 3:

With (1:20) dilution, replicate 1 is found to contain 98 spermatozoa in 15 rows (grids 5, 4 and 6), while replicate 2 contains 114 spermatozoa in 15 rows (grids 5, 4 and 6). Volume of ejaculate 2.5ml

- Sum (98 + 114) = 212 in 30 rows
- Difference (114–98) is 16
- See table
- The values are accepted.
- \[ C = \frac{(212/30) \times 1/20 \times 20}{1/1} = 7.07 \text{spermatozoa/nl} \]
  \[ = 7.1 \times 10^6 \text{spermatozoa per ml of semen} \]

- Total sperm number = 7.1 x 2.5 x 10^6 spermatozoa per ejaculate
  \[ = 18 \times 10^6 \text{spermatozoa per ejaculate} \]

Note: In this example, the sample has been overdiluted, since fewer than 200 spermatozoa were found in grids 5, 4 and 6; a 1+4 (1:5) dilution would have been more appropriate.
Example 4: With a 1:5 dilution, replicate 1 is found to contain 224 spermatozoa in four rows, while replicate 2 contains 268 spermatozoa in four rows.

- The sum = (224 + 268) = 492 in eight rows
- Difference (268–224) is 44.
- From Table, this is seen to exceed the difference expected by chance alone (43), so new replicate dilutions are made.

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<td>563–587</td>
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</tbody>
</table>
Example 5: With a 1:5 dilution, replicate 1 is found to contain 224 spermatozoa in eight rows, while replicate 2 contains 213 spermatozoa in eight rows. Volume of ejaculate 3ml

- Sum (224 + 213) = 437 in 16 rows
- Difference (224–213) is 11.
- From Table this is seen to be less than that found by chance alone (41), so the values are accepted.
- \[ C = \left(\frac{437}{16}\right) \times \frac{1}{20} \times 5 = 6.825 \text{ spermatozoa/nl, or } 6.8 \times 10^6 \text{ spermatozoa per ml of semen} \]
- Total sperm count = \(6.8 \times 3 \times 10^6\) spermatozoa per ejaculate
  \[ = 20.4 \times 10^6 \text{ spermatozoa per ejaculate} \]
Calculation of sperm concentration for low sperm counts - all grids; 1:2 dilution

- Spermatozoa concentration = \( \frac{\text{total number of sperms counted in both replicates}}{\text{volume in which they are found}} \times \text{dilution factor} \)

- i.e, \( SC = \frac{N}{n} \times \text{dilution factor} \)

- i.e, \( C = \frac{N}{n} \times \frac{1}{100} \times 2 \)

- i.e, \( C = \frac{N}{n} \times \frac{1}{50} \) spermatozoa/nl.
When no spermatozoa are observed in either wet preparation, the sample can be centrifuged to determine if any spermatozoa are present in a larger sample.

- Remove a 1ml aliquot of semen and centrifuge at 3000g for 15 minutes.
- Decant most of the supernatant and resuspend the sperm pellet in the remaining approximately 50 µl of seminal plasma.
- Place one 10 µl aliquot of the pellet on each of two slides and create two wet preparations.

The presence of spermatozoa in either replicate indicates **cryptozoospermia**.

The absence of spermatozoa from both replicates suggests **azoospermia**.
<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspermia</td>
<td>no semen (no or retrograde ejaculation)</td>
</tr>
<tr>
<td>asthenozoospermia</td>
<td>percentage of progressively motile (PR) spermatozoa below the lower reference limit</td>
</tr>
<tr>
<td>asthenoteratozoospermia</td>
<td>percentages of both progressively motile (PR) and morphologically normal spermatozoa below the lower reference limits</td>
</tr>
<tr>
<td>azoospermia</td>
<td>no spermatozoa in the ejaculate (given as the limit of quantification for the assessment method employed)</td>
</tr>
<tr>
<td>cryptozoospermia</td>
<td>spermatozoa absent from fresh preparations but observed in a centrifuged pellet</td>
</tr>
<tr>
<td>haemospermia (haematospermia)</td>
<td>presence of erythrocytes in the ejaculate</td>
</tr>
<tr>
<td>leukospermia (leukocyto-spermia, pyospermia)</td>
<td>presence of leukocytes in the ejaculate above the threshold value</td>
</tr>
<tr>
<td>necrozoospermia</td>
<td>low percentage of live, and high percentage of immotile, spermatozoa in the ejaculate</td>
</tr>
<tr>
<td>normozoospermia</td>
<td>total number (or concentration, depending on outcome reported)* of spermatozoa, and percentages of progressively motile (PR) and morphologically normal spermatozoa, equal to or above the lower reference limits</td>
</tr>
<tr>
<td>oligoasthenozoospermia</td>
<td>total number (or concentration, depending on outcome reported)* of spermatozoa, and percentage of progressively motile (PR) spermatozoa, below the lower reference limits</td>
</tr>
<tr>
<td>oligoasthenoteratozoospermia</td>
<td>total number (or concentration, depending on outcome reported)* of spermatozoa, and percentages of both progressively motile (PR) and morphologically normal spermatozoa, below the lower reference limits</td>
</tr>
<tr>
<td>oligoteratozoospermia</td>
<td>total number (or concentration, depending on outcome reported)* of spermatozoa, and percentage of morphologically normal spermatozoa, below the lower reference limits</td>
</tr>
<tr>
<td>oligozoospermia</td>
<td>total number (or concentration, depending on outcome reported)* of spermatozoa below the lower reference limit</td>
</tr>
<tr>
<td>teratozoospermia</td>
<td>percentage of morphologically normal spermatozoa below the lower reference limit</td>
</tr>
</tbody>
</table>

*Preference should always be given to total number, as this parameter takes precedence over concentration.

Note: The suffix “sperma” refers to the ejaculate and “zoospermia” to the spermatozoa. Thus, the following terms should not be used: asthenospermia, asthenoteratozoospermia, cryptozoospermia, oligoasthenozoospermia, oligoteratozoospermia, oligospermia, teratospermia.
Sperm Morphology
Normal morphology of spermatozoa

- **Head**: consists of nucleus with condensed chromatin and some nuclear vacuoles.

- **Acrosome**: anterior 2/3rd of the head shows an acrosome cap,
  - 40-70% of head
  - secretes enzymes that dissolve the cells of corona radiata and zona pellucida of the ovum during fertilization.

- **Middle piece** contains mitochondria → provides energy.

- **The tail** used for motility.
Fig. 2.10 Morphologically “normal” spermatozoa

(a, b) Shorr-stained spermatozoa recovered from the zona pellucida in vitro. (c) Papanicolaou-stained spermatozoa recovered from endocervical mucus after intercourse. Very few defects on the sperm head, midpiece or principal piece are observed. Tails may be curved but not sharply angulated.

(a, b) Reproduced from Liu et al. (2003) by permission of the European Society of Human Reproduction and Embryology. (c) Reproduced from Menkveld & Kruger (1990) by permission.
Steps of Determination of Sperm Morphology

- Determination of sperm morphology comprises the following steps:
- Preparing a smear of semen on a slide.
- Air-drying, fixing and staining the slide.
- Mounting the slide with a coverslip if the slide is to be kept for a long time.
- Examining the slide with bright field optics at ×1000 magnification with oil immersion.
- Assessing approximately 200 spermatozoa per replicate for the percentage of normal forms or of normal and abnormal forms.
- Comparing replicate values to see if they are acceptably close: if so, proceeding with calculations; if not, re-reading the slides.
Preparation of semen smears

**Normal semen sample**

- Undiluted semen
- Feathering method - 10µL + 45°
  - angle + 1 second
- Low sperm concentration:
  - Centrifuge – 600rpm x 10mins

**Viscous semen sample**

- Washed semen sample (0.2-0.5ml semen + 10ml saline → centrifuge at 800rpm x 10mins)
- Pipette method by Pasteur pipette
- Sperm morphology affected
Staining

- Air dried — 4 hours in pap and shorr stain
- Fixation — ethanol / methanol
- Recommended stains: Papanicolaou, Shorr, Diff-Quik stain (rapid method)
- Mounted
- Examine the morphology of at least 200 sperms
- **Normal** > 4% of sperm should have normal morphology.
Interpretation

- Head - pale blue in acrosomal region and dark blue in the post-acrosomal region.
- Midpiece - red
- Tail - blue or reddish
- Excess residual cytoplasm, usually located behind the head and around the midpiece
  - stained pink or red (Papanicolaou stain)
  - reddish-orange (Shorr stain)
Classification of normal sperm morphology

- Spermatozoa consist of a head, neck, middle piece (midpiece), principal piece and endpiece. As the endpiece is difficult to see with a light microscope, the cell can be considered to comprise a head (and neck) and tail (midpiece and principal piece).

- For a spermatozoon to be considered normal, both its head and tail must be normal.

- All borderline forms should be considered abnormal.

- The head should be smooth, regularly contoured and generally oval in shape. There should be a well-defined acrosomal region comprising 40–70% of the head area (Menkveld et al., 2001). The acrosomal region should contain no large vacuoles, and not more than two small vacuoles, which should not occupy more than 20% of the sperm head. The post-acrosomal region should not contain any vacuoles.
Classification of normal sperm morphology

- The midpiece should be slender, regular and about the same length as the sperm head. The major axis of the midpiece should be aligned with the major axis of the sperm head. Residual cytoplasm is considered an anomaly only when in excess, i.e. when it exceeds one third of the sperm head size (Mortimer & Menkveld, 2001).

- The principal piece should have a uniform calibre along its length, be thinner than the midpiece, and be approximately 45 m long (about 10 times the head length). It may be looped back on itself, provided there is no sharp angle indicative of a flagellar break.
Classification of abnormal sperm morphology

- Human semen samples contain spermatozoa with different kinds of malformations.
- Defective spermatogenesis and some epididymal pathologies are commonly associated with an increased percentage of spermatozoa with abnormal shapes.
- The morphological defects are usually mixed. Abnormal spermatozoa generally have a lower fertilizing potential, depending on the types of anomalies, and may also have abnormal DNA.
- The following categories of defects should be noted:
  - Head defects: large or small, tapered, pyriform, round, amorphous, vacuolated (more than two vacuoles or >20% of the head area occupied by unstained vacuolar areas), vacuoles in the post-acrosomal region, small or large acrosomal areas (<40% or >70% of the head area), double heads, or any combination of these.
  - Neck and midpiece defects: asymmetrical insertion of the midpiece into the head, thick or irregular, sharply bent, abnormally thin, or any combination of these.
Classification of abnormal sperm morphology

- Principal piece defects: short, multiple, broken, smooth hairpin bends, sharply angulated bends, of irregular width, coiled, or any combination of these.

- Excess residual cytoplasm (ERC): this is associated with abnormal spermatozoa produced from a defective spermatogenic process. Spermatozoa characterized by large amounts of irregular stained cytoplasm, one third or more of the sperm head size, often associated with defective midpieces (Mortimer & Menkveld, 2001) are abnormal. This abnormal excess cytoplasm should not be called a cytoplasmic droplet (Cooper, 2005).
Fig. 2.13 Schematic drawings of some abnormal forms of human spermatozoa

A. Head defects
(a) Tapered  
(b) Pyriform  
(c) Round  
   - No acrosome  
   - Small  
(d) Amorphous  
(e) Vacuolated  
(f) Small acrosomal area

B. Neck and midpiece defects
(g) Bent neck  
(h) Asymmetrical  
(i) Thick insertion  
(j) Thin

C. Tail defects
(k) Short  
(l) Bent  
(m) Coiled  
(n) > one third head

Adapted from Kruquer et al., 1993 and reproduced by permission of MO Medical.
Immunological Analysis

1. Sperm MAR test – direct, indirect
2. Immunobead test
Biochemical Analysis
Clinically used markers to determine secretory capacity of accessory glands

- Prostate gland function
  - **Zinc**
  - **Citric acid**
  - **Acid phosphatase**
  - **α-glutamyl transpeptidase**

- Seminal vesicles
  - **Fructose**
  - **Prostaglandins**

- Epididymis
  - **Neutral Alphagalactosidase**
  - Free L-carnitine
  - **glycerophosphocholine (GPC)**

Infection causes decrease in the secretion of these markers.
Sperm function tests

1. Post coital (Sims-Huhner test)
2. Cervical mucous penetration test
3. Hamster egg penetration test
4. Hypoosmotic swelling of flagella
5. Computer assisted semen analysis
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lower reference limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen volume (ml)</td>
<td>1.5 (1.4–1.7)</td>
</tr>
<tr>
<td>Total sperm number (10^6 per ejaculate)</td>
<td>39 (33–46)</td>
</tr>
<tr>
<td>Sperm concentration (10^6 per ml)</td>
<td>15 (12–16)</td>
</tr>
<tr>
<td>Total motility (PR + NP, %)</td>
<td>40 (38–42)</td>
</tr>
<tr>
<td>Progressive motility (PR, %)</td>
<td>32 (31–34)</td>
</tr>
<tr>
<td>Vitality (live spermatozoa, %)</td>
<td>58 (55–63)</td>
</tr>
<tr>
<td>Sperm morphology (normal forms, %)</td>
<td>4 (3.0–4.0)</td>
</tr>
</tbody>
</table>

Other consensus threshold values

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>≥7.2</td>
</tr>
<tr>
<td>Peroxidase-positive leukocytes (10^6 per ml)</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>MAR test (motile spermatozoa with bound particles, %)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Immunobead test (motile spermatozoa with bound beads, %)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Seminal zinc (μmol/ejaculate)</td>
<td>≥2.4</td>
</tr>
<tr>
<td>Seminal fructose (μmol/ejaculate)</td>
<td>≥13</td>
</tr>
<tr>
<td>Seminal neutral glucosidase (mU/ejaculate)</td>
<td>≥20</td>
</tr>
</tbody>
</table>
THANK YOU