

Validation of a single-step procedure for the objective assessment of sperm motility characteristics

A. HINTING*, F. SCHOONJANS, and F. COMHAIRE
Department of Internal Medicine, State University Hospital, Ghent, Belgium

Summary

A relatively cheap method is described for the objective assessment of sperm concentration and motility characteristics. The method uses a digitizing tablet with cursor, a micro-computer and a phase-contrast or dark-field microscope equipped with a drawing tube. With this technique the following are accurately assessed: sperm concentration, percentage motility, motility grading, concentration of grade a motile spermatozoa, sperm velocity, linear velocity, linearity and angularity. The data are acquired in less than 5 min. Validation studies reveal this method to be accurate, reproducible (coefficient of variation of motility characteristics = <7%) and clinically useful.

Keywords: semen analysis, sperm analysis, sperm motility, sperm velocity, motility, velocity.

Introduction

Semen analysis is the cornerstone of the evaluation of male reproductive function and fertility. In most laboratories semen analysis is performed with subjective methods and the results depend on the skill and judgement of the observer. The lack of standards for interpretation contributes to great variation among observers and laboratories performing semen analyses (Comhaire *et al.*, 1987a). The availability of a standard and objective method to provide proper information and interpretation is needed urgently.

Several studies have shown that motility characteristics of sperm are of utmost importance for man's fertility *in vivo* (Zaini, Jennings & Baker, 1985). The percentage and concentration of sperm with rapid linear progressive motility has the strongest power to discriminate between semen of fertile, subfertile and infertile men (Comhaire, Vermeulen & Schoonjans, 1987b). Moreover, the results of sperm velocity measurements correlate well with the *in-vitro* fertilizing potential using either human (Holt, Moore & Hillier, 1985) or hamster ova (Aitken *et al.*, 1982).

*Home address: Department of Biomedics, Faculty of Medicine, Airlangga University, Surabaya, Indonesia.

Correspondence: Dr F. H. Comhaire, Department of Internal Medicine, State University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium.

The technology for reliable assessment of sperm motility characteristics has not been widely available. During the last decade, several sophisticated and costly systems have been developed to establish more precise and objective sperm analysis. In general, these methods involve several steps, including recording by photography or video-micrography followed by analysis of the sperm tracks, making these methods tedious and time-consuming (Holt *et al.*, 1985). A simple and inexpensive method has been described by Overstreet *et al.* (1979). This method is, however, time-consuming and rather cumbersome (Mathur *et al.*, 1986).

Computerized systems using image analysis have recently been made available. Provided with constant supervision and adaptation, these systems seem to be adequate, but they are extremely expensive and have not yet been validated sufficiently (Mathur *et al.*, 1986). Another method using the Doppler principle and laser-light requires highly sophisticated and expensive equipment. However, this system fails to provide information about individual sperm and lacks standardized interpretation.

The development of a relatively simple, inexpensive, rapid, accurate and reproducible method for the objective assessment of motility characteristics as well as other routine semen variables is still needed. This report deals with the set-up and validation of such a method.

Materials and methods

Equipment

The method is based on the use of a digitizing tablet with cursor equipped with a light point and four buttons, and a micro-computer (Fig. 1). A zoom drawing tube is introduced between the objective and the eyepiece of a phase-contrast or dark-field microscope. The drawing tube permits simultaneous observation of the microscopic field and the digitizing tablet, which is placed beside the microscope. A square of 7×7 cm with a grid is drawn on the tablet and this is observed at the centre of the microscopic field. The zoom of the drawing tube is adjusted so that the superimposition of the square on the microscope slide corresponds exactly to $100 \times 100 \mu\text{m}$.

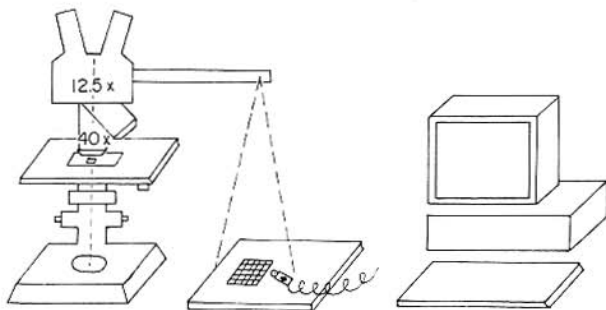


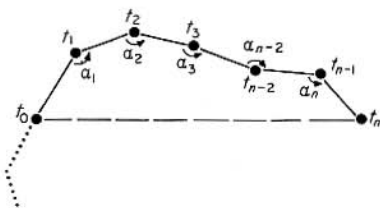
Fig. 1. Diagram of the microscope equipped with a drawing tube, digitizing tablet with cursor, and micro-computer (Autosperm*, Amsaten Corp., Keistraat 139, B-9720 De Pinte, Belgium).

Sample preparation

An aliquot of 11.5 μl of thoroughly mixed, undiluted fresh semen is placed on a microscope slide and covered with a standard cover-slip of 24 \times 24 mm to give a uniform specimen depth of 20 μm (Overstreet *et al.*, 1979).

Techniques of analysis

Objective method. Analysis is performed at a magnification of 40 \times 12.5 and at room temperature (20°C). The technician starts at the upper left corner of the large square and scans the whole grid in a defined sequence until all sperm within the square have been analysed. The movement of each sperm encountered is tracked by the cursor on the digitizing tablet whilst the first button of the cursor is depressed. Sperm with non-progressive motility are identified by pressing the first button of the cursor and leaving the cursor on the spot. Immotile sperm are recorded by pressing the second button of the cursor. Changing to another square is recorded by pressing the third button. The fourth button is touched to stop the procedure.



..... : delay - not evaluated

$$\text{Velocity: } \frac{\sum_{i=0}^{n-1} |r_i r_{i+1}|}{\text{time}} \mu\text{m/sec}$$

$$\text{Linear velocity: } \frac{|r_0 r_n|}{\text{time}} \mu\text{m/sec}$$

$$\text{Linearity index: } \frac{\text{linear velocity}}{\text{velocity}} \times 100 \%$$

$$\text{Angularity: } \left(1 - \frac{\sum_{i=1}^n \cos(\alpha_i)}{n} \right) \times 50 \%$$

$$\text{Angular velocity: } \frac{\text{velocity} \times \text{angularity index}}{100} \mu\text{m/sec}$$

Fig. 2. Diagram of the sperm track generated by the digitizing tablet and the calculation of sperm motility characteristics.

The computer has been programmed to calculate the following variables (Fig. 2):

- (i) velocity (in $\mu\text{m/sec}$), which is the total distance covered by the sperm per unit of time;
- (ii) linear velocity ($\mu\text{m/sec}$), which is the shortest distance between the start and the end point, calculated per unit of time;
- (iii) linearity index (%), which is the quotient of linear velocity divided by velocity, multiplied by 100;

- (iv) angularity index (%), which is the deviation of linearity (straight line) calculated as the mean of the cosine of angles (Fig. 2 α), transformed to a percentage (a straight track has an angularity index of 100%);
- (v) angular velocity ($\mu\text{m}/\text{sec}$), which is calculated by multiplying angularity index by the velocity, divided by 100;
- (vi) sperm concentration ($\times 10^6/\text{ml}$);
- (vii) motility (%), which is the number of motile sperm divided by the total number of sperm (motile + immotile), multiplied by 100;
- (viii) proportion of grade a, b, c and d sperm (%) (WHO, 1987);
- (ix) concentration of grade a sperm per ml.

Semi-subjective method. In this method, originally described by Hellinga (1976), the microscope preparation is made in the same way as for the objective method. The microscope field is scanned systematically over many fields and the motility of each sperm encountered is classified. The categories used for classifying sperm motility have been designed a, b, c and d and are defined in accordance with the World Health Organization recommendations (WHO, 1987):

- (a) the sperm has a rapid linear progressive motility;
- (b) it has a slow or sluggish linear or non-linear movement;
- (c) it has non-progressive motility;
- (d) the sperm is immotile.

One hundred successive sperm are classified, thus yielding a percentage value for each motility category. The numbers of sperm in each category are counted with the aid of a laboratory counter.

Validation methods

In order to assess the minimum time of tracking sperm necessary to enable reliable measurement of motility characteristics, each track of 20 motile sperm was displayed as a series of coordinates at intervals of 0.4 sec. The computer was programmed to calculate the length of the track on the basis of the coordinates generated by the digitizing tablet. The mean velocity of the sperm was calculated between every pair of coordinates. Initially the delay time was determined, corresponding with the slowness of the hand in following movement of the sperm. This delay being ignored, the minimum time of tracking sperm necessary to achieve stable results was assessed.

The minimum number of motile sperm to be tracked for reliable assessment of motility characteristics, sperm concentration and percentage motility was then calculated.

The influence of sample depth was assessed by comparing motility characteristics at depths of 25, 20 and 10 μm in 10 different semen samples.

The objective motility characteristics of sperm grades a and b were assessed. Two highly experienced laboratory technicians were requested to each select and track 200 type a and 200 type b sperm in 10 different semen samples. The cumulative frequency distribution curves of sperm motility characteristics were drawn for sperm types a and b, and from these curves Receiver Operating Characteristic (ROC) curves were constructed. The criteria values giving the best

discrimination between the two types of motility were deduced from the ROC curves (Turner, 1978; Robertson & Zweig, 1981; Comhaire *et al.*, 1987b). Briefly, the curves are constructed from the cumulative frequency distribution of the result of the different motility characteristics in two separate groups of sperm by plotting the proportion of subjects in the first group (sperm with type a motility) against the proportion in the second group (sperm with type b motility). If the distribution of the result of a particular test does not differ in both groups, the ROC curve will coincide with the diagonal. The greater the difference of the distribution of the test result in the two groups, the further the curve will shift from the diagonal to the upper left corner. Hence, the distance from the diagonal to the ROC curve is a measure of the power of that particular variable to discriminate between type a and type b motile sperm. The measuring value located at the greatest distance from the diagonal is the criterion value that permits the best separation between the two types of motility.

The correspondence between the motility grading using the semi-subjective and objective method was studied. Furthermore, the sperm concentration calculated with the objective method was compared with the concentration assessed by the haemocytometer method.

Finally, the inter-assay variability of results obtained with the semi-subjective and objective methods was estimated in 10 preparations from the same sample analysed by the same observer.

Results

All motility variables differed significantly from the mean during the initial 0.8 sec of cursor movement (Fig. 3). Therefore, a delay time of 0.8 sec was incorporated before the data were accepted for motility calculation. The velocity recorded from the 3rd to the 9th coordinate did not differ significantly from the mean, but the linearity index only stabilized after the 8th coordinate. Hence, each sperm should be tracked for at least 3.6 sec, of which the initial 0.8 sec are to be ignored, to achieve a reliable measurement. The interval for data acquisition should be from 0.8 to 3.6 sec.

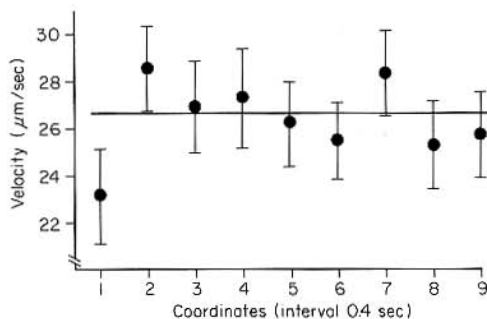


Fig. 3. Velocity (mean \pm SEM) of the sperm calculated between every pair of coordinates.

The mean and SEM for velocity and linear velocity, obtained by tracking different numbers (between 10 and 50) of sperm, were calculated. The percentage

difference of results after calculation of different sample sizes is illustrated in Figs 4 and 5. Tracking of 20 motile sperm appeared to be sufficient to achieve a reliable estimation of motility characteristics with less than 10% variability.

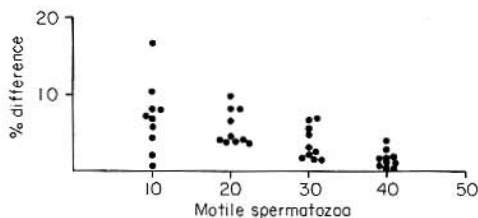


Fig. 4. Percentage difference of the mean value for sperm velocity from the overall mean value.

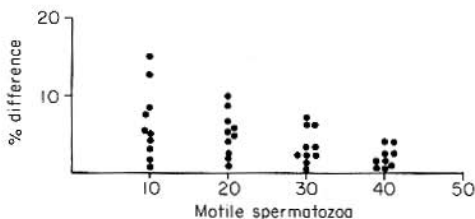


Fig. 5. Percentage difference of the mean value for sperm linear velocity from the overall mean value.

In order to assess sperm concentration and per cent motility adequately, 50 sperm must be analysed before 10% precision is achieved.

Whereas sperm velocity, linear velocity and angular velocity did not differ in preparations with a chamber depth of 25 (not shown), 20 and 10 μm , linearity and angularity indices were reduced significantly using a chamber depth of 10 μm (Fig. 6). It therefore seems that a chamber depth of 20 μm permits optimal rolling of sperm, resulting in less angular movement that more closely reflects physiological sperm movement.

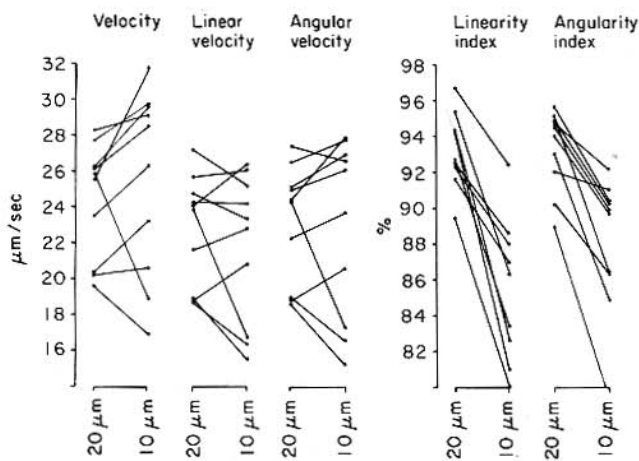


Fig. 6. Values for sperm motility characteristics obtained with different preparation chamber depths.

Figure 7 shows the cumulative frequency distribution curves for linear velocity of sperm grades a and b. The results obtained by the two laboratory technicians were virtually identical, and the ROC curves (Fig. 8) revealed that linear and angular velocity had the strongest power to discriminate between the two types of motility, followed by velocity. The indices had less discriminating power.

When using the criterion value deduced from the ROC curves as the cut-off point, sperm with a linear velocity of $\geq 22 \mu\text{m}/\text{sec}$ were categorized as grade a.

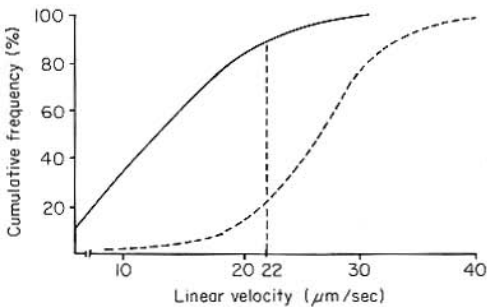


Fig. 7. Cumulative frequency distribution of linear velocity from grade a (---) and grade b (—) sperm.

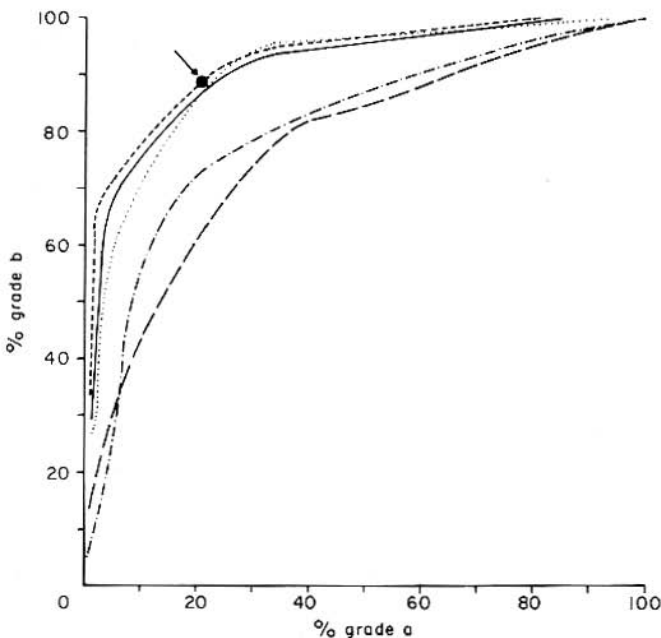


Fig. 8. ROC curve of grade a and grade b sperm for sperm motility characteristics. The criterion value corresponding with the measuring value located the farthest away from the diagonal is indicated by an arrow. (—) Linearity index, (-.-.-) angularity index, (. . .) velocity, (—) angular velocity, (- - -) linear velocity.

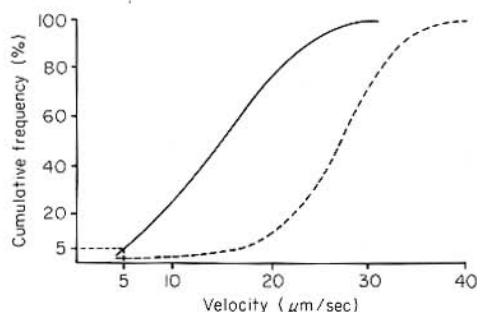


Fig. 9. Cumulative frequency distribution of velocity from grade a (---) and grade b (—) sperm.

The 5th percentile of velocity of grade b sperm, being 5 $\mu\text{m}/\text{sec}$, was used as the cut-off point between sperm grades b and c (Fig. 9). Hence the criteria for motility classification were as follows:

grade a: linear velocity $\geq 22 \mu\text{m}/\text{sec}$;

grade b: linear velocity $< 22 \mu\text{m}/\text{sec}$ and velocity $\geq 5 \mu\text{m}/\text{sec}$;

grade c: velocity $< 5 \mu\text{m}/\text{sec}$;

grade d: immotile sperm.

Using these criteria, the correlation of the values for grade a sperm motility and percentage motile sperm, as assessed by the semi-subjective and objective methods, was excellent (Figs 10 and 11), and the results obtained in both methods were not significantly different (Wilcoxon test).

Similarly, an excellent correlation was found between sperm concentration measured using the objective method and using a haemocytometer (Fig. 12).

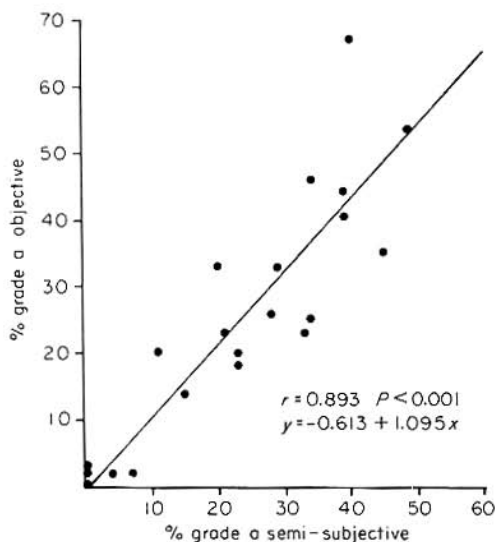


Fig. 10. Correlation between the percentage grade a sperm assessed using the objective and semi-subjective methods.

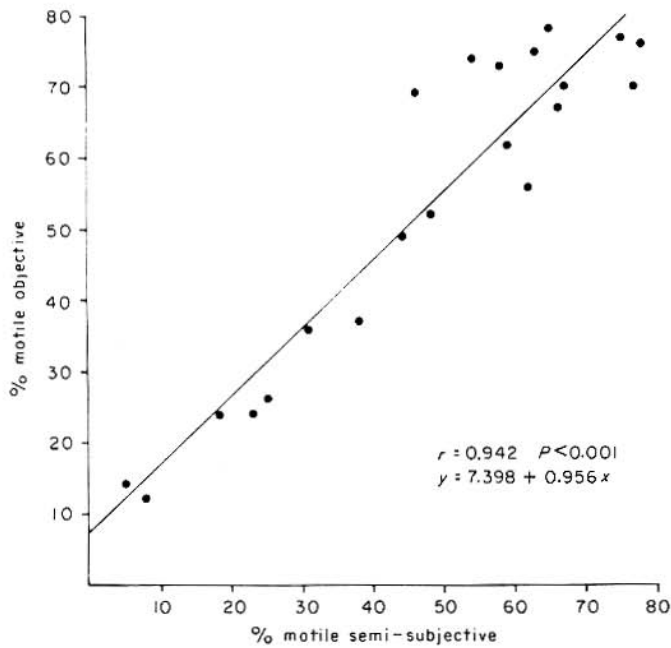


Fig. 11. Correlation between the percentage motile sperm assessed using the objective and semi-subjective methods.

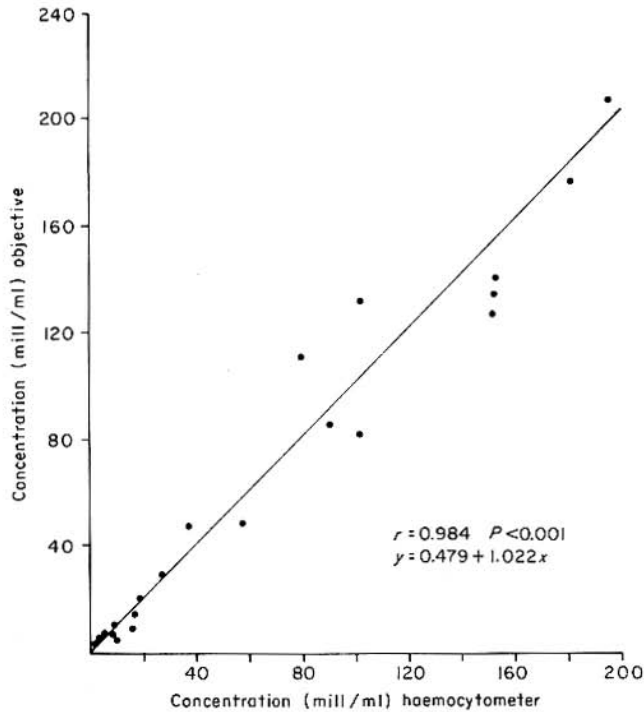


Fig. 12. Correlation between sperm concentration as determined using the objective and haemocytometer methods.

The inter-assay coefficient of variation was assessed by testing 10 aliquots of the same semen sample using both the objective and semi-subjective methods (Tables 1 and 2). Both methods yielded reproducible results with somewhat lower variability using the objective than the semi-subjective technique.

Table 1. Variability of sperm motility characteristics and concentration using the objective method

Parameter	Coefficient of variation (%)
Velocity	6.3
Linear velocity	5.5
Angular velocity	6.4
Linearity index	1.1
Angularity index	1.5
Grade a motility	7.7
Grade b motility	12.6
Grade c motility	71.3*
Grade d motility	5.1
% concentration	7.9

* Mean close to 0.00.

Table 2. Variability of sperm motility grading using the semi-subjective method

Motility grade	Coefficient of variation (%)
a	8.1
b	14.6
c	61.7*
d	17.2

* Mean close to 0.00.

Discussion

Thorough validation of a simple, single-step method for objective assessment of sperm motility characteristics reveals this method to give reproducible and accurate results. Furthermore, sperm concentration and the percentage and concentration of sperm grades a, b, c and d, as well as per cent sperm motility, were assessed accurately at the same time. The complete objective evaluation of sperm characteristics takes no more than 5 min per sample, since only 50 sperm have to be analysed. This sample size is identical to that which was recommended independently by Overstreet *et al.* (1979).

The reproducibility of results was, if anything, better with the objective method than with the semi-subjective method. It should be underscored that the latter was performed by highly trained technicians with more than 10 years of experience in semen analysis.

In summary, the proposed method for objective semen analysis has wide application both in a routine setting and for research purposes. It is relatively simple, rapid, inexpensive, accurate and reproducible.

References

- Aitken, R. J., Best, F. S. M., Richardson, D. W., Djahanbakch, O. & Lees, M. M. (1982) The correlates of fertilizing capacity in normal fertile men. *Fertility and Sterility*, **38**, 68-76.
- Comhaire, F. H., de Kretser, D. M., Farley, T. T. & Rowe, P. J. (1987a) (eds) Towards more objectivity in diagnosis and management of male infertility. *International Journal of Andrology*, Suppl. 7.
- Comhaire, F. H., Vermeulen, L. & Schoonjans, F. (1987b) Reassessment of the accuracy of traditional sperm characteristics and adenosine triphosphate (ATP) in estimating the fertilizing potential of human semen *in vivo*. *International Journal of Andrology*, **10**, 653-662.
- Hellings, G. (1976) *Clinical Andrology*. William Heinemann Medical Books Ltd, London.
- Holt, W. V., Moore, H. D. M. & Hillier, S. G. (1985) Computer-assisted measurement of sperm swimming speed in human semen: correlation of results with *in vitro* fertilization assays. *Fertility and Sterility*, **44**, 112-119.
- Mathur, S., Carlton, M., Ziegler, J., Rust, Ph. & Williamson, H. O. (1986) A computerized sperm motion analysis. *Fertility and Sterility*, **46**, 484-488.
- Overstreet, J. W., Katz, D. F., Hanson, F. W. & Fonseca, J. R. (1979) A simple inexpensive method for objective assessment of human sperm movement characteristics. *Fertility and Sterility*, **31**, 162-172.
- Robertson, E. A. & Zweig, M. H. (1981) Use of receiver operating characteristic curves to evaluate the clinical performance of analytical systems. *Clinical Chemistry*, **27**, 1569-1574.
- Turner, D. A. (1978) An intuitive approach to receiver operating characteristic curve analysis. *Journal of Nuclear Medicine*, **19**, 213-251.
- World Health Organization (1987) *Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*. Cambridge University Press, Cambridge.
- Zaini, A., Jennings, M. G. & Baker, H. W. G. (1985) Are conventional sperm morphology and motility assessments of predictive value in subfertile men? *International Journal of Andrology*, **8**, 427-435.

Received 2 July 1987; accepted 15 December 1987