# Estimates of Sperm Motility

5

N. Srivastava and Megha Pande

**Abstract**

Spermatozoa motility is a very important attribute most commonly exploited for discriminating between a good and a bad semen sample at the fresh as well as post-cryopreservation stage. Accurate measurement of motility provides crucial information about viability of spermatozoa as well. In the chapter on sperm motility, principles governing various motility parameters, protocols and finer practical hints have been provided for the investigator to carry out experimentation accurately. We have provided protocols of estimating sperm motility using CASA for an effective and objective assessment as well. This section includes various advantages and disadvantages of CASA vis-à-vis other motility parameters as well.

**Keywords**

Mass activity • Individual progressive motility • Post thaw • Incubation test • CASA

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## 5.1 Introduction

Motility, the easiest and most certain way of determining the viability, is also the most desirable spermatozoa characteristics to decide the fate of the sample for further processing. It is expressed as either mass activity or individual progressive motility in fresh semen. In post-thaw semen, motility estimates are crucial criteria to discriminate between a good or bad batch of semen.



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Though the estimation of motility appears easy to perform, the procedure selected needs to be meticulously followed to achieve repeatable results. For example, immediate examination of undiluted ejaculates is required to limit the deleterious effects of dehydration, pH or changes in the temperature on motility. On the other hand, diluted semen sample can be examined up to 2h at RT and 24 h at +5 °C. Moreover, an investigator is well advised to remember that the extender may slightly alter the motility specially the velocity parameter. This is because the speed of a sperm cell is never constant and is affected mostly by its physiology and environment. The average speed of a bull sperm varies from 68 to 162 μm/s.

Considering the importance of the motility estimates in spermatology, this chapter provided details of various parameters of sperm motility and their procedures and finer points, crucial for accurate measurements of sperm motility. Because various means of estimating motility suffer from inaccuracies of subjective analysis, this chapter has outlined protocol for evaluating sperm motility by computer-aided sperm analysis (CASA) system as well. However, the nature of automated evaluation means that compensation for preparation defects and artefacts cannot be attempted. Therefore, while using CASA, even small differences in background shading relative to cell staining can result in incorrect classification or an inability to identify the epithelial cell as a spermatozoon, with a consequent bias in the results. It is imperative, therefore, that procedures and instruments must be standardized and quality control maintained to ensure comparable and reliable results.

In addition to various estimates of motility, this chapter also includes a small subsection on microscopy and preparation of wet smear for accurate evaluation. Investigators are also advised to go through the subsection 'Points to Ponder' meticulously to minimize subjective error. A comparative evaluation of advantages of motility estimates as compared to CASA has been included in the relevant section of the chapter.

## 5.2 Microscopy for Motility Evaluation

A phase-contrast microscope is necessary for accurate evaluation of semen because the refractive index of spermatozoa differs only slightly from that of the surrounding medium. A biotherm to keep the microscope stages at 36–38 °C is an essential part of the microscope.

**Points to Ponder**

(a) Always operate the microscope and its accessories through a voltage stabilizer.
(b) Keep the lenses of the microscope clean by wiping through a lens-cleaning paper.
(c) Xylol can be used to remove greasy materials; however, excess of xylol should not be applied.

Glass slides of 1.2 mm thickness are suitable for the most of the phase-contrast microscope. Thoroughly wash and rinse in distilled water and dry well to remove all greasy materials from glass slides. Coverslips of 0.18 mm thickness either round or square can be used.

## 5.3 Preparing a Wet Smear for Motility Estimates

Preparation of a good wet smear is of utmost importance for arriving at an accurate result.

(a) Gently mix the semen sample well.
(b) Remove an aliquot of semen immediately after mixing.
(c) Always remix the semen sample before removing duplicate aliquots.
(d) Place a standard volume of sample (10 μL) onto a clean grease-free glass slide.
(e) Cover the sample with a coverslip: the weight of the coverslip spreads the sample.
(f) Assess the freshly made wet preparation as soon as the sperm drifting stops.

**Points to Ponder**

(a) It is important to remove aliquot of semen sample immediately after mixing, as this allows sample preparation before the spermatozoa settle out of suspension.
(b) Standardize dimension of the coverslip with the volume of sample. This allows analyses on a preparation of fixed depth of about 20 μm and allows the spermatozoa to swim freely.
(c) For 10 μL sample, a coverslip of 22 × 22 mm provides a chamber approximately 20 μm deep.
(d) To avoid observing sperm on borders (with effects of drying on motility), always look for spermatozoa in an area at least 5 mm from the edge of the coverslip.
(e) Many a times, semen samples are too thick for coverslip, mostly with round ones, to spread it evenly. Press coverslip lightly to achieve uniform spread.
(f) A chamber depth of less than 20 μm constrains the rotational movement of spermatozoa [1, 2].
(g) Too deep a chamber makes sperm evaluation difficult as they move in and out of focus. In such samples, flipping movements of sperm cells are observed.
(h) Avoid the formation and trapping of air bubbles between the coverslip and the slide.
(i) When the semen sample is not homogenous, the number of sperm cells per visual field will vary considerably. Mix the semen sample again and prepare a new slide.
(j) Lack of homogeneity may also result from abnormal consistency, aggregation of spermatozoa or sperm agglutination.
(k) Systematically scan the slide, either in a horizontal or vertical manner of field selection, to avoid repeatedly viewing the same area.
(l) Always develop a reasonable speed of counting of sperm to avoid overestimating the number of motile spermatozoa.
(m) Count only intact spermatozoa (defined as having a head and a tail). Do not count motile pinheads.

**Depth of a Wet Preparation**

The depth (D, μm) of a prepared sample is obtained by dividing the volume of the sample (V, μL, $mm^3$) by the area over which it covers (A, μm, $mm^2$): D= V/A. While preparing for microscopic examination, a sample volume of 10 μL, covered with coverslips of 22 × 22 mm (484 $mm^2$), 18 × 18 mm (324 $mm^2$) and 20 × 25 mm (500 $mm^2$), provides a chamber depth of 20.7, 20.1 and 20.0 μm, respectively.

## 5.4 Estimates of Motility

### 5.4.1 Mass Activity

The motility of spermatozoa at the time of collection of semen as seen under low-power microscope without coverslip is termed variously as mass/gross activity or initial motility of spermatozoa. It is considered as a significant predictor of fertilizing ability of spermatozoa [3]. The mass activity is observed as 'a mass of swirling wave like motion produced by live progressively motile spermatozoa'. When semen is fresh, there is continuous movement of dark streaks and swirls, which appear and disappear extremely rapidly [4]. These eddy and waves are due to rapid movement of viable spermatozoa, which helps in initial grading of ejaculates (Table 5.1).

**Table 5.1** Initial grading of semen depending on mass activity

| Grade | Semen characteristics |
|---|---|
| +5 | 80–100% cells showing progressive movements with rapid waves, individual sperms not seen |
| +4 | 60–80% cells showing progressive movements with eddies towards extremities, individual sperms not seen |
| +3 | 40–60% cells showing progressive movements with slow waves, individual sperms can be seen |
| +2 | 20–40% cells showing progressive movements but no waves |
| +1 | 10–20% cells showing progressive movements but no waves |
| 0 | No motility at all |

**Materials**

Freshly collected ejaculate, microscope with thermal stage, slide

**Procedure**

(a) Take a clean grease-free slide and mount on a microscope with thermostat at 37 °C.
(b) Place a drop of freshly collected neat semen on the slide.
(c) Observe under 10× without coverslip.
(d) Another method is 'hanging drop method'.
(e) Take a micro-slide of 20 mm diameter and 0.5 mm depth.
(f) Place a small drop of semen in the cavity of micro-slide and observe as above.

The minimum requirement for a good ejaculate for further processing is a mass activity of +3 or above.

**Points to Ponder**

(a) Always use prewarmed slides for examination of mass activity.
(b) Switch on the microscope and stage warmer at least 10 min before for the stage to equilibrate a constant required temperature of 37 °C.
(c) The estimates of mass activity are not very precise.
(d) Some percentage of spermatozoa which are weakly motile may be exaggerated under the influence of actively motile sperm.

### 5.4.2 Estimates of Sperm Motility by Direct Count

The most prominent behaviour of the spermatozoa is their unrelenting motility. Motility is the most frequently and easily observable activity of the spermatozoa before further grading of sample. A progressively motile spermatozoon is a sperm cell:

(a) That moves across the microscopic field reasonably rapidly.
(b) With each back and forth lash of the tail, the head must rotate 360°.

**Table 5.2** Types of spermatozoa motility in diluted semen (1:1) at 20–40×

| Motility attribute | Description |
|---|---|
| Progressive | Sperm travelling straightforward head first |
| Circular | Movement narrowed to radius approximate length of sperm |
| Oscillatory | Side-to-side motion in static position, associated with aged semen |
| Reverse | Sperm in backward motion |

In diluted semen, general pattern of sperm motility appears in a long semi-arc manner.

In fresh semen, individual progressive motility (IPM) of 70% and above is only selected for further processing and freezing (Table 5.2).

**Procedure**

(a) Dilute the fresh ejaculate in the collection tube with the extender (1:1) kept at 37 °C in a water bath.
(b) Take an empty straw, cut at both ends, and dip in the diluted sample.
(c) Take a small glass tube kept at 37 °C in water bath and wipe away water.
(d) Transfer contents of the straw (i.e. sample) in above glass tube while holding the tube firmly in the fist to maintain temperature.
(e) Place a small drop of diluted semen on a clean grease-free warm slide.
(f) Gently place a coverslip over the semen drop.
(g) Assess the motility by examining the best possible field in phase-contrast (PC) microscope under 20×.
(h) Follow laboratory protocol to discriminate between freezable or non-freezable ejaculate.

**Observation**

(a) To evaluate individual progressive motility, observe first if more than half of the spermatozoa within the field of vision are in forward propulsion or not, and then turn attention to the other half and estimate its proportion to total (x) (see 'Background Information').

(b) Motility is rated from 0 to 100% in blocks of 10.
(c) Motility percentage indicates corresponding number of progressively motile spermatozoa (excluding circular, oscillatory, reverse and nonmotile sperms).

**Points to Ponder**

(a) Observe individual progressive motility of spermatozoa immediately after completion of basic semen evaluation of sample.
(b) As this test requires screening individual cells, hence the slide prepared should consist of a single layer of spermatozoa viewed through 20–40×.
(c) Circular or reverse motion indicates effect of cold shock or non-isotonic solution.
(d) Reflection of light by sperm indicates excessive fluid between slide and coverslip. This appears as flipping of spermatozoa.
(e) When sperm cells appear to move in a two-dimensional pattern, it indicates less fluid between slide and coverslip.
(f) Avoid the formation and trapping of air bubbles between the coverslip and the slide. Achieve this by gently placing the coverslip in a slating manner and pressing it lightly using the index finger.

### 5.4.3 Estimates of Sperm Motility Using Haemocytometer

**Principle**

Individual progressive motility is an important indicator of fertilizing ability of spermatozoa. In addition to direct visual count, evaluation of individual progressive motility using haemocytometer confers advantage of differentiating between progressively motile and weakly motile spermatozoa. This is a two-step procedure: in the first step, a haemocytometer is used to count weakly and nonmotile spermatozoa; in the next step, second haemocytometer is charged with same semen sample, followed by killing of cells by freezing and finally counting of the total number of spermatozoa. Difference of value between these two provides a fair estimate of total progressively motile spermatozoa, expressed as per cent.

**Materials**

Semen, diluting fluid, test tubes, Neubauer haemocytometer (NH), microscope, pipettes, refrigerator

**Formal Saline**

100 μL formalin.
Dilute to 10 mL normal saline.

**Diluting Fluid**

0.5 g eosin.
1 mL formal saline.
Dilute to 100 mL DW.

**Procedure**

(a) Take two haemocytometers and charge them with test semen sample.
(b) Place one haemocytometer (marked A) in refrigerator chamber of the fridge for 1h.
(c) After 1 h calculate total sperm concentration from first haemocytometer (A).
(d) Calculate only weakly and nonmotile sperm from second haemocytometer (B).
(e) Follow the procedure for loading RBC chamber and sperm count as described in chapter on concentration estimation.

**Calculation**

$$\mathrm{IPM}(\%) = \frac{A - B}{A} \times 100$$

where

IPM = individual progressive motility in per cent
$A$ = total concentration of spermatozoa
$B$ = total number of weakly and nonmotile spermatozoa

**Points to Ponder**

(a) During the time haemocytometer 'A' is kept in refrigerator, count the total number of weakly motile and nonmotile spermatozoa from haemocytometer 'B'.
(b) While placing the haemocytometer, ensure that the coverslip is not disturbed in any way.
(c) Examine different fields to calculate as many weakly and nonmotile spermatozoa as possible.
(d) Other methods of killing sperm are heating at 50 °C for 5 min or using 1% chlorazine.

### 5.4.4 Post-thaw Motility

The motility of spermatozoa in a fresh sample is not a good predictor of either fertility or freezability [3], but nonetheless provides a quick estimate of viable sperm number while such estimates suffering from inaccuracies of the method. On the other hand, motility of post-thaw semen is an important assay to select batches of good semen [5]. Therefore, post-freeze assessment of motility must be done as objectively as possible.

**Materials**
Slides, coverslip, scissor, dry warm towel, tissue paper, water bath, microscope

**Procedure**

(a) Empty content of frozen semen straw in a prewarmed (34 °C) Eppendorf tube.
(b) Thaw at 37 °C for 30 s.
(c) Place a small drop of diluted semen, using original straw, on a clean grease-free warm slide.
(d) Gently place (in a slanting manner) a coverslip over the semen drop.
(e) Press the coverslip lightly to evenly distribute spermatozoa.
(f) Assess the motility by examining the best possible field in PC microscopes under 20×.

**Observation**
Observational points are essentially the same as detailed above. However, while selecting a sample (batch of frozen straw), a cut-off point of 50% is used. That is, at least 50% or more sperm cells show progressive motility in at least two out of three fields examined (see 'Background Information').

**Points to Ponder**
As given in earlier sections

### 5.4.5 Motility Index

Motility index (MI) is another parameter to relate motility with fertility of semen samples. Various workers have reported highly significant correlation of MI with fertility and cold shock resistance of spermatozoa.

**Materials**
Semen, sodium citrate, test tubes, Neubauer haemocytometer (NH), microscope, pipettes, stopwatch

**Sodium Citrate (2.95%)**
0.29 g sodium citrate.
Dilute to 10 mL DW.

**Procedure**

(a) Dilute semen sample with 2.9% sodium citrate to get $10\times10^6$ sperm per mL (A).
(b) Charge haemocytometer as described before.
(c) Focus on any one WBC chamber.
(d) Count total number of spermatozoa crossing on either side of the 0.25 mm line/min.
(e) Repeat counting in different lines of the same chamber in microscopic field four times.
(f) Take average of the four counts (B) for calculation.

**Calculation**

$$\text{MI} = \frac{B}{A \times 10} \times 100 \text{ million}$$

where

MI = motility index
$A$ = number of spermatozoa per mL
$B$ = number of spermatozoa crossing the line/min

**Points to Ponder**
As listed before in the chapter

## 5.5 Computer-Aided Sperm Analysis (CASA)

The application of computer science in measuring sperm concentration was not feasible because of difficulties in distinguishing spermatozoa from particulate debris [6]. However, advances particularly in the use of fluorescent DNA stains and tail detection algorithms allowed accurate and more objective measurement of sperm concentration as well as progressively motile spermatozoa using computer-aided sperm analysis (CASA) [7]. In brief, image analyser or CASA is an automated sperm-tracking computer-aided device, essentially focusing through microscope providing successive images of motile sperms within a static field of view, and is calculated by distance travelled over period. In most of the systems currently being used, standard video image acquisition rates, i.e. frames/sec of 25, 30, 50 or 60 Hz, are used.

With the adequate care in preparing specimens and applications of instruments, CASA can now be used in day-to-day semen biology [8]. There are several CASA machines available, which can measure one or all of the parameters like sperm motility and kinematics, concentration and morphology as well. The advanced instruments have several advantages/disadvantages over subjective methods [reviewed by Amann and Weberski, 8] as given below:

**Advantages**

- It has high precision.
- It provides quantitative data on the kinematic parameters of spermatozoa (forward progression and hyperactivated motility, characteristic of capacitated cells).
- It is best used for kinematic analysis of spermatozoa, as it can detect motile cells.
- CASA confers greater objectivity, precision and reproducibility than manual systems [9].
- In CASA, precision and reproducibility can be less than 7% [10], much superior to manual evaluation even by an experienced technician.
- It reduces the burden of measuring sperm tracks where individual track data can be generated.
- The method is quick, and many samples can be screened in a short time.
- The expert for second opinion can always verify the data generated.

**Disadvantages**
Even the computer-aided analysis system is as intelligent as the programmer is. Along with several advantages, CASA has some clear disadvantages as well. They are:

- Determination of percentage motility may be unreliable, as they depend on determining the number of immotile spermatozoa, and debris may be confused with immotile spermatozoa.
- Tracking of minimum of 200 sperm cells for estimating at least one attribute is required. This implies that many more number of sperm cells will be needed if other analyses of variability within a specimen are planned.
- Needs standardization of the number of spermatozoa in each specimen for motility estimates.
- Linking with computer software that permits data organization and statistical analysis is a must.
- Different CASA instruments use different mathematical algorithms to compute many of movement variables. Therefore, the greatest

disadvantage in application of CASA remains unreliability of comparative measurements across all instruments.

### Factors Affecting Performance of CASA Instruments

Several factors, which affect the performance of CASA instrument, are frame rate, sample preparation, sperm concentration and counting chamber depth [2].

### Procedure

### Setting Up of CASA Instrument

(a) Each CASA instrument requires customized setting up for its anticipated use for optimum performance.
(b) It is best to follow settings provided by the manufacturer.
(c) Investigator must crosscheck that the instrument is performing to the required degree of repeatability and reliability.
(d) Always arrange appropriate quality control materials, e.g. video recording.

### Sample Preparation and Assessment

(a) Ensure maintenance of sample at 37 °C for motility estimates.
(b) Evaluate motility characteristics and sperm concentration in undiluted semen.
(c) For sperm motility estimates, prepare sample concentration of 2–50 × $10^6$ cells/mL.
(d) Dilute the original semen sample using normal saline with the sperm-free seminal plasma to bring the concentration below 50 × $10^6$/ mL.
(e) Fill both chambers (7 μL sample each) of the dual-chamber system (Leja or Makler's, Fig. 5.1) and assess after 1 min equilibration.
(f) Examine several representative fields: 6 fields per chamber (12 fields in total) give reliable results.
(g) Count at least 200 spermatozoa in each chamber.
(h) Start from left upper corner and end in right lower corner of the chamber.
(i) Analyse samples either directly or from a video recording.
(j) Follow setting provided by the manufacturer for type of recording device to be used and the illumination setting for maximum contrast between sperm heads and background.
(k) Follow a sperm for a minimum of 1 s to achieve satisfactory results (Fig. 5.2)


**Fig. 5.1** Above photograph depicts the Makler counting chamber and cover glass with grid. This is a simple-to-use device for rapid and accurate sperm count and motility evaluation

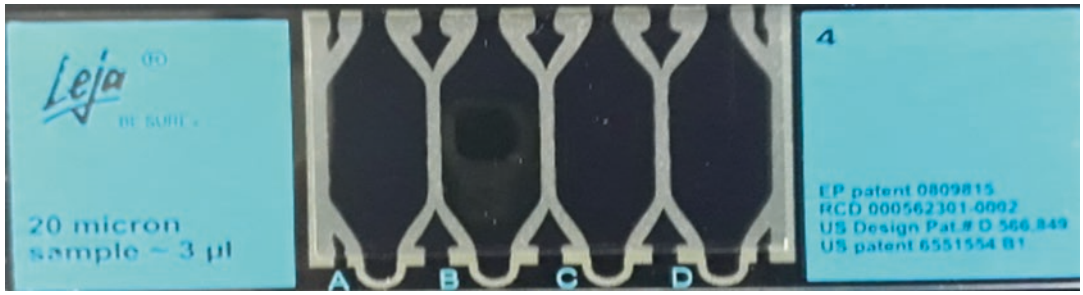


**Fig. 5.2** Four-chambered Leja slide is shown above. These are disposable and non-toxic slides used for sperm counting and motility analysis in animal semen biology

**Table 5.3** Common convention of sperm identification in CASA system

| Sperm type | Identification | Description |
|---|---|---|
| Progressive | A single cyan track | Progressive cells move faster than the minimum 52 on the *Analysis Setup* screen |
| Static | A single red dot | No red dots should be present on any of the motile sperm or debris |
| Slow | A purple track | These are slow-moving cells at a speed lower than the VAP *cut-off* and *VSL cut-off*. |

### Sperm Identification in CASA

In commonly used sperm identification system in CASA, motile cells are identified by a single green or cyan (light-blue) track. Sperm departing the area during analysis are identified by a blue track, and sperm eliminated by the anti-collision algorithm are marked with yellow or white track. Exclude these sperms from the count, whereas count all sperms entering the area and assign green or cyan tracks. This convention of sperm identification (Table 5.3 and Fig. 5.3) avoids counting errors.

### Points to Ponder

(a) Collison of spermatozoa may occur in samples with >50 million cells per mL which may induce error.

(b) Disposable, dual-counting chamber system, 20 µm deep, gives reliable results.

(c) Following sperm cell for a time of 1 s is sufficient for the basic CASA measurements [11].

(d) For better standardization and accuracy of results, analyse video recordings (from videotape, CD-ROM or DVD).

(e) Transform mathematically the measurements on a single spermatozoon before doing statistical analysis.

(f) Because the distributions of many of the sperm movement parameters are not Gaussian (not in a standard bell curve distribution), therefore the median, rather than the mean, is more appropriate as a summary of the central tendency of each variable.

(g) While using disposable chambers, it is important to evaluate the sample at several different distances from the site of loading the chamber as the distribution of spermatozoa throughout the chamber will be non-uniform [12].

(h) Centrifugation of semen sample may affect sperm morphology. Mention its use in the results.

### CASA Terminology

The CASA system has become common in most of the semen biology laboratories across the countries; however, terminology remains standard for all of them. Some standard terminology for variables measured by CASA systems has been provided in Table 5.4 and the illustration in Fig. 5.4.

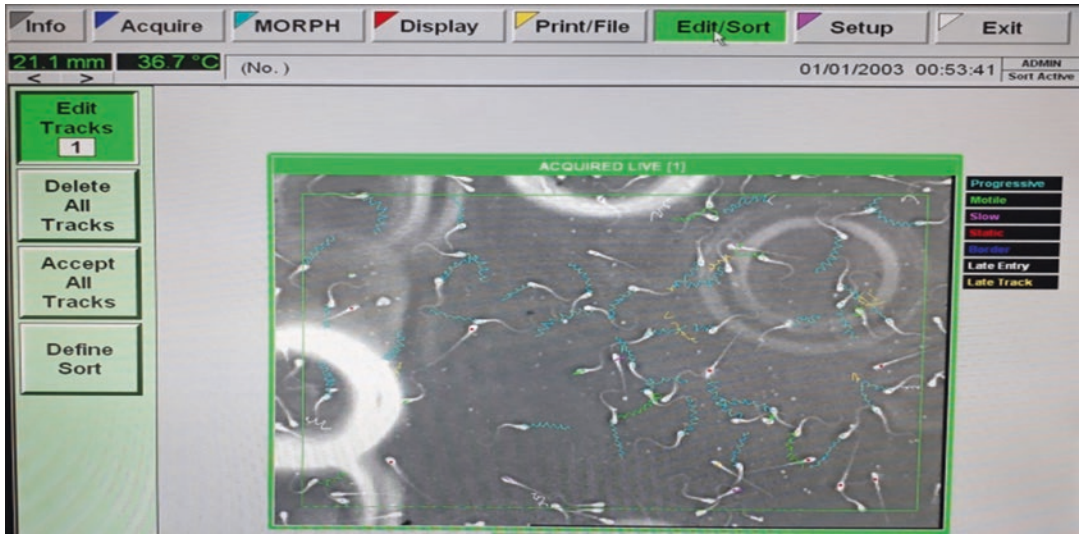


**Fig. 5.3** Common convention of sperm identification in CASA system

**Table 5.4** Standard terminology followed in the CASA system

| Sl | Terminology and unit | What it means |
|---|---|---|
| 1. | VCL, curvilinear velocity (μm/s) | Time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope. It is a measure of a cell vigour. |
| 2. | VSL, straight-line (rectilinear) velocity (μm/s) | Time-averaged velocity of a sperm head along the straight line between its first detected position and its last |
| 3. | VAP[a], average path velocity (μm/s) | Time-averaged velocity of a sperm head along its average path |
| 4. | ALH[b], amplitude of lateral head displacement (μm/s) | Magnitude of lateral displacement of a sperm head about its average path |
| 5. | LIN, linearity | The linearity of a curvilinear path, VSL/VCL |
| 6. | WOB, wobble | A measure of oscillation of the actual path about the average path, VAP/VCL |
| 7. | STR, straightness | Linearity of the average path, VSL/VAP |
| 8. | BCF, beat-cross frequency (Hz) | The average rate at which the curvilinear path crosses the average path |
| 9. | MAD, mean angular displacement (degrees) | The time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory |

[a]VAP is computed by smoothing the curvilinear trajectory according to algorithms in the CASA instrument; since these algorithms vary between instruments, therefore values may not be comparable among systems
[b]ALH is expressed as a maximum or an average of such displacements. Since different CASA instruments compute ALH using different algorithms, therefore values may not be comparable among systems

## 5.5.1 Applications of CASA

### 5.5.1.1 Sperm Concentration and Motility

Application of fluorescent DNA stains with CASA allows precise assessment of the sperm cell concentration and percentage motility, provided technique is adhered to scrupulously. However, validation against haemocytometer is essential. This is because the CASA instrument detects and counts fluorescent sperm heads. Without microscopic evaluation, there is no way of knowing if the spermatozoa are intact (i.e. the head is attached to a tail).

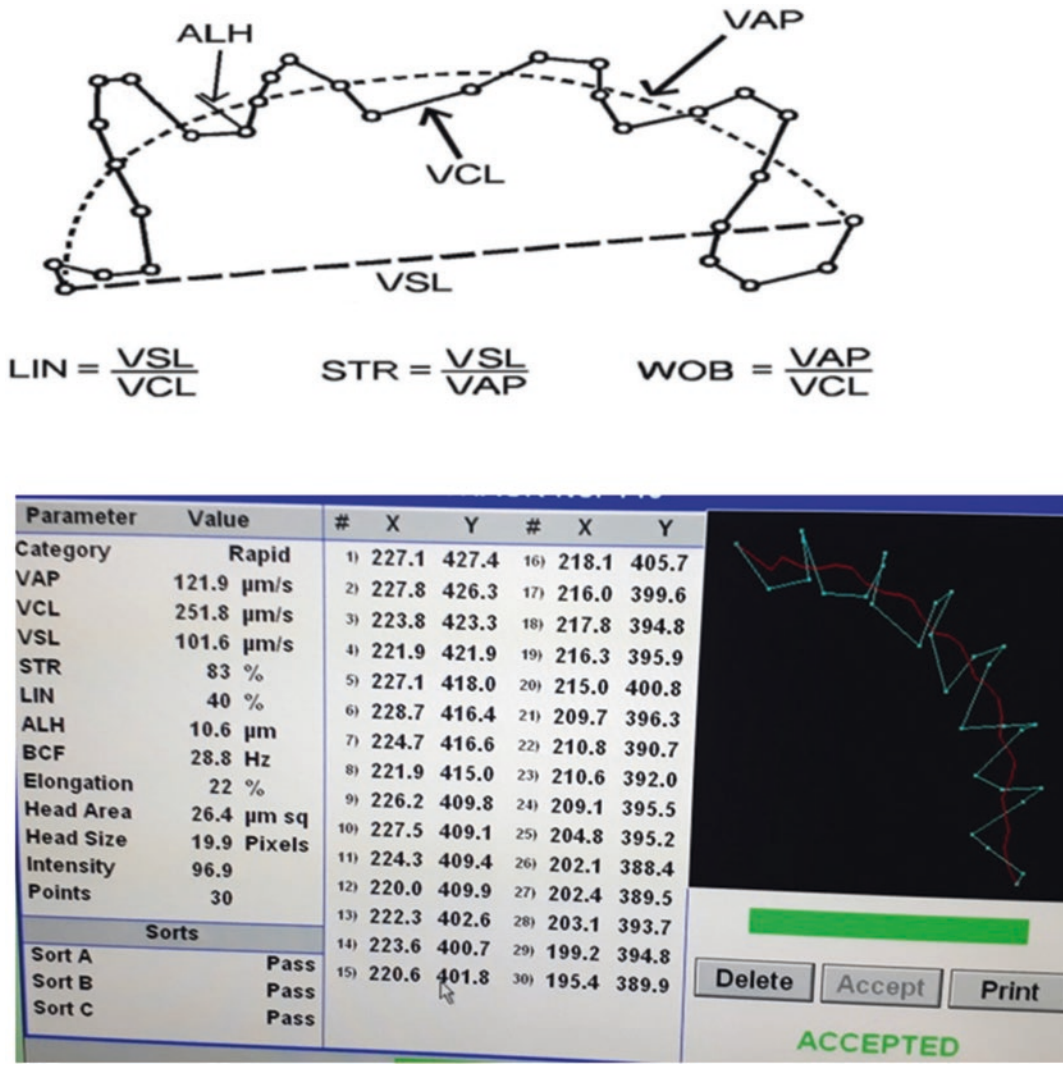


**Fig. 5.4** Sperm kinetic parameters evaluated by CASA

#### 5.5.1.2 Computer-Aided Sperm Morphometric Assessment (CASMA)

There are several CASA systems for quantifying the morphology of the sperm head, midpiece and principal piece. However, tail defects can be more directly assessed by using CASA to measure motility and motion. In the image analysis, the system generally classifies the sperm head and midpiece as normal or abnormal and gives the mean and standard deviation or median for head ellipticity and regularity, head and midpiece dimensions and a stain-dependent measurement of the acrosome area. However, CASMA system can suffer from inaccuracies because of several factors such as sample preparation, staining, focus and illumination [13] and technical difficulties in correctly differentiating sperm heads from seminal debris, particularly at low sperm concentration [14].

##### 5.5.1.2.1 Information

Motility estimates of an ejaculate are very important criteria before an ejaculate at initial stage (individual progressive motility) or a batch of semen after cryopreservation (post-thaw evaluation) is discarded. For deciding about a

**Table 5.5** Discrimination of progressively motile spermatozoa from others

| Grade | Description |
|---|---|
| Progressive (P) | Spermatozoa moving actively, either linearly or in a large circle, regardless of speed |
| Non-progressive (NP) | All other patterns of motility with an absence of progression, for example, swimming in small circles, the flagellar force hardly displacing the head or when only a flagellar beat is observed |
| Nonmotile (M) | No movement |

**Table 5.6** Seminal attributes at post-thaw stage for selecting a batch of straws

| Parameter | Threshold value |
|---|---|
| Live sperm count per straw | 20 million |
| Post-thaw motility | ≥50% |
| Acrosome integrity | ≥65% |
| Hypo-osmotic swelling | ≥40% |
| Microbial load | <5000 CFU/ mL |

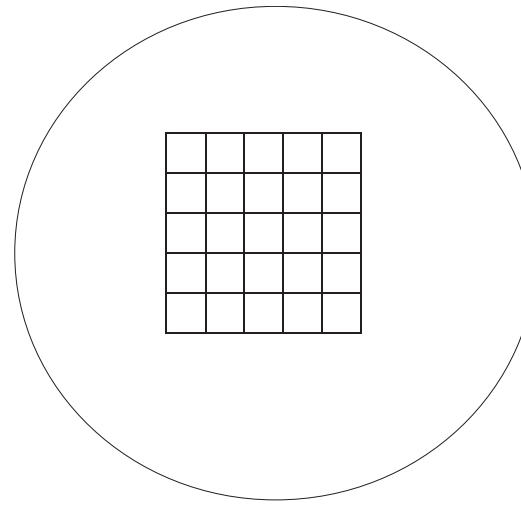

**Fig. 5.5** Eyepiece reticle with grid

sample for its freezability, different laboratories as well as experts follow different discrimination point. For example, in some laboratories, presence of any one, out of several, field of vision showing 70% progressive motility is sufficient to select that particular ejaculate for further processing. On the other hand, others go for three out of five fields to select or cull ejaculates. In our considered opinion to maintain quality and because in most of the semen processing laboratories bovine ejaculates are not sparse, it is best to examine five random fields and select semen sample if it shows at least three fields with more than 70% sperm cells with progressive motility.

Another alternate simple procedure for deciding about cut-off point is to distinguish spermatozoa with progressive or non-progressive motility from those that are immotile. This is graded as follows (Table 5.5).

Strict adherence of criteria outlined below at post-thaw stage is very crucial to maintain quality of cryopreserved semen (Table 5.6).

**Increasing Accuracy of the Motility Estimates**

Estimates of motility, being an important assay in semen biology, and accuracy of the method employed are of utmost importance. This can be achieved as follows:

**Use of an Eyepiece Reticle with Grid**

This is recommended to limit the observational area viewed and allows the same area of the slide to be assessed during different stages of counting. For example, count progressively motile sperm cells first, followed by NP and M spermatozoa. The use of grid allows examination of several areas of the preparation.

Investigators commonly overestimate sperm motility. To avoid this, reverse the order of analysis (NP and IM first) avoiding to the extent possible potential sources of bias for motility (Fig. 5.5).

**Sampling Error in Duplicates**

Sometimes, 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.

In counting of duplicates of a sample, larger than acceptable differences suggest the following error points:

- That there has been miscounting or errors of pipetting
- That the cells were not mixed well, with non-random distribution in the chamber or on the slide

In all such cases, discard the first two values and reassess. Avoid, at all cost, counting a third sample and take the mean of the three values, or take the mean of the two closest values.

**Application of Statistical Methods**

Accuracy of estimates depends not only on the number (N) of spermatozoa counted but also on the true, but unknown, percentage (p) (binomial distribution) [15]. Thus, in such cases, the approximate standard error (SE), for percentage between 20 and 80, is

$$\mathrm{SE} = \sqrt{\left(\left(p\left(100 - p\right)\right)/N\right)}$$

For data outside above range, use angular transformation (arc sin square root), with a standard deviation of 1/(2_N) radians, which depends only on the number of spermatozoa counted and not the true percentage.

$$z = \sin^{-1}\sqrt{\left(p/100\right)}$$

**Rounding of Percentage Value**

One way of writing decimal percentage value is to round to the nearest whole number. The convention is to round 0.5% to the nearest even number, e.g. 42.5% is rounded up to 42%, but 7.5% is rounded up to 8%. Remember that the rounded percentages may not add up to 100%.

## Literature Cited


1. Le Lannou D, Pichón JP, Quero JC (1992) Effects of chamber depth on the motion pattern of human spermatozoa in semen or in capacitating medium. Hum Reprod 7:1417–1421
2. Kraemer M, Fillion C, Martin-Pont B, Auger J (1998) Factors influencing human sperm kinematic measurements by the Celltrak computer-assisted sperm analysis system. Hum Reprod 13:611–619
3. Salisbury GW, VanDemark NL, Lodge JR (1985) Physiology of reproduction and artificial insemination of cattle, 2nd edn. WH Freeman and Company, pp 268–274
4. Kumar S, Singh LP (2003) Thawing of frozen semen and evaluation of quality of post-thawed semen. Anupam Printinmg Press, Bareilly, pp 71–72.
5. Edwin J, Ulganathan V, (1988) Deep freezing of semen. TNAU publication, Coimbatore-03
6. Eshre (1998) Guidelines on the application of CASA technology in the analysis of spermatozoa. Hum Reprod 13:142–145
7. Garrett C, Liu DY, Clarke GN, Rushford DD, Baker HWG (2003) Automated semen analysis: "zona pellucida preferred" sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples. Hum Reprod 18:1643–1649
8. Amann RP, Waberski D (2014) Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology 81(1):5–17
9. Menkveld R, Stander ESH, Kotze TJW, Kruger TF, Zyl JA (1990) The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. Hum Reprod 5:586–592
10. Garrett C, Baker HWG (1995) A new fully automated system for the morphometric analysis of human sperm heads. Fertil Steril 63:1306–1317
11. Mortimer D (1994) Laboratory standards in routine clinical andrology. Reprod Med Rev 3:97–111
12. Douglas-Hamilton DH, Smith NG, Kuster CE, Vermeiden JPW, Althouse GC (2005) Capillary-loaded particle fl uid dynamics: effect on estimation of sperm concentration. J Androl 26:115–122
13. Menkveld R, Lacquet FA, Kruger TF, Lombard CJ, Sanchez Sarmiento CA, DE Villiers A (1997) Effects of different staining and washing procedures on the results of human sperm morphology by manual and computerized methods. Andrologia 29:1–7
14. Coetzee K, DE Villiers A, Kruger TF, Lombard CJ (1999) Clinical value of using an automated sperm morphology analyzer (IVOS). Fertil Steril 71:222–225
15. WHO laboratory manual for the examination and processing of human semen. Cooper TG (ed), 5th edn. World Health Organization 2010, Switzerland


## Key References


16. Salisbury GW (1985) See above [3] In-depth discussion and excellent background information related to estimation of motility of spermatozoa
17. Kumar and Singh (2003) See above [4] For procedures related to routine semen evaluation
18. Edwin and Ulganathan (1988) See above [5] For background information and detailed description of routine semen evaluation
19. WHO laboratory manual, 5th edn (2010). See above [15] For excellent explanation of semen evaluation protocols including protocols and application of CASA