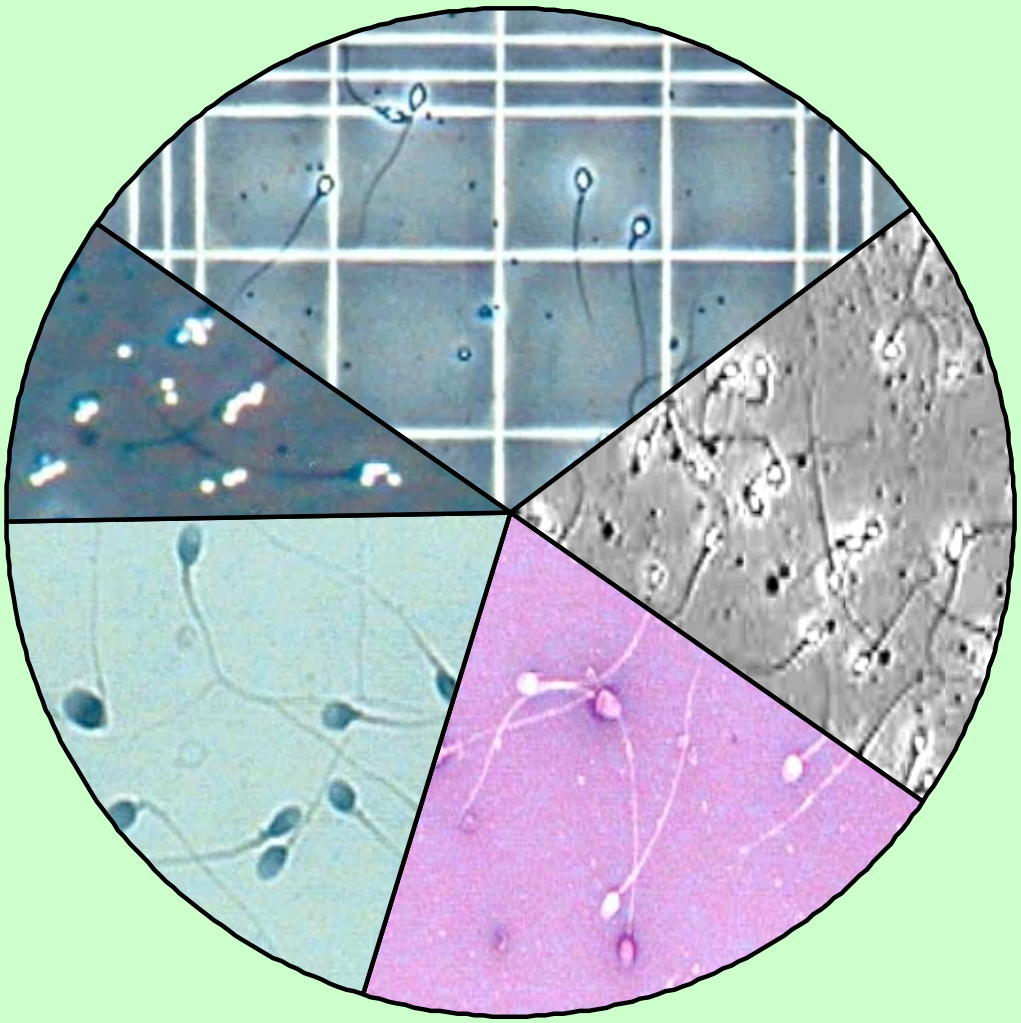
Manual on Basic Semen Analysis 2002

Final version 2002-03-11



This manual is revised to comply with the 1999 edition of the WHO laboratory manual

NAFA

ESHRE

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Foreword

The Nordic Association for Andrology (NAFA) decided in Turku in August 1995 to form the *NAFA andrology laboratory quality group on semen analysis*. The mission of this group was to develop a detailed, technical manual with methods based on the WHO recommendations (1992). The aim was that the manual should constitute a basis for external quality control and for scientific collaboration in this field.

Members in the NAFA group were: Ulrik Kvist (co-ordinator), Aleksander Giwercman, Trine B Haugen, Jyrki Suominen, and Lars Björndahl (secretary). Other contributors were: Birute Zilaitiene, Margus Punab, Øystein Magnus, Åse Strutz, Trine Henrichsen, Turid Vollen, Ingmarie Sundgren, Inger Söderlund, Maiken Simonsen, Lene Andersen, Majbrit Kvist, and Antero Horte.

This manual was first published in 1997. A second edition was published in 1998 and a third, re-edited to comply with the 1999 edition of the WHO recommendations, in 2000.

At the annual business meeting of ESHRE Special Interest Group on Andrology (SIGA) in Bologna, July 2000, it was decided that efforts should be made to make a joint NAFA-ESHRE Manual on Basic Semen Analysis. A working group with representatives of the ESHRE SIGA was formed, consisting of Usha Punjabi, David Mortimer, Christopher Barratt, and Lars Björndahl for ESHRE, and the NAFA andrology laboratory quality group on semen analysis. Ulrik Kvist co-ordinated the work and Lars Björndahl acted as secretary.

This NAFA-ESHRE manual is an application of the guidelines of the WHO (WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction, 4th edition, Cambridge University Press, Cambridge, ISBN 0-521-64599-9) to facilitate the establishment of common, standardized methods and materials in andrology laboratories in the European countries, this being a prerequisite for external quality control and scientific collaboration. No single method is completely ideal, and the group has discussed different possibilities to reach consensus on methods, which are possible to use in a clinical andrology laboratory and still are acceptable from a methodological point of view (elimination of sources of errors).

The methods in the manual also comply with the curriculum of the standardised training course in Basic Semen Analysis developed and implemented by the ESHRE-SIGA. Such courses have for instance been given as the joint ESHRE-NAFA courses in Stockholm (1995, 1996 and 2001), Århus (1997), Göteborg (1998), Oslo (1998), and Helsinki (2001).

Addresses

NAFA – Nordic Association for Andrology, President Marita Räsänen

http://www.ki.se/org/nafa

ESHRE – European Society of Human Reproduction and Embryology

SIGA – Special Interest Group on Andrology; co-ordinator Herman Tournaye

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Semen analysis - overview

Background and principles

From the results of semen analysis we can never predict whether a specific man can become a biological father or not. There are no specific properties one can measure in the whole population of spermatozoa that specifically reflect the fertilizing capacity of the very small population spermatozoa, which is able to reach the site of fertilization. However, analysis of semen can give us information about problems in the genital organs of the male; semen analysis can thus be used to focus the continued infertility investigation. However, the results of semen analysis have been used to categorize men into groups with different probabilities of achieving pregnancy within a certain time period.

The aim of the basic semen analysis is to evaluate descriptive parameters of ejaculates obtained by masturbation. The qualities that are assessed are visual appearance, smell, liquefaction, viscosity, volume, sperm concentration and total number of spermatozoa, sperm motility, and sperm vitality. Furthermore, differential count with respect to sperm morphology, assessment of sperm agglutination/aggregation, and assessment of presence of debris and other cell types in semen are also performed.

Safety in the andrology laboratory

All staff should be aware that semen samples might contain harmful viruses and should therefore be handled with due care. Safety guidelines as pointed out in Appendix II of the WHO 1999 Manual should be strictly observed.

The semen sample

Analysis of an ejaculate, obtained with masturbation, begins at the laboratory 30 minutes after ejaculation. Special rooms should be provided for sample collection. Before sample collection, the WHO manual recommends a maximum interval of abstinence between 2 and 7 days, but recommends that the interval should be “as constant as possible”. Therefore, standardization of ”abstinence time” to 3-4 days is strongly advised[1]. Some men have difficulties producing a semen sample at the laboratory. In these cases the man may collect a first sample at home and deliver it to the laboratory within one hour. In some cases it may be necessary for the man to use a special, non-spermicidal condom to collect a sample at intercourse.

Instructions to the patients

An appropriate request form should accompany the sample containing relevant clinical details. Before producing semen sample the patient should get verbal as well as written information about the purpose of the investigation and other important facts to avoid problems with the analyses. A short explanation of the male reproductive organs for laymen could be included in the written material. The patient should be informed about the relevance of the abstinence time and the importance of collecting the complete ejaculate. If the sample cannot be produced in the laboratory it should be transported protected from cooling (close to body) and delivered to the laboratory preferably within 30 minutes, but at least within one hour after ejaculation

[1] The time of sexual abstinence, given in days, is very imprecise. It is therefore advantageous if the time of abstinence is given in hours. When results are to be used in studies relating to abstinence time it is mandatory to register it in hours.

Calibration

Calibration shall always be done after changes have been done to the equipment or any time errors are suspected.

Automatic pipettes used for measurements (sperm concentration, biochemistry) should be calibrated at least twice a year. Protocols for these measurements and their results should be kept in a special binder.

Balances for weighing semen and reagents should be calibrated at least every year by a technologist or engineer certified by the balance manufacturer. Results are stored in a special binder.

Procedure

Overview of procedure

In the table below, the steps in basic semen analysis are given in an appropriate order to allow efficiency and quality in the laboratory work. Details for the individual steps are given in the text below. For *microbiological culture*, a sterile sample container and sterile disposable pipettes must be used. Some steps are not mandatory, either because of agreed limitations (*), or because steps are optional (#) according to the WHO manual (i.e. each laboratory can chose whether to perform them or not).

Time after ejaculation	**Task**
0 - 5 minutes	Register, weigh[1] and label sample container; label corresponding laboratory documents[2]
	Put sample on an orbital mixer in the incubator (+37°C)
20-25 minutes	Assess liquefaction, visual appearance and smell
30 minutes	Assess semen viscosity[3].
	Examine wet preparation for: sperm motility and sperm aggregation/agglutination; other cells and debris
	Perform antibody test
	* Make smears for eosin-nigrosin stain if <40% motile spermatozoa and assess sperm vitality
	Make dilutions for determination of sperm concentration
	Save 100 µL semen for assessment of inflammatory cells
	Make smears to stain for morphological assessment
	# Save and centrifuge semen for later biochemical analyses
Later	Count spermatozoa in the improved Neubauer chamber
	* If >1 million round cells/mL in semen: assess inflammatory cells
	# Perform biochemical analyses of secretory contribution from prostate (e.g. zinc), seminal vesicles (e.g. fructose) and epididymis (e.g.α-glucosidase)
	Stain smear for morphology assessment
	Assess morphology (differential counting)

[1] Semen volume can also be determined by a graded pipette with 0.1 mL accuracy; weighing is then not necessary

[2] Labels should in general comprise two unique identifiers, e.g. name and a unique number

[3] The pipette used for determination of semen volume could be used to assess semen viscosity when semen is allowed to drop from the pipette

Details of procedure

Register, weigh and label sample container

Initially, the sample should be registered. Sample container must be uniquely labelled[1] concomitantly with the requisition, sample record form, and optional questionnaire. If the sample is produced at the laboratory, the container should be weighed and marked before sample collection. When samples are not collected at the laboratory, the weight of empty containers should be marked on the container before distribution to remitting physicians/clinics or directly to patients. When the sample is collected, the time for ejaculation should be clearly marked on the container, as well as written in the protocol. The net weight of the sample (total weight of sample and container minus container weight) should be noted in the protocol as semen volume (unit mL; 1 decimal).

Put sample on an orbital mixer in the incubator (+37°C)

The sample should without delay be placed on a moving tray in an orbital mixer[2] (+37°C). The time of placement in the incubator should be written on the sample record form. The sample should be left in the incubator until 25-30 minutes after ejaculation, so that examination can begin at 30 minutes after ejaculation. Take the container out of the incubator and check that the sample is well mixed by ”swirling” the sample around the bottom of the container for some 20 seconds. If the sample was been produced outside the laboratory, it must be warmed up in the incubator for 5-10 minutes before examination.

Assessment of liquefaction, visual appearance and semen viscosity

Inspect the **visual appearance** of the sample with respect to *colour* (without remark **or** reddish-brown)[3]; *opalescent or clear*, presence of *gel particles or mucous streaks*. Check that **liquefaction** is complete. If not (gel particles or mucous streaks), put the specimen back into the incubator for some minutes (the examination must, however, begin within 60 minutes after ejaculation - see *Treatment of some viscous samples* below). Deviations from normal findings are noted on the sample record form. If the smell of the sample differs strongly from the majority of samples, this is also written on the sample record form.

The viscosity of the sample is assessed by estimation of how fast the sample runs out of a pipette. Fill a pipette[4] (e.g. a 5-mL pipette) with semen and let the semen flow back into the container. If the droplets form ”threads” that are more than 2 cm long, note ”increased viscosity” on the sample record form.

Treatment of viscous samples

For samples with moderate to very high viscosity a comment of the finding in the sample record form as free text is sufficient. High viscosity interferes with determination of sperm motility, concentration and antibody coating of spermatozoa. For samples where high viscosity hinders examination, addition of a known volume of saline, phosphate buffer solution (PBS) or culture medium and careful mixing with a wide bore pipette should give a homogenous dilution for examination. The original volume must be calculated and used to express the original sperm concentration in undiluted semen. If spermatozoa are to be used for assisted fertilization, one must avoid contamination of the sample and the medium used for sperm preparation for assisted fertilization should be used for dilution. **N.B.** When semen samples are diluted the motility characteristics of the spermatozoa will be affected.

Wet preparation

Examination of a wet preparation

Put 6 µL of well-mixed semen on a clean microscope slide and put a cover glass on top (18 x 18 mm, #1½)[5]. This gives the preparation a depth of about 20 µm. Examination of this wet preparation should begin as soon as the ”flow” in the preparation has ceased. If drifting does not cease within 60 seconds, the prepara-

[1] Labelling should, in general, comprise two unique identifiers, e.g. name and a number that is unique for the sample

[2] It is important to check that the sample containers used allow a complete mixing of the samples with the orbital mixer used.

[3] A yellowish colour of the ejaculate usually depends on an increased content of flavo proteins of seminal vesicular origin, indicating long time of sexual abstinence, or from B-vitamins. Also icterus may give a yellow colour. A reddish or brown discolouring mostly depends on presence of erythrocytes (haemoglobin).

[4] If volume is not measured by weight, the pipette used for assessing the volume could be used

[5] If a 22 x 22 coverslip is used, the semen volume on the microscope slide should be 10 µL.

tion must be discarded and a new one prepared. Phase contrast optics are needed. If there is on average less than 1 spermatozoon per field of vision (40X objective with wide fields eyepieces), the sample should be treated as *suspected azoospermia*. For details and motility determination on **video monitor**, see **Chapter 3** in this manual

Azoospermia or severe oligozoospermia

If there are no or only very few spermatozoa in the wet preparation you should note * (asterisk) in the sample record form and as a free comment write how many motile and immotile spermatozoa that were observed in the wet preparation. If no motile spermatozoa or only a few spermatozoa were found in the wet preparation the sample should be centrifuged[6], and the pellet examined under the microscope (40 X objective, phase contrast optics). If motile or immotile spermatozoa can be identified when the whole area of a coverslip (at least 400 fields in a 22 x 22 mm coverslip) is scanned, the number of spermatozoa and whether they were motile or immotile should be noted on the sample report form.

Assessment of sperm motility

The first aspect of sperm function to assess in the wet preparation is *sperm motility*. This assessment should begin immediately to avoid temperature drop or dehydration of the preparation. For detailed methods, see **Chapter 3** in this manual.

Sperm aggregation and sperm agglutination

Sperm aggregation or agglutination is determined in 10 randomly chosen fields, away from the coverslip edges. The average percentage (estimated to nearest 5%) of spermatozoa trapped in clumps is estimated. **Agglutination** means spermatozoa adhering without other cells and debris. Multivalent antisperm antibodies cause sperm agglutination. If the clumps are very large, it can be difficult to determine whether the binding patterns are specific (e.g. head-to-head or tail-to-tail). If cells, debris, and immotile spermatozoa are included, the clumping is most probably caused by **aggregation**. The agglutinates are caused by sperm antibodies and often contain a certain proportion motile spermatozoa, while aggregates usually contain only dead spermatozoa. Small aggregates of dead spermatozoa and other material are often found in semen from normal men, while presence of large aggregates - often containing hundreds of spermatozoa, is abnormal. When the presence of sperm aggregates or agglutinates becomes clearly increased this should be noted as a free comment in the report.

Other cells and debris

Other cells and debris that can be found in semen are assessed in several fields and unusual findings are expressed as free comments in the report with a few different, standardized expressions:

- **No debris** is a very unusual situation, **some debris** is typical, but **moderate contamination with debris** is not necessarily abnormal. **Larger amounts of debris** are, however, abnormal. Be careful to differentiate between particulate debris and motile bacteria!
- **Red blood cells** (erythrocytes) should not be found in semen, although a few can be present without indicating pathology.
- **Epithelial cells** (squamous, cubic and transitional) are usual in semen in low numbers. Increased presence is not related to any specific functional impairment or presence of infection.
- **"Round cells"** are often seen in semen and it is important that leukocytes are differentiated from immature gametes or large cell bodies (usually without nucleus) with cytoplasm exfoliated from the seminiferous epithelium of the testis. Also cells of prostatic origin appear round in the ejaculate. If there are more than 1 million round cells/mL, counted in the Neubauer chamber (at the same time as sperm concentration is determined), a special method for detection of leukocytes should be used to determine the presence of ”inflammatory cells”.
- **Bacteria and protozoa** are usually not present in semen, but if signs of microorganisms are found, this must be noted in the report.

Smears for eosin-nigrosin stain

According to the WHO manual, if the proportion of motile spermatozoa is less than 50%, the proportion vital (living) spermatozoa should be determined. The reason for assessing the proportion living spermatozoa is to differentiate between dead spermatozoa and live, immotile spermatozoa. Clinically, this difference is only of interest when there are no

[6] At least at 1000 g for 15 minutes

or very few motile spermatozoa. Therefore, a decision limit at less than 40% motile spermatozoa will not lead to any missed diagnosis, but will decrease work load by reducing the number of vitality assessments of samples with living spermatozoa. For a detailed method for assessment of **sperm vitality** see **Chapter 4** in this manual.

Antibody test

The secretory part of IgA-type antibodies bind to cervical mucus. Presence of spermatozoa bound IgA therefore results in a reduced ability to penetrate cervical mucus.

Antibodies on sperm

IgG and IgA on spermatozoa can be assessed directly in semen with e.g. SpermMAR™ for IgG and IgA, and after washing with the Immunobead™ test.

Antibodies in seminal plasma

In semen samples lacking spermatozoa the presence of antisperm antibodies can be assessed indirectly by exposing donor spermatozoa, without antibodies, to seminal plasma from a man with suspected presence of antibodies. The donor spermatozoa are then processed as in a direct sperm antibody assay.

All methods are dependent on motile spermatozoa. At least 200 motile spermatozoa should be assessed.

Results from SpermMAR™ and Immuno-bead™ are not fully concordant. A positive finding of IgA (i.e. more than 50% of motile spermatozoa with beads attached) should be verified with a sperm-mucus interaction test.

Dilution for determination of sperm concentration

It is essential for the accuracy of the sperm concentration assessment that the semen sample is gently, but thoroughly mixed before an exact volume is withdrawn with a positive displacement pipette. See **Chapter 2** in this manual for a detailed method for assessment of **sperm concentration**.

Save 100 μL for assessment of inflammatory cells

To evaluate if a high concentration of round cells (>1 million round cells/mL) in semen is due to inflammatory cells, a specific method (cytological or immunocytochemical) for detection of leukocytes should be used.

Make smears to stain for morphological assessment

Two smears are made on clean microscope slides from each semen sample for morphological evaluation. An aliquot (8-10-15 μL - volume adjusted to the sperm concentration to reduce overcrowding of the spermatozoa) of well-mixed semen is placed on the slide and the droplet is spread with the edge of a cover slip[7]. The smears are left to air dry and then fixed immediately and stored for later staining for assessment of **sperm morphology** (see **Chapter 5** in this manual). Smears from samples with suspected azoospermia should be fixed in separate vials with fresh solutions to avoid the risk of contamination with spermatozoa from other smears.

Save and centrifuge semen for later biochemical analyses

The remaining semen volume is centrifuged (3000 g, 15 min) and the supernatant (seminal plasma) put into a labelled test tube for later biochemical analyses. The test tube is sealed and put in sample number order in a separate rack in the freezer (-20°C). Methods for biochemical analyses are not included in this manual.

Counting in an improved Neubauer haemocytometer

See **Chapter 2** in this manual.

Staining of smear for morphology assessment

See **Chapter 5** in this manual

[7] An alternative method, using two opposed microscope slides smearing the semen droplet in between them and then pulling the slides apart, could also be used. Irrespective of which method is used the smears must be even and thin to allow good staining and easy examination.

Equipment and materials

The equipment and materials listed here are for semen collection and examination of the wet preparation in general. In the following chapters, equipment and materials for the specific methods described are listed separately.

Sample collection container (e.g. Sarstedt: 100 mL; # 75.563; Lid: #76.564)
Special condom for semen collection (Milex products Inc., Chicago, Illinois 60631, USA; Male Factor Pack®, Hygiene®, FertiPro N.V., Belgium)
Phase contrast microscope (40X phase objectives)
Microscope slides (standard size); *Coverslips* (18 x 18 mm, # 1½ [thickness]; 22 x 22 mm; # 1½)
Pipettes for making

wet preparation: air displacement (positive displacement not needed): 6 µL
morphology smears: air displacement (positive displacement not needed): 8-15 µL
vitality smears: air displacement (positive displacement not needed): 15-50 µL

Pipette tips: plastic, for air displacement pipettes, volume 5-50 µL
Centrifuge (1000-3000 g)[1]
Laboratory balance, top loading 150 or 300 g capacity; resolution 0,01 g)
Test tubes for determination of inflammatory cells, e.g. styrene tubes 55 mm long, 11 mm OD
Test tubes with lid for freezing (biochemistry), e.g. styrene tubes 55 mm long, 11 mm OD
Incubator or *heated chamber* (+37°C)
Plastic gloves for laboratory use

[1] The relative centrifugal force [RCF] (g) is calculated from the formula $g=1118 \bullet 10^{-8} \bullet R \bullet \cdot N^2$ where R = distance in cm from centre of rotor to the point where the RCF is required (ie. in the bottom of the tube); N = revolutions per minute

Sperm concentration

Principles

To count diluted and immobilized spermatozoa is easy using the microscope. The problem is to ensure that these spermatozoa are representative and that the aliquot examined is adequate and representative. This means that the spermatozoa should not be allowed to disappear by sedimentation, aggregation or by adhesion to the walls of the sample container, pipette tips or test tubes, and that there are no dilution errors. Thorough mixing is essential both before the semen is sampled for dilution, and before an aliquot is taken from the sperm dilution to fill the counting chamber.

Furthermore, the dilution must be exact. Positive displacement pipettes are mandatory to minimize the error when sampling a fixed volume from the well-mixed semen for dilution. The reason for using a positive displacement pipette is that this pipette takes an exact volume. Ordinary pipettes, which displace a volume of air, are calibrated to take an exact volume of water. Since the viscosity of semen is higher than water and varies from one sample to another air displacement pipettes will take different volumes samples from different semen specimens.

In addition, to achieve a correct volume in the counting chamber, the coverslip must be mounted properly (tight), and the chambers must be correctly filled.

The sperm concentration (10^6/mL semen) is calculated by dividing the number of spermatozoa counted in the counting chambers, with a factor depending on the dilution and the number of squares counted. The total sperm number (10^6/ejaculate) is the product of ejaculate volume and sperm concentration.

Assessment of appropriate dilution

The wet preparation is used to estimate the concentration and select the most appropriate dilution. With the recommended method for making the wet preparation (6 µL semen, cover slip 18 x 18 mm, "thickness" # 1½), the depth of the sample volume, surveyed in one field of vision, is about 20 µm. Provided the diameter of the field of vision is 500 µm[1], the given numbers of spermatozoa per field of vision can be used to chose the appropriate dilution of the sample as given in Table 1 (4 spermatozoa per field approximately correspond to the concentration of 1 million sperm/mL). In older microscopes, the field diameter may be substantially smaller and then fewer spermatozoa are seen per field of vision (e.g. if the diameter is 250 µm, 1 spermatozoon per field of vision corresponds to 1 million sperm/mL, and the "spermatozoa per field of vision" observed in the microscope should be multiplied by four before going to Table 1).

Table 1: Dilution of the ejaculate

Spermatozoa per field of vision 40X objective	Dilution	Semen µL	Diluent µL
Swim up	1 + 1 (1:2)	100	100
< 15	1 + 4 (1:5)	100	400
15-40	**1 + 9 (1:10)**	**50**	**450**
40-200	**1 + 19 (1:20)**	**50**	**950**
> 200	1+ 49 (1:50)	50	2450

Thus*, standard dilutions* are 1 + 19 and 1 + 9; for *swim up* preparations with < 10 millions/mL a dilution 1+ 1 could be used. When *azoospermia* is suspected, a pellet (after centrifugation) is examined with regard to the presence of motile and immotile spermatozoa in the pellet (see "Azoospermia..." on page 4).

[1] The correct area of the microscopic field can be assessed with a stage micrometer, i.e. a microscope slide with a graded scale, usually with indications of 0.1 mm and 0.01 mm. Measure the diameter of the field and calculate the field area with the formula Area = $\pi \cdot r^2$ where r = diameter / 2.

Dilutions can be kept for a maximum of four weeks in tested vials at +4°C, but should preferably be assessed the same day. The problems that can occur upon prolonged storage are sperm clumping and sperm adhesion to the test tube walls.

Procedure

(1) For every semen sample one dilution is prepared according to Table 1. The exact volume of liquefied semen is withdrawn from the well-mixed semen sample with *a positive displacement pipette* and added to the diluent in a test tube with a tight lid.

(2) Mount the coverslip[1] on the counting chamber (improved Neubauer haemocytometer). Interference patterns (>10 Newton's rings/fringes or iridescence lines) should be seen between the glass surfaces of both areas where the cover glass attaches to the Neubauer slide. If too few lines are visible, then the distance between the cover glass and the Neubauer slide is increased, and thereby the volume of the chamber is too large and the result of the assessment will be incorrect.

(3) The tubes containing the diluted sample should be mixed for at least 10 seconds (on a vortex mixer) immediately before filling the counting chamber. After mixing, an aliquot of about 6-10 μL[2] is taken with a pipette to one side of an improved Neubauer haemocytometer. Then a second aliquot is taken to the other side. Each chamber should be completely filled. If a chamber is overfilled, it must be discarded and a new chamber filled. Removal of superfluous volume from the chamber must *not* be done, since this can change the sperm concentration in the chamber. The counts from these two aliquots are then compared.

(4) Let the chamber rest for 10-15 minutes in a humid box to allow the spermatozoa to sediment to the grid of the counting chamber.

(5) Count the spermatozoa with a 20-40 X-objective[3] (phase contrast) according to the following criteria: One "large square" in an improved Neubauer chamber is limited on all sides by triple lines (see also Figure 1: the visual appearance of the central grid of an improved Neubauer haemocytometer). To define each large square, you should always use the uppermost lines and the leftmost lines as limits.

a) Determine the number of squares that should be counted. First count the number of spermatozoa in the upper, left corner square.

< 10 sperm	count all the grid (25 squares in each chamber)
10-40 sperm	count 10 squares in each chamber
> 40 sperm	count 5 squares in each chamber (e.g. 4 corners and centre).

The purpose is that typically 200 spermatozoa should be counted in each chamber and that this number is sufficient for a comparison between the two counts.

b) If a free sperm head can be recognized with certainty, it is recommended that it should be included in the sperm count assessment. "Pin heads" should be counted separately and commented in the report form. Immature germ cells should not be counted (these will be assessed in differential morphology count), but round cells and inflammatory cells should be counted separately in the counting chamber.

c) "Borderline cases": only spermatozoa whose head is located on the upper or left limiting lines (marked "OK" in Fig. 1) should be counted as "belonging" to that square. Thus, do ***not*** count spermatozoa located on the lower or right limiting lines (marked "out" in Fig. 1).

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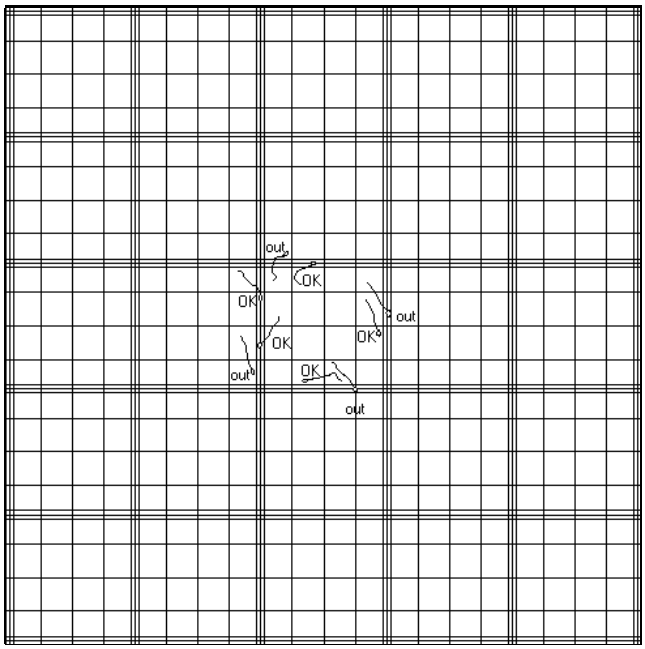
[1] This must be the special, thick coverslip for haemocytometers to achieve correct depth in the chamber.

[2] the exact volume depends on what volume is needed to fill the area under the coverslip properly.

[3] Note that a 40x objective with long working distance is necessary when using a Neubauer chamber.

Figure 1: The central field of a counting chamber in an improved Neubauer haemocytometer



One chamber consists of the 25 ”large squares” seen in the figure. Each ”large” square is surrounded by triplet lines and contains 16 small squares. These small squares can be used when there are many spermatozoa to count in the large square. With a 40X objective, only about one large square can be seen in the same field of vision. Each counting chamber (25 large squares) measures 1 mm x 1 mm and has a depth of 0.1 mm (100 μm). Thus the total volume in one chamber is 0.1 mm^3= 0.1 μL = 100 nL. (A Makler chamber also measures 1 mm x 1 mm, but has a depth of only 0.01 mm. Thus the total volume in a Makler chamber is one tenth of the Neubauer = 0.01 μL = 10 nL).

Calculations

The counts from the two aliquots are compared as described in Appendix I. Calculate the total number of counted spermatozoa (sum) and the difference between the two counts. The assessments are accepted if the difference between the two counts is equal to or less than the value obtained in Appendix I. If not, discard the counts, vortex the sperm dilution thoroughly, fill two new chambers and make new assessments.

The *sum* of the two counts (total number of spermatozoa in both chambers) is divided by the appropriate factor (Table 2) to get the sperm concentration in the semen sample (expressed as millions sperm/mL).

N.B. In this manual, the total number of spermatozoa counted is divided by the factors given in Table 2. In the WHO manual the total number of counted spermatozoa is first divided by two to obtain an average and then this average is divided by another factor. Hence, **the factors given in the WHO manual are only half of the values given here.** The procedure given here saves one division step and thereby reduces possible arithmetic error.[1]

*Table 2: factor to divide the **total number of spermatozoa counted***

Dilution	No of counted squares 25	10	5
1+1	100	40	20
1 + 4	40	16	8
1+9	**20**	**8**	**4**
1+19	**10**	**4**	**2**
1+49	4	1,6	0,8

[1] If your two counts are accepted, there are three calculations to obtain the sperm concentration in millions/mL semen. These steps can be done step by step, but preferably combined into one single calculation.

(1) Firstly, you calculate the mean number of counted spermatozoa by dividing the sum of counted spermatozoa by two (x ½)

(2) Secondly, the sperm concentration in the chamber (i.e. in the diluted sample) must be calculated. To do this you must know the volume you have examined in the chamber. The volume of one large square in the improved Neubauer haemocytometer is 4 nL (200 μm x 200 μm x 100 μm (depth)). Thus:

Number of squares	5	10	25
Volume in nL	20	40	100

The mean number of counted spermatozoa is thus divided by the volume within which they were counted
(x 1/20; 1/40; 1/100)

The sperm concentration obtained is the number of spermatozoa per nL, which equals millions of spermatozoa/mL (spermatozoa/10^{-9} L = spermatozoa x $10^6/10^{-3}$ L)

(3) Thirdly, you have diluted the original semen sample and to get the sperm concentration in semen you multiply with the dilution factor: (x 2; 5; 10; 20; 50)

Dilution	1+1	1+4	1+9	1+19	1+49
Factor	x 2	x 5	x 10	x 20	x 50

These three steps can be carried out in a single division, where the total number of spermatozoa in the two chambers is divided by a single combined factor that is given in Table 2.

Results

Sperm concentration is noted in the sample record form, and the total number of spermatozoa in the ejaculate is calculated (multiply the concentration by the ejaculate volume). Besides incomplete sample collection and varying abstinence time, there are many other external factors that influence the total number of spermatozoa in an ejaculate, e.g. fever, some drugs and occupational exposure. For an appropriate evaluation of the quantitative aspects of sperm production, the results from the laboratory must contain data on sperm concentration, ejaculate volume (or total sperm number), abstinence time, and if the sample was collected completely.

Regarding the report of the result "no spermatozoa" it is preferred that the result is not presented as 0.0 million spermatozoa/mL, but that an asterisk (*) is written in the place for concentration and total sperm number. As a free comment, it should be stated if non or perhaps single motile or immotile spermatozoa have been found in the ejaculate or in the centrifugation pellet. If no spermatozoa have been found in the 100 µL pellet, the maximum number of spermatozoa that still may be present in the ejaculate should be given (details on calculations are given in the last sections of this chapter), e.g. no spermatozoon found; it is likely to be less than 188 spermatozoa in the entire sample.

Quality Control

Duplicate counting should be done to detect random errors in sampling and counting chamber.

Calibration of the positive displacement pipettes (some times called PCR pipette) and ordinary pipettes for measurements of diluents should be done regularly. Pipettes showing erroneous results should be amended according to instructions of the manufacturer or sent for repair and adjustments. Results of calibration controls as well as repairs and adjustments should be recorded in a separate list for each pipette.

Great care is needed when mixing and diluting semen, mixing the diluted sample and preparing counting chambers (attachment of coverslip). Counting chambers should be checked for accuracy when new and regularly after use (risk for wear and changed chamber depth).

Reagents

Diluent: 50,0 g $NaHCO_3$
10,0 mL 36-40% formaldehyde solution (a saturated formaldehyde solution)

- Dissolve constituents in distilled water (>10 MΩ/cm) and dilute to 1000 mL.
- **Filter** (to eliminate crystals) to a clean bottle
- Store at + 4°C (can be stored for at least 12 months).
- Should not contain solid particles

Equipment and materials

Vortex mixer
Phase contrast microscope (40X or 20X objective, and 10X to find focus easily)
Humid box: any box with lid and gauze or paper that should be kept wet, some sort of support to place the counting chamber on above the moist material. **N.B.** the counting chamber must rest *horizontally*!
Calculator, laboratory counter, centrifuge, a working sheet / calculation sheet
Counting chamber: "improved" Neubauer haemocytometer with proper coverslip
Positive Displacement Pipette: e.g.: Finnpipette PCR 20-200 µL; tips: e.g. Labsystems 9403 080
Calibrated, adjustable pipette: e.g. Finnpipette Digital 200- 1000 µL; tips: e.g. blue Treff, or Finntip
Test tube Trombo-test-tube **or** "Stockholm tube" (KEBO 104.112-250)
Lid to Trombo-test-tube **or** lid to "Stockholm tube" (KEBO 103.811-12)

The confidence of sperm counts

What does it mean when no spermatozoon is found?

If, theoretically, an entire semen sample is examined and no spermatozoon is found, then the result is 0 spermatozoa. However, in a wet preparation or in a counting chamber only a small part of the whole sample is examined. Therefore, the finding "no spermatozoa" means that the sample with a certain probability contains less than a certain concentration of spermatozoa. The precision of an assessment depends on the true sperm concentration and how large a proportion of the original sample that was examined under the microscope. The larger proportion, the higher the precision will be. An example: if a semen sample is diluted 1+1 and assessed in an improved Neubauer haemocytometer (two chambers) you have examined 100 nL of the original sample. If the same sample is examined undiluted in a Makler chamber you have examined 10 nL. In the wet preparation you may examine some 10 fields, which corresponds to a volume of 40 nL. Table 3 shows the risk to find no spermatozoon at various true sperm concentrations. The risk to find no sperm is less than 5% only at sperm concentrations ≥ 300 000/mL for 10 nL-chambers, ≥ 75 000/mL for wet preparations (40 nL), and ≥ 30 000/mL for 100 nL-chambers.

These numbers also mean that when no spermatozoa have been found there is a risk of 5% to ignore a sperm concentration of 290 000/mL and 50% to ignore 70 000/mL when examined in the smaller, 10 nL-chambers. This emphasizes the need to investigate a centrifuged sperm pellet when no spermatozoa have been found in the wet preparation.

Table 3: The risk to find no spermatozoon at various sperm concentrations assessed in 100 nL- and 10 nL-chambers and in a wet preparation (approximately 40 nL). The risk to find no spermatozoa is less than 5% at sperm concentrations ≥ 300 000/mL for the 10 nL-chambers, ≥ 75 000/mL for the wet preparation and ≥ 30 000 for the 100 nL-chambers.

True sperm conc. (sp-zoa/mL)	**1 Makler No dilution** (10 nL of sample analyzed)	**Wet preparation** (10 fields ≈ 40 nL)	**Neubauer (2 chambers) 1+1 dilution** (100 nL of sample analyzed)
	Risk to find no spermatozoon		
1 000	0.99	0.96	0.90
10 000	0.90	0.67	0.37
30 000	0.74	0.30	**< 0.05**
70 000	0.50	0.06	
75 000	0.47	**< 0.05**	
100 000	0.37		
200 000	0.14		
300 000	**< 0.05**		

Table 4: There is always a risk to find no spermatozoon even when a sample does contain spermatozoa. In this table the critical sperm numbers are given for when the risk to find no spermatozoon goes under the limit of 5%, in relation to the number of fields examined. Thus, the risk to find no spermatozoon is less than 5% when the sample contains 188 spermatozoa and 400 fields have been examined. If the sample contains fewer spermatozoa, e.g. 100, the risk to find none is greater than 5% (20%). Field diameter 500 µm, preparation depth 20 µm.

	No of fields assessed:		
	4	*40*	*400*
Critical number of spermatozoa	18 750	1 875	188

Confidence in assessment of a 10 µL droplet from a centrifuged sperm pellet

Under the 40X objective one microscopic field corresponds to 4 nL[1]. Each time the coverslip is scanned during screening in the microscope some 40 fields will be "passed". Scanning up and down 10 times means that some 400 such fields are screened. When 400 fields have been scanned a total volume of about 1600 nL (4 x 400) have been assessed, i.e. about 1/62 of the total pellet volume of 100 µL (100 000 nL). If there are more than 188 spermatozoa in the centrifuge pellet, the risk to find no spermatozoon is less than 5%. However, with lower concentration the probability to find no spermatozoon is higher than 5%. Therefore, with no spermatozoon found, after examining a

[1] If the aperture has a diameter of 500 µm you view an area of 196 427 μm^2 and with a 10 µL droplet under a 22x22 coverslip the depth is 20.7 µm giving a viewed volume of 4 058 442 $\mu m^3 \approx 4$ nL

total volume of 1600nL, you can answer that the 100 µL-pellet, and thus the sample, contains less than 188 spermatozoa (Table 4).

Counting spermatozoa selected for assisted fertilization

Spermatozoa selected by gradient centrifugation or swim-up are to be assessed after 1+1 dilution (to immobilise the spermatozoa) in the improved Neubauer haemocytometer. Both chambers are counted.

Spermatozoa selected by swim up or gradient centrifugation are used for ART and for various experimental studies. Whenever the results are to be related to, or evaluated as, a function of sperm number (e.g. in publications), Good Laboratory Praxis (GLP) calls for a certainty of ± 10%, in the sperm concentration assessment. A concentration of 4.0 mill/mL should then mean 4.0 mill/mL ± 10%, i.e. 3.6 to 4.4 mill/mL with 95% confidence. This certainty is easy to reach - it is simply a question of counting at least 400 spermatozoa. One improved Neubauer haemocytometer (both chambers) takes 2 x 100 nL of a sperm suspension diluted 1+1 (to immobilize the spermatozoa). The Makler chamber takes 10 nL of undiluted sample. Thus, in the Neubauer haemocytometer there are ten times more spermatozoa to count than in the 10 nL-chamber. This is of special importance for the low sperm concentrations obtained after sperm selection. With the recommended routine assessment of sperm concentration, counting all spermatozoa in both sides of an improved Neubauer haemocytometer gives accurate results from 4 mill/mL and above. In contrast, counting all spermatozoa in a 10 nL-chamber gives accurate results within ± 10%, only when the sperm concentration is 40 mill/mL and above. To obtain a result within ± 10% for samples in the concentration range of 4 mill/mL you need to assess 10 of the 10 nL-chambers.

Table 5 gives the 95% confidence interval for the sperm concentrations obtained after counting all spermatozoa in one Neubauer haemocytometer and one 10 nL-chamber. When the variation exceeds ±10%, awareness of the uncertainty should be given as the range in the report form. For instance, 2 mill/mL obtained from an assessment in an improved Neubauer chamber is reported as 2 mill/mL (1.7-2.3) or ±14%. If GLP, i.e. a uncertainty < ±10%, is desired assessment of two Neubauer chambers is necessary. If the same result is obtained after assessment in one 10 nL-chamber, the result should be expressed as 2 mill/mL (1.1-2.9) or ±44%. To achieve the result 2 mill/mL ±10% with the 10 nL-chamber, 20 assessments are needed. If your IVF protocol aims at adding e.g. 50 000 spermatozoa to every oocyte and an assessment in one 10 nL-chamber gives the result 1 mill/mL (0.4-1.6), ±62%, you have actually 20 000-80 000 spermatozoa in a 50 µL droplet. Only in 24% of the outcomes you add 50 000 ±10%, i.e. 45 000-55 000 spermatozoa. In most cases (76%), you add either more than 55 000 or less than 45 000 spermatozoa.

Table 5: Expression of results for sperm concentration with 95% confidence interval due to number of assessed spermatozoa in a 10 nL-chamber (e.g. a Makler chamber) and in an improved Neubauer haemocytometer (two chambers)

One 10 nL-chamber Undiluted sperm suspension					**One Neubauer haemocytometer** Two chambers, sample diluted 1+1 to immobilize spermatozoa				
Number of counted spermatozoa	Sperm conc.	95% of all assessments lie in the interval (Mill/mL)			Number of counted spermatozoa	Sperm conc.	95% of all assessments lie in the interval (Mill/mL)		
	Mill/mL	**Low**	**High**	**± %**		**Mill/mL**	**Low**	**High**	**± %**
1	0.1	0.0	0.3	148	10	0.1	0.0	0.2	62
2	0.2	0.0	0.5	119	20	0.2	0.1	0.3	44
3	0.3	0.0	0.6	107	30	0.3	0.2	0.4	36
4	0.4	0.0	0.8	98	40	0.4	0.3	0.5	31
5	0.5	0.1	0.9	88	50	0.5	0.4	0.6	28
6	0.6	0.1	1.1	80	60	0.6	0.4	0.8	25
7	0.7	0.2	1.2	74	70	0.7	0.5	0.9	23
8	0.8	0.2	1.4	69	80	0.8	0.6	1.0	22
9	0.9	0.3	1.5	65	90	0.9	0.7	1.1	21
10	1.0	0.4	1.6	62	100	1.0	0.8	1.2	20
11	1.1	0.4	1.8	59	110	1.1	0.9	1.3	19
12	1.2	0.5	1.9	57	120	1.2	1.0	1.4	18
13	1.3	0.6	2.0	54	130	1.3	1.1	1.5	17
14	1.4	0.7	2.1	52	140	1.4	1.2	1.6	17
15	1.5	0.7	2.3	51	150	1.5	1.3	1.7	16
16	1.6	0.8	2.4	49	160	1.6	1.4	1.8	16
17	1.7	0.9	2.5	48	170	1.7	1.4	2.0	15
18	1.8	1.0	2.6	46	180	1.8	1.5	2.1	15
19	1.9	1.0	2.8	45	190	1.9	1.6	2.2	14
20	2.0	1.1	2.9	44	200	2.0	1.7	2.3	14
25	2.5	1.5	3.5	39	250	2.5	2.2	2.8	12
30	3.0	1.9	4.1	36	300	3.0	2.7	3.3	11
35	3.5	2.3	4.7	33	350	3.5	3.1	3.9	11
40	4.0	2.8	5.2	31	**400**	**4.0**	**3.6**	**4.4**	10
45	4.5	3.2	5.8	29	**> 400**				**< 10**
50	5.0	3.6	6.4	28					
55	5.5	4.0	7.0	26					
60	6.0	4.5	7.5	25					
65	6.5	4.9	8.1	24					
70	7.0	5.4	8.6	23					
75	7.5	5.8	9.2	23					
80	8.0	6.2	9.8	22					
85	8.5	6.7	10.3	21					
90	9.0	7.1	10.9	21					
95	9.5	7.6	11.4	20					
100	10.0	8.0	12.0	20					
150	15.0	12.6	17.4	16					
200	20.0	17.2	22.8	14					
250	25.0	21.9	28.1	12					
300	30.0	26.6	33.4	11					
350	35.0	31.3	38.7	11					
400	**40.0**	**36.1**	**43.9**	10					
>400				**<10**					

Sperm motility

Principles

Ideally, all assessments are made on a video monitor (see below) in order to minimize differences in assessing videotaped samples for QC purposes, and daily routine assessments of "live" samples. If video equipment is not available assessments are made by eye via the oculars and using a 40X phase contrast objective.

Assessment of sperm motility should begin immediately to avoid artefacts caused by either a temperature decrease or dehydration of the preparation.

Sperm motility is determined by counting all motile and immotile spermatozoa in several randomly chosen fields (but not fields close to coverslip edges) using a 40X objective. If more than 25% of spermatozoa are involved in clumping motility assessment is only done on free spermatozoa, and a comment is given on this in the report form. Spermatozoa with pinheads, "free tails", should not be counted.

Procedure

Wet preparation

Put 6 µL well mixed undiluted semen (+37°C) on a clean microscope slide (+37°C) and put on a coverslip (18 x 18 mm, "thickness" #1½). This gives a preparation depth of about 20 µm. Examination of this "wet preparation" should begin as soon as the "flow" has ceased. If the flow has not stopped within 60 seconds a new aliquot should be prepared and examined.

If coverslips of the size 22 x 22 are used, the volume must be 10 µL to give a depth of about 20µm.

Counting

At least 200 spermatozoa are classified in duplicate, i.e. at least 400 spermatozoa in total (for criteria and a detailed method for assessment of sperm motility: see below, *Assessment of sperm motility*). At least five fields should be assessed in each count.

Within each field, all *rapidly progressive* spermatozoa (WHO class a) and *slowly progressive* spermatozoa (WHO class b) are counted first - and care should be taken to count only, and all, cells which are present in the field at the same time. **N.B.** If the number of progressively motile spermatozoa in the field is very high, a smaller part of the field should be used; a graticule or reticle in the ocular is of great help.

When all progressive spermatozoa have been counted, *non-progressive* spermatozoa (WHO class c) and *immotile* spermatozoa *(WHO class d)* are counted in the same field.

The four categories are expressed as percentages (rapid, slow and non-progressive, and immotile.

Duplicated counting is important to detect and minimise random errors due to variations due to variation when withdrawing aliquots from the semen sample, and when making and assessing the wet preparations. Assessment of sperm motility should therefore be repeated on a second aliquot prepared in the same way. The averages for two counts are calculated and given as results. If the difference between the two motility counts is too large, you have detected a random error and two new assessments should be done (see below for details on method and comparisons of duplicate counts).

Sperm motility on video monitor

On the monitor screen, either a black mask with a circular opening or an acetate transparency with a circular field drawn upon it should be mounted. In the circular field so defined, a grid with squares corresponding to 25 x 25 µm in the specimen can be attached to facilitate estimation of velocity.

Use the 10X-phase contrast objective and corresponding condenser phase ring. Check in the oculars that the sample is focused correctly.

Adjust the focus while examining the picture on the screen.

Assessment of sperm motility

Sperm motility is assessed by categorisation into four groups of motility at 37°C:

WHO category	*"code"*	*corresponding velocity*
rapid progressive	a	≥25 µm/s (≥ 1 monitor square, or a 5 sperm head lengths)
slow progressive	b	5-24 µm/s
non progressive	c	<5 µm/s (< 1 sperm head length)
immotile	d	-

(1) Chose randomly a field of vision (if the sperm concentration is very high, only count those spermatozoa in a smaller field, e.g. in the most central four squares). First, count all rapid and slow progressive spermatozoa. Thereafter, count non-progressive and immotile spermatozoa in the same field.

(2) Count at least five different fields. At least 200 spermatozoa should be counted in each preparation.

(3) Repeat the assessment of the motility of at least 200 spermatozoa in a new, separate preparation from the same semen sample.

Calculations

For each of the two preparations, the total number of spermatozoa in each motility group is divided with the total of number of spermatozoa assessed to obtain percentages for the four groups (a-d).

To check that the difference between the two counts is within an acceptable range, the following calculations are done:

- Calculate the percentage of spermatozoa in each motility group (a-d).
- Calculate the average in each group for the two assessments.
- Then, select the group of motile spermatozoa with the highest average proportion (i.e. one of group a, b, c, or d) and calculate the difference in proportion between the two assessments in this group.
- The assessments are accepted if the difference between the two motility percentages is equal or below the value obtained in Appendix II. If not, discard the assessments and make two new wet preparations and recount.

When two accepted counts have been achieved, the average proportions (a-d) are rounded to integers (no decimals). 0.5% is rounded to the nearest *even* number. Finally, the sum of percentages (a-d) should add up to 100%. If necessary the group with the highest proportion is adjusted (decreased or increased) to obtain a sum of 100%.

Results

Percentages for the four categories of sperm motility, percentage motile spermatozoa (a+b+c), and (optionally) percentage progressive spermatozoa (a+b) are written on the sample report form.

Quality control

Duplicate counting is used to decrease the risk of random errors.

In semen samples recorded on videotapes the velocity of individual spermatozoa can be measured ”frame by frame” to classify spermatozoa into the different groups (a-d) more objectively.

Some aspects of Internal Quality Control can be covered by repeated examination of videotapes with known values by all technologists in each laboratory.

One technologist at each laboratory, assessing videotapes sent out to participating laboratories, is the basis for External Quality Control.

Equipment

- Phase contrast microscope (10X objective) with +37°C microscope stage. Triocular tube (oculars and photo tube with additional 10X magnification).
- Black & white (or colour) video camera, e.g. Panasonic: (1/2 inch; WV-BL200); connect to text generator.
- Monitor 13 cm (black & white; e.g. Panasonic WV5340 or WV5370 [Size normal]). A black mask with a circular opening (∅ about 13 cm)
  CONNECTIONS:
  In: connect from video recorder;
  switch: 75 Ω.
- A total magnification on the monitor: 100 μm = 75-88 mm (further magnification is usually needed ”above” the 10X magnification in the objective; typically about another 10X in the photo tube). Check the overall on-screen magnification with a micrometer scale.
- Micrometer scale mounted on a microscope slide (for calibration).
- Microscope slides; cover glass 18 x 18 mm, #1½
- Videotapes (30-60 min recording time; high quality).
- 4-channel cell counter.

Video recording

Extra equipment needed for video recording:

- Text generator connected to video recorder (e.g. Hama Video Script 550)
  Input: Video camera.
  Output: connect to video recorder (SCART multi pin socket: In).
- Video Cassette Recorder (VHS) with possibility to move frame by frame (1/25 s) - ”ordinary home video” and two SCART multi pin sockets (In and Out).
  Input from text generator.
  Output to monitor.
- Videotapes (30-60 min recording time; high quality).

Procedure

1. Turn on camera, monitor, video recorder, and text generator. The text generator starts to show a demonstration - turn it off by pressing three times on MODE.
2. Put the videotape to be used into the video recorder. Wind the tape first forward and then back to start again before first recording.
3. Reset the counter so that used time is zero. Check that the video recorder shows ”used time” on the panel.
4. First record the image of a micrometer scale, to allow ”calibration”. Record for about 10 s, then set recorder for ”pause”.
5. Set the beam splitter so that the light does not go to the camera, i.e. make the picture black. Get the ”start text” from the sign generator (e.g. ”Andrology laboratory, XXXXX Hospital, Quality control”). When the text is visible on the monitor,

start recording for about 10 s. Record a further 5 s without text but with black background.

6. Now put an aliquot of the sample on the microscope slide (pre-heated to 37°C) and focus the microscope. Set the beam splitter so that the light goes to the camera and adjust the focus. Deselect the camera at the beam splitter.
7. Note the start time and number for the sample to be recorded. Get the ”sample text” from the text generator (e.g. ”Sample 1”).
8. With the sample text turned on, start recording for 5 s.
9. Turn off the sample text and reset the beam splitter so that the light goes to the camera.
10. Check focus. Record for 10-15 s. Estimate how many spermatozoa can be counted in that field. Repeat step 10-12 until more than 200 spermatozoa that can be assessed have been recorded.
11. Change the field of view in the microscope, then set the beam splitter so that the light goes to the camera.
12. Record 5 s with black background after each field of view has been recorded.
13. After one sample has been completed, make a longer period of ”black recording” (10 s).
14. Repeat from step 7 until the tape is completed (about 10-12 samples should be possible with samples having reasonable sperm concentrations in a 60 minute videotape).
15. Record a message ”End of tape” directly after last field of the last sample.

Sperm vitality

Principles

A cell with an intact cell membrane does not take up the stain Eosin Y, while a dead cell (i.e. one with damaged cell membrane) takes up the red stain. Nigrosin is used as a background stain to provide contrast for the unstained (white) live cells.

Procedure

(1) Mix one drop (50 µL) of undiluted, well-mixed liquefied semen with one drop (50 µL) of eosin-nigrosin staining solution (e.g. in a porcelain spotting plate) and incubate for 30 seconds.
(2) Then place a drop of the solution (12-15 µL per microscope slide) on a clean microscope slide, and make a smear[1].
(3) Let the smear air dry and examine directly or mount permanently the same day, and examine after the mountant has dried (e.g. overnight). Mounted smears are stored at room temperature.
(4) At least 200 spermatozoa should be assessed[2] at 1000X (or 1250X) magnification under oil immersion with a high-resolution 100 X objective (not phase contrast) *with correct adjustment* of the bright field optics (Köhler illumination). Spermatozoa that are white (unstained) are classified as "live" and those that show any pink or red colouration are classified as "dead".
(5) WHO does not specify duplicate counting for vitality assessment, but if this is done, comparison should be done as for motility or morphology (comparison of proportions; Appendix II).

Calculations and Results

The result is the proportion of vital ("live") spermatozoa, expressed as an integer percentage (i.e. without decimals).

The proportion of live spermatozoa is usually somewhat higher than the proportion of motile spermatozoa in the sample.

Reagents

Reagents:
0.67 g eosin Y (C.I. 45380, Europe M 15935), 10 g Nigrosin (C.I. 50420, Europe M 15924) and 0.9 g sodium chloride in 100 mL distilled water

- Dissolve 0.67 g eosin Y and 0.9 g sodium chloride in 100 ml distilled water under gentle heating and add 10 g nigrosin
- Bring the solution to boil and allow to cool to room temperature
- Filter the solution through filter paper (eg. Munktell Class II)
- Store in a sealed glass bottle
- The staining solution should be at room temperature when used

Mounting:

- Use Merck Entellan-medium or other equivalent medium for quick and permanent mounting.

Equipment and materials

Microscope with 100X objective (oil immersion)
Microscope slides (standard size) and cover slips (e.g. 24 x 60 mm)

[1] The drop is smeared by sliding a cover slip in front of the drop. If two smears are made simultaneously by spreading between two microscope slides without feathering, one drop of 20-30µL is used. Don't make the smears too thick, since the background stain could be too dark and cover the sperm.

[2] Since this method dilutes the original semen sample 2 times, patience is needed to find 200 sperm in samples with low sperm concentration.

Sperm morphology

Principles

The morphology seen with the microscope is not the true morphology of a living spermatozoon, but an image we create. This image comprises a number of factors: spermiogenesis, sperm transport, maturation and ageing, time in seminal plasma, smearing technique, fixation, staining, mounting, and the optics and illumination used (i.e. the quality of the microscope). The criteria for classification are only the final step by which we describe the image created during preparation. With standardized and quality controlled methods, we can minimize technique dependent sources of errors and focus our efforts on classifications of variations in sperm morphology.

It is of great importance that the preparations (smearing and staining) are of high quality when assessing sperm morphology. Even small, technique-dependent artefacts influence the appearance of the spermatozoa.

Although human spermatozoa show large variations in morphology, observations on spermatozoa obtained from post-coital cervical mucus have helped to define the morphology of an ideal spermatozoon. Presumably, the fertilizing spermatozoon is selected among these ideal spermatozoa.

Papanicolaou stain (haematoxylin, orange G6, and EA50) gives a clear difference between basophilic and acidophilic cell constituents and thereby enables a detailed examination of chromatin pattern, which is useful in the evaluation of sperm morphology and assessment of presence of immature spermatozoa. Cytoplasmic staining can vary between red and green dependent on ionic strength, pH, and composition of the cell department and the stain (OG6 and EA50).

Nuclear staining: there are several **haematoxylin stains** depending on how the haematoxylin is oxidized and which type of mordant (the reagent that facilitates binding of the dye to cell structures) is used. *Haematoxylin itself is in fact colourless*. It is the oxidized form, haematein that gives the staining. The colour of haematein depends on pH. Under acid conditions it is red, whilst under alkaline conditions it is blue. Consequently, the pH during staining affects the final results. Spontaneous oxidation of haematoxylin to haematein takes weeks. Therefore haematoxylin is oxidized artificially by adding an oxidizing agent; usually sodium iodate or mercuric oxide and most of the commercially available haematoxylins are artificially oxidized and contain a mixture of haematoxylin and haematein. Over-oxidation can occur and then the coloured haematein is transformed to colourless oxyhaematein. This explains the spontaneous fading of colour in haematoxylin-stained smears during long-term (years) storage.

Haematoxylin and haematein are charged positively but only weakly, and it is therefore necessary to use "mordants" which behave as binding reagents, linking haematoxylin stain to the cells. The type of mordant also has an effect on the colour of the stained cells. Mordants are metallic compounds bound both to haematoxylin stain and to negatively charged tissue components, usually the phosphoric acid residues of the DNA, but also to carboxyl groups. Aluminium ammonium sulphate is the most commonly used mordant and it is a component of both Harris' and Mayer's haematoxylins.

Smears stained with Harris' haematoxylin (the usual haematoxylin in the Papanicolaou method) are stable for months to years if stored in the dark. However, the colour intensity of haematoxylin will fade during a prolonged storage as explained above.

Cytoplasmic staining: this is done with OG6 (orange G6), which is an acidic solution allowing binding of OG6 to negatively charged cytoplasmic proteins.

Cytoplasmic and nucleolar staining: EA 50 is a polychromatic dye mixture: Light green (SF yellowish), Eosin (Eosin Y) and Bismarck brown Y. Light green and Eosin are acidic dyes: Light green binds to side-chains of basic proteins. Eosin is a xantheine dye; negatively charged in aqueous solutions; stains cytoplasmic components, nucleoli (which do not occur in spermatozoa) and cilia. Its absorption maximum is 515-520 nm.

Background for assessment of sperm morphology

Each spermatozoon without morphological "defects" is defined as *ideal*. All deviations from the *ideal* morphology are classified as defects. The presence of defects in each region of the spermatozoon is expressed as "defects per 100 spermatozoa" for that region. A spermatozoon with a head defect, a neck/mid-piece defect and a tail defect is registered as having defects in all three sperm regions (i.e. three defects), but is still only one defective cell. Therefore, the total number of defects will be higher than the number of defective cells.

Only intact spermatozoa, i.e. those with both head and tail, are counted. Free sperm heads are counted separately. If there are many free tails or "pin heads" (>20 % in relation to spermatozoa) this should be noted separately. Immature germ cells should be counted separately and not as spermatozoa.

What is an ideal spermatozoon?

An ideal, mature spermatozoon, as adopted by the WHO 1999, has an oval shaped head with a regular contour (4.0-5.0 μm long and 2.5-3.5 μm wide) with a pale anterior part (acrosome; 40-70% of the head area) and a darker posterior region. The length-to-width ratio of the head should be 1.50 to 1.75. The sperm tail should be attached in a symmetrically situated fossa in the base of the head. The base of the head should be broad and not arrow-like. Only one tail should be attached (about 45 μm long), not coiled, nicked or bent over itself. Immediately behind the head the first part of the tail, the mid piece, should be somewhat thicker (maximum width = 1 μm) and about 7-8 μm long. A "normal" cytoplasmic droplet has a smooth outline (not irregular), appears at the base of the head and its size is less than 1/3 the of a normal sperm head.

All borderline cases should be classified as defective. The criteria are supposed to conform to the "strict criteria" for morphological normality published by Menkveld *et al* (Human Reproduction, **5**: 586-592, 1990).[1]

Counting of defect forms

This assessment relates only to the main regions of the spermatozoon (i.e. head, neck/mid-piece, tail). Differentiation between different abnormalities within the head, or between different tail defects is not performed. If a specific abnormality is predominant, this should be commented upon in the report form. The following four main categories are assessed:

Head defects

Includes *large*, *small*, *tapered* (i.e. length-to-width ration >2), *pear-shaped (pyriform)*, *round*, *amorphous*, *vacuolated* (>20% of head area is unstained vacuoles), *small acrosomal area* (<40% of head area), *double heads* or *combinations* of these defects. "Pin head" or "micro head" spermatozoa are not counted.

Defective neck and mid-piece

Includes *bent tail* (midpiece and tail form an angle > 90° to the long axis of the sperm head = grown from the wrong centriole?), *asymmetrical tail insertion*, *thick or irregular mid piece*, *thin mid-piece* (absence or displacement of mitochondrial sheath) or combinations of these abnormalities.

Tail defects

Includes *short*, *double (multiple)*, *hairpin, broken*, *bent* (angle >90°), *irregular width*, or *coiled tail* - or combinations of these abnormalities. Free tails are not counted. A high frequency of coiled tails can indicate that the spermatozoa have been subjected to hypo-osmotic stress. Tail coiling is also related to sperm ageing. A frequency of coiled tails above 20 % should be commented on in the report form.

Cytoplasmic droplet

Cytoplasmic residues can persist protruding from the base of the head at the neck-midpiece region. Droplets with a smooth outline and a size not exceeding 1/3 of the sperm head are classified as normal. Consequently, if the size exceeds 1/3 of the head or if the contour of a droplet is irregular (stained red or green) it is an abnormal cytoplasmic residue and, classified as cytoplasmic droplet. Some immature spermatozoa can have a cytoplasmic droplet at other positions along the tail.

[1] On page 19 in the WHO manual (1999) the size of a normal cytoplasmic droplet should be smaller than ½ the area of a normal head area. On page 21, the given limit is one-third. The recommendation of this manual is the limit of one-third of the normal head size.
In the WHO manual the figure legends (Fig. 2.9-2.10) are not congruent with the text on page 19-21 in the WHO manual. This manual recommends that the criteria given in the present text should be applied.

Specific defects

Among domestic animals, different ”sterilizing” defects have been described, where practically all the spermatozoa produced by an individual animal have a specific structural defect that impairs sperm function. A few equivalent cases have been described in men; the best known is probably the so-called "round head defect" or "globozoospermia". This defect is seen in some men and affects all the spermatozoa in their ejaculates.

Preparation of smears

Slides for morphology smears should be completely free from grease to ensure that the smears stick to the glass. If smears fall off the slides during fixation and staining, of the slides being used should be pre-cleaned with 95% (or absolute) ethanol to allow the smears to be attached firmly to the slides.

If a smear is made from a sperm suspension prepared by washing and swim-up technique, it is difficult to make an even and firmly attached smear on the slide if the solution does not contain proteins. In these cases, use slides, which have been pre-coated with albumin or poly-L-lysine, or add albumin to the sperm solution (final concentration 1% w/v).

To make a smear

To make a smear, a ca 6 µL aliquot of semen is placed on the slide. The aliquot is then **pulled** out into a smear with a second slide or a coverslip. This must be done with minimal force otherwise the sperm tails might be broken. Two smears are made from each sample. If the sperm concentration is judged to be below 20 million/mL then 10-20 µL of semen are used. Especially with viscous samples, alternative methods could be used. Either the sample is diluted with a 170 mM NaCl solution and smears are done as above, or one drop of semen is put on a slide, and another slide is put on top of the first. Then the slides are slided apart.

Let the smears air-dry[1]. As soon as the moisture of the smear has evaporated, the smear should be fixed.

Fixation of smears

Fixation: 15 minutes in 95% ethanol. Smears could be kept for days in this ethanol until staining.[2]

One (of the two) smears from each sample is kept as reserve; the other is stained and assessed.

[1] A **wet** fixation is used for most of smears in cytological diagnostic cytology, i.e. the still wet smear is immersed in, or sprayed with, the fixative. In this way the shrinkage of the cells can be avoided. This method can also be used with good results for semen smears (but note that, as a complication, the cells can float off from the slide).
Semen smears can be completely **air dried** before fixation, but if smears are left dry for long periods morphological changes might appear in spermatozoa (e.g. a gap between the tail and head, or loosening of the acrosome can be caused by shrinkage due to too intensive drying). Therefore, semen smears should be transferred into the fixative immediately after the moisture from the fresh made smear has evaporated. Furthermore, this ”incomplete” air-drying usually allows a better attachment of the smear on to the slide.

[2] **Ethanol** (undiluted spirit; **94-96% ethanol**) is a satisfactory fixing fluid for semen smears. These smears can be stained after 15 minutes of fixation. Semen smears can be stored in ethanol for several days or even weeks (optimally 70% for long storage). Ethanol fixation causes a **dehydration** of cells. Therefore, the cells in a fixed *and dried* smear must be **rehydrated** before staining.
The previously used ethanol-ether fixative does not give any benefit for the analysis of sperm morphology, and ether is relatively expensive, much more inflammable and a health hazard. **Acetic acid**, which is one component of the fixative recommended by WHO, causes an increase in water content of the cells and might improve the nuclear staining, but gives a lighter cytoplasmic staining.
Where **spray fixatives** are used for fixation of smears, the fixative is usually sprayed on a wet smear. The spray usually contains 50% ethanol or iso-propanol, and also polyethylene glycol ("Carbovax"), which forms a **protective coat** on the smeared cells which preserves the smear e.g. during transportation from a too vigorous drying. *If polyethylene-glycol is used, it must be washed off before the slide is immersed in the haematoxylin stain*: the first container with 50% ethanol (i.e. before haematoxylin) must be replaced after every set of slides to make sure that the polyethylene-glycol is excluded.

Staining of smears

Fill staining dishes or jars with the different solutions (see the sequence below). Use a fume hood for safety.

Place the microscope slides into each solution according to the following schedule (one "dip" corresponds to immersion of about 1 second):

Stain or reagent	Exposure	Explanations and comments
ethanol 50%	10 dips or 10 seconds	• Smears transferred directly from a 95% ethanol fixation solution (without drying) must be transferred through **at least one container** with 50% ethanol. • **Air-dried smears**. Rehydration of **air-dried smears** need longer time, 2-3 minutes in 50% ethanol if "dry" time has been long (days or weeks).
Distilled water	10 dips	
Harris' haematoxylin	3 minutes (exactly)	Fixed and **dried smears** can be transferred directly to the haematoxylin, container, but the incubation time usually must be increased. Haematoxylin is a nuclear stain. Observe that haematoxylin is "consumed". If nuclear staining is weak, exposure time can be increased, or, preferably, a fresh stain solution used.
Running tap water	5 minutes	Removal of unbound haematoxylin
Acid ethanol (0.25% HCl in 70% ethanol)	2 dips[1]	After staining, the haematoxylin is bound over the entire cell. Acid treatment removes unspecific staining since colour bound to cytoplasmic components is replaced with hydrogen ions (H^+). However, too long acid treatment also decreases the amount of colour in the nucleus. After acid treatment the slides should be put under tap water immediately to increase pH and thus to prevent disappearance of all the haematoxylin. (The intensity of the staining can be checked here, before continuing the staining procedure. If the nuclei appear too dark this step (acid ethanol) can be repeated). Too weak stain indicates that exposure to haematoxylin was insufficient.
Running tap water (or alkaline water)	5 minutes	The colour of haematoxylin is dependent on the pH. After acid treatment haematoxylin is reddish. Washing the slides in tap water for several minutes increases pH and haematoxylin bound to the nucleus will return the bluish colour.[2]
Distilled water	1 dip	**Preparation for cytoplasmic staining** To stain cytoplasmic components (see below), dehydration of the cells using a series of increasing strength ethanol solutions, is necessary because the staining solutions (Orange G6 and EA-50) are only soluble in alcohol.
Ethanol 50%	10 dips	
Ethanol 70%	10 dips	
Ethanol 80%	10 dips	
Ethanol 95%	10 dips	
Orange G6	2 minutes	**Cytoplasmic staining**
Ethanol 95%	10 dips	These steps are to remove surplus Orange G6. If exposed to ethanol for too long cytoplasmic orange G6 will be released.
Ethanol 95%	10 dips	
EA-50	5 minutes	**Cytoplasmic and nucleolar staining**: A small amount of acid, e.g. acetic acid, in the stain mixture, increases the presence of positively charged components of the tissue which then binds more eosin. Excessive stain is washed away by ethanol (EA 50 is dissolved in 95% ethanol-methanol).

If the colours fade on the slides change reagents (and certainly at least after 25-30 staining series)![3]

[1] Suitable time for acid treatment can be tested, but usually 2 dips are enough to remove unspecific stain (this can be checked directly in the microscope).

[2] Sometimes the tap water is too acidic (pH 4-5), which means that this change of colour (bluing) does not occur. In these cases the smears are immersed either in Scott's solution (contains sodium bicarbonate 3,5 g, magnesium sulphate 20 g, tap water 1000 ml and 1 small crystal of Thymol to avoid microbial growth). Another method to alkalinize the water is to dip the slide once in a solution made by adding 2-3 droplets of liquid ammonium (NH_4OH) in 200 mL tap water. This quickly develops the blue colour and also shortens the procedure.

[3] The stain intensity decreases gradually when sets of slides are stained. This means that, for example, after 8-10 sets of slides one could increase the staining time and/or add a portion of fresh stain solution to the dish or jar. In all three staining

Mounting of stained smears

Mounting allows the storage of smears for long periods of time with maintenance of acceptable quality. Smears to be mounted should be mounted directly using a permanent mountant. Let the mounted smear dry at least over-night before examining it. Mounted smears can be stored for at least several months at room temperature. Smears that are not for mounting can be assessed as soon as they are air-dried. The use of alcohol-soluble mounting media is recommended instead of xylene, which constitutes a health hazard.

Step	Exposure	Explanations and comments
Ethanol 95%	5 dips	Dehydration steps to prepare for mounting with inorganic solvent
Ethanol 95%	5 dips	
Ethanol 95%	5 dips	
Ethanol 98-99,5% (”absolute ethanol”)	2 minutes	
Mounting[4] 2-3 small Droplets	• Mountex • Eukitt	When the mounting medium is soluble in ethanol (Mountex, Eukitt), the medium can be put directly on smears, which are still moist by ethanol.
	• DePex • Pertex	If the mounting media is *insoluble* in ethanol, (DePex, Pertex Permount) let the smear air dry thoroughly (ethanol evaporates) and then add the medium and mount. If smears are not completely dried, ethanol has to be removed, by dipping the smears first in an ethanol-xylene solution (v/v) and then in two changes of 100% xylene. Bioclear® can also be used instead of xylene for removal of ethanol. Ethanol must be thoroughly removed. Check the slide against light to be sure that there is no liquid phase due to presence of ethanol. If ethanol drops are left on the slide to be mounted with a medium insoluble in ethanol, it results in Eosin rich background areas. However, when smears are air dried there is neither need for xylene nor Bioclear®.
Cover slip 24 x 60 mm		• A cover slip of 24 x 60 mm size is most convenient, and it can be placed directly on the smear after 2 or 3 small drops of mounting medium have been added. • Place the cover slip so, that the first contact with the mounting medium begins from one end of the cover slip. In this way air-bubbles can be avoided. Pressing gently on the top of the cover slip and wiping off the extra mounting medium can also remove bubbles. Allow the mounted smear to dry before examining under the microscope, usually over night. If there is an urgent need for sperm morphology results, microscopy can be undertaken after 30 minutes of drying. However, any pressure or sliding of the coverslip should be avoided

Assessment of sperm morphology

At least 200 spermatozoa should be assessed. Each smear is assessed using a microscope with a total magnification 1000-X using a high qualitative 100X *non*-phase contrast objective under oil immersion and correctly adjusted bright field (Köhler illumination). The objective should be a plane-corrected objective. Phase contrast objectives do not provide sufficient resolution for assessment of sperm morphology due to the presence of the phase ring on the back face of the lens. Only consider intact spermatozoa, i.e. a sperm head with a tail. This includes situations where the amount of cytoplasm and a short tail length indicate that the spermatozoon is immature (i.e. is still at the elongating spermatid stage).

solutions (haematoxylin, Orange G6 and EA50, sedimented material appears and the solutions should be filtered to avoid these sediments being deposited on the slides.

[4] The last step of the staining procedure is to mount the smear, i.e. the slide is covered by a cover slip, which is "glued" in place by a mounting medium.

Immature germinal cells and mature spermatozoa

The presence of immature forms should also be noted. An immature germ cell is a "round cell". Spermatids in the ejaculate can have between one and four distinct blue-reddish, round nuclei located peripherally in the cell. Spermatocytes have only one centrally located nucleus.

In this way, immature forms can be expressed per 100 spermatozoa and their concentration in the original semen sample can be calculated from the sperm concentration. The formula for this calculation is:

Concentration of immature germ cells =
*number of immature cells counted per 100 spermatozoa (%) / 100 * sperm concentration*

Quality control

The WHO manual recommends that duplicate counts of 200 spermatozoa (i.e. 2 x 200 sperm) be performed if the diagnosis and treatment of the patient depends crucially on this analysis. ESHRE/NAFA recommend that duplicate counts of 200 spermatozoa should also be performed when the derived data are to be used in scientific publications. In routine practice, only 200 spermatozoa are assessed, and not in duplicate (i.e. 1 x 200 spermatozoa). When duplicate counts are performed, calculations are performed as follows:

(1) First calculate the average % normal and % abnormal and select the larger of these two groups for duplicate comparison.

(2) Calculate the difference between the two assessments in this group.

(3) If the difference is smaller than the value obtained from Appendix II, the assessments can be accepted. If the difference is larger than the value, two new assessments should be made.

If the smear is technically difficult to read, the smear kept in reserve should be stained with fresh solutions and assessed.

If the smear has disappeared during the staining process, one reason could be that the smear was too thick, another could be that the slides was not clean enough. Small, black-blue crystals on the stained smear indicate that the haematoxylin solution had not been filtered before use.

Calculations and Results

Transfer the following data to the report form:

1. % ideal spermatozoa (corresponding to WHO "% normal")
2. % head defects, % neck/mid piece defects, % tail defects, % cytoplasmic droplets
3. Teratozoospermia Index (TZI^{92}: average number of defects per defective spermatozoon; calculated by dividing the sum of the % defects (head defects, neck/midpiece defects, tail defects, and cytoplasmic droplet, i.e. all four groups of defects) by the % abnormal.[1]

Reagents

N.B. Distilled water means water, which has been deionized or double-distilled (the lack of ions should give a resistance of >10 MΩ/cm).

Fixation: Ethanol, 95% (see below).

Staining: ***Ethanol:*** different strengths of ethanol in water solution are used: 50%, 70%, 80%, 95%, 99,5% v/v. These solutions are prepared by volumetric dilution of 99,5% ethanol with distilled water and kept in tightly stoppered glass bottles at room temperature. *Do not store them in refrigerator due to explosion hazard!*

Acid ethanol:
150 mL 70% ethanol (v/v)
1.0 mL concentrated HCl (36 %)
50 mL distilled water

[1] The 1999 WHO manual classifies the calculation of a Teratozoospermia Index (TZI) as "optional". The recommendation of ESHRE/NAFA is that the Teratozoospermia Index should be calculated including all groups of defects, as defined by WHO 1992. However, to avoid confusion with the former index, which included also cytoplasmic droplets, ESHRE/NAFA suggests that the TZI should be given with the index 92 – TZI^{92} – to clearly refer to the earlier WHO recommendation.

Stains: **Harris Haematoxylin, EA50 and OG6** can be bought ready to use and stored at room temperature protected from light. Haematoxylin should be filtered (e.g. Reeve 802 filter paper) immediately before each use.
A maximum of 25-30 staining series can be performed with each batch of working stain solutions. If the intensity of colours becomes too weak or decreases rapidly on the slides (especially the blue colour), reagents should be exchanged for fresh prepared solutions. After 10 staining filtration should be used to remove debris (detached fragments of smears) and sedimented stain particles.

Mounting: Mountex, Eukitt, DePex, Permount, Pertex (See Table ”Mounting of stained smears”).

Equipment and materials

Microscope: 100X oil immersion, an objective corrected for irregularities in the surface of the lens, *not* phase contrast.
Microscope slides (standard size), cover slips (24x60 mm)
Conventional cytological equipment for the fixation and staining of smears

Antisperm antibodies

Principles

The spermatozoon evokes an immune-response when exposed to the systemic immune defense system. Therefore, trauma, e.g. vasectomy, in the male genital apparatus or inflammatory reactions in the male or female genital tracts can evoke the production of sperm directed antibodies. Depending on the nature and the localization of the sperm antigen and on the concentration of antibodies, different effects can be seen:

(1) Agglutination. The effect can be seen as agglutinates of moving spermatozoa in the semen sample.

(2) Cytotoxic effect. With serum (i.e. with active complement) spermatozoa will be killed. The presence of antibodies exerting a cytotoxic effect might be suspected if motility and vitality decline rapidly in a semen sample. This is a rare phenomenon and probably needs high concentrations of antibodies.

(3) Other effects as hampering passage through cervical mucus, and zona binding and passage.

Antibodies detected on the sperm surface are not always directed towards antigens originating from the spermatozoon, but may be directed towards loosely attached molecules originating from the accessory sex glands.

Antisperm antibodies

A description of methods for detecting sperm surface antibodies of IgG and IgA type is given below. Antibodies of IgG type can appear in the genital ducts by transudation from blood. Immunological cells "homed" into the mucosal linings of the genital tracts (e.g. the epididymis or the cervix) can produce antibodies of the IgA type.

Antibodies of the IgA-type have a special structure (secretor-part) that can bind to cervical mucins. If IgA is present on the sperm surface this means that the spermatozoa, via the IgA-antibody, can become attached to the cervical mucus. This can be seen as a "shaking phenomenon" of spermatozoa in cervical mucus. Antibodies binding to the sperm head can interfere with zona binding and the acrosome reaction, and thereby interfere with fertilization. Pregnancy has been achieved after *in vitro* fertilization in cases with up to 80% of sperm heads being covered with antibodies. In other cases, intra-cytoplasmic sperm injection has been used to overcome a tentative, immunological infertility, where up to 100% of spermatozoa have antibodies bound to their surface. Almost all cases with locally produced antisperm IgA-antibodies also secrete IgG-antibodies. Screening for sperm antibodies on spermatozoa has therefore often employed a test for antisperm IgG-antibodies. However, there are now tests available, by which surface bound IgA-antibodies can be assessed directly in the semen sample.

Tests for anti-sperm antibodies on sperm surface

IgG and IgA on spermatozoa can be assessed directly in semen with SpermMAR™ reagents for IgG and IgA. After washing, IgA and IgG can be identified on spermatozoa by the Immunobead™ test.

IgM–type antibodies are very rare, and considered of only limited importance in infertility. Their presence can reduce the availability of spermatozoa by heavy sperm agglutination.

Tests for free antibodies, directed against the sperm surface

For detection of free antibodies directed against spermatozoa in samples lacking spermatozoa and in serum or cervical mucus, donor spermatozoa without antibodies are used to bind any free antibodies in the examined fluids. Then, the antibodies bound to these donor spermatozoa are detected as described above for the direct tests.

Cervical mucus can be solubilized enzymatically (bromelain) or by sonication.

SpermMAR™ Tests

Principles

Direct SpermMAR™ IgG Test

The test uses latex particles coated with human IgG. Normally when spermatozoa are mixed with these latex particles nothing happens. However, in the presence of *anti-human* immunoglobulin, there are two possibilities. If the spermatozoa do not have antibodies on their surface they will be seen swimming without attached particles, whereas the particles (which do have antibodies on their surface) will be clumped together due to the antiserum. In contrast, if the spermatozoa have anti-bodies on their surface, the anti-human immunoglobulin will bind together the IgG localized on the particles and the spermatozoa. Motile spermatozoa, swimming with attached particles, will then be seen.

Direct SpermMAR™ IgA Test

The SpermMAR™ IgA Test contains latex particles that carry antibodies directed towards human IgA, i.e. *anti*-human IgA. Thus, after mixing spermatozoa and these anti-IgA coated particles, the particles will bind to spermatozoa *if* the spermatozoa have IgA on their surface.

Indirect SpermMAR™ Tests

Antibodies bound to donor spermatozoa, incubated in the fluid to be analyzed, are detected as described above for the direct tests.

Procedures

Direct SpermMAR™ IgG Test

(1) Put as separate equal-sized drops on a microscope slide: one droplet[1] of fresh unwashed semen, one droplet of IgG-coated latex particles and one droplet of antiserum to human IgG.
(2) Mix first, using a yellow pipette tip, the two droplets containing semen and IgG-coated particles. Then mix in the third droplet of antiserum. Put the coverslip on top. Assess after 3 and 10 minutes, under a 40X phase contrast objective, examining at least 200 motile spermatozoa (with beating tails) and classifying whether they carry particles.

Direct SpermMAR™ IgA Test

(1) Put as separate equal-sized drops on a microscope slide: one droplet[2] of fresh unwashed semen and one droplet of the anti-IgA-coated latex particles.
(2) Mix using a yellow pipette tip and put the coverslip on top. Assess after 3 and 10 minutes under 40X phase contrast objective and examine at least 200 motile spermatozoa (with beating tails): classify whether they carry particles.

Indirect SpermMAR- tests

(1) The indirect SpermMAR test is used to detect antisperm antibodies in fluids without spermatozoa (serum should be heat inactivated: 56°C, 45 minutes in plastic tube)
(2) Prepare motile donor spermatozoa by swim-up or density gradient centrifuga-tion. Adjust the final motile sperm con-centration to 20 million/mL.
(3) Dilute the sample to be tested serially, e.g. to 1/16 for the IgG-test and ¼ for the IgA-test. Dilute with swim-up medium, e.g. Earle's medium (pH 7.4) without albumin or serum.
(4) Mix 100 µL of the sperm suspension with 100 µL of the diluted test speci-men and incubate for 60 min at 37°C.
(5) Add 2 mL of medium, mix well, and centrifuge for 10 min at 400 *g*.

[1] With a droplet volume of 3,5 µL (semen, particles and antiserum) use a cover slip of 22 x 22 mm # 1,5 thickness. If 10 µL droplets are used as recommended by the manufacturer, a larger coverslip should be used (e.g. 24 x 40 mm # 1,5 thickness) otherwise the depth of the preparation will be too large and force the observer to continuously change focus.

[2] See footnote #1 (above).

(6) Remove the supernatant and resuspend the sperm pellet with 50 µL medium.
(7) Proceed as described above for the respective direct SpermMAR™-tests, steps 1 and 2, but use one droplet of the prepared sperm suspension instead of fresh semen.

Calculations and Results

Count at least 200 motile spermatozoa (with beating tails). The result is given as the percentage of motile spermatozoa carrying attached particles. If 50% or more of spermatozoa have latex particles attached, an immunological problem is probable.

Reagents

Reagents for SpermMAR™ IgG and IgA Tests are available from FertiPro N.V., Beernem, Belgium. Kits contain all the materials needed. For a large-scale andrology laboratory, bottles containing 0.7 mL of latex particle solutions with IgG or anti-IgA and 0.7 mL antiserum solutions (anti human IgG) can be ordered separately.

Immunobead™ Test

Principle

Antibodies bound to the human sperm surface can be visualized by other antibodies, which have been raised against human IgG, IgA or IgM immunoglobulin molecules. Immunobeads are plastic particles with attached *antihuman Ig antibodies*. Thus, anti-IgG, anti-IgA or anti-IgM Immunobeads detect spermatzoa with anti-sperm antibodies of the IgG-, IgA- and IgM-isotypes, respectively. For screening, Immunobeads designed for total B-cell labelling ("GAM" beads) can be used since these beads are coated with antibodies directed against all three immunoglobulin isotypes.

Indirect test

Antibodies bound to donor spermatozoa, incubated in the fluid to be analyzed, are detected as described above for the direct tests.

Procedures

Direct Immunobead-Test™

(1) For each Immunobead type, add 0.2 mL of stock bead suspension to 10 mL of Buffer I in separate conical-based centrifuge tubes.
(2) Determine the amount of semen to be used according to Table 6, and transfer the volume of semen needed to a tube and add up to 10 mL with Buffer I.
(3) Centrifuge all tubes at 500 *g* for 6 min at room temperature.
(4) Tubes with spermatozoa: Discard the supernatants. Resuspend the sperm pellets in 10 mL of fresh Buffer I and centrifuge again as above. Discard supernatants and resuspend sperm pellets in 200 µL of Buffer II.
(5) Tubes with beads: Discard the supernatants and resuspend the beads in 200 µL of Buffer II.
(6) Put 5 µL droplets of each Immunobead type on clean microscope slides. Add 5 µL of washed sperm suspension to each droplet of beads and mix well using a yellow pipette tip. Place a 22 x 22 mm # 1.5 coverslip on each of the mixtures.
(7) Leave the slides 10 min at room temperature in a moist chamber and then assess under a 20X phase contrast objective.

Table 6: Semen volume needed for analysis

Sperm Conc. (10^6/mL)	Motility grades a+b, (%)	Amount of semen to be used (µL)
>50		200
20-50	>40	400
20-50	<40	800
<20	>40	1000
<20	<40	2000
<10		>2000

Indirect Immunobead-Test™

(1) Wash normal donor spermatozoa twice in Buffer I as described above, steps 2 through 4, or prepare them initially by swim-up procedure or density gradient centrifugation procedure and wash thereafter. Adjust the washed sperm suspensions to a final motile sperm

concentration of 50 million/mL in Buffer II.

(2) Dilute 10 µL of the fluid to be tested with 40 µL of Buffer II and mix with 50 µL of the washed donor sperm suspension. Incubate at 37°C for 60 min.

(3) Wash the spermatozoa twice as described above (steps 2 through 4) and perform the test as described in step 6 above.

Calculations and Results

Assess only motile spermatozoa and score the percentage that has two or more attached Immunobeads (ignore binding to the tip of the tail). Count at least 200 motile spermatozoa in duplicate for every preparation. Record the percentage of spermatozoa carrying attached beads, the immunoglobulin class (IgG or IgA) and the site of binding (head, mid-piece, tail)

Reagents:

- Immunobeads: anti-IgG, -IgA and IgM beads (Irvine Scientific, Santa Ana, California 92705, USA; Laboserv GmbH, Am Boden 25-26, 35460 Staufenberg, Germany; Labsystems AB, Sweden).
- For screening, beads for total B-cell labelling can be used.
- Reconstitute the Immunobeads according to manufacturers instructions. Beads can be kept for several months at +4°C in the original buffer which contains preservative (azide).
- Stock buffer: Tyrode's solution or Dulbecco's phosphate-buffered saline (PBS) can be used. (Table 7),
- Buffer I (0,3% BSA): Buffer for bead washing (10 mL) and sperm washing (2 x 10 mL for each semen sample). Add 0.6 g BSA (bovine serum albumin, Cohn fraction V) to 200 mL Stock Buffer. 200 mL Buffer I is enough to wash and run 6 unknown samples, one positive and one negative control and two sets of IgA and IgG beads)

Table 7: Tyrode's and Dulbecco's solutions

Constituents	Tyrode's solution (g/L)	Dulbecco's PBS (g/L)
$CaCl_2$	0.2	0.1
KCl	0.2	0.2
NaCl	8.0	8.0
NaH_2PO_4	0.05	--
$MgCl_2$ ' $6H_20$	0.2	0.1
$NaHCO_3$	1.0	--
$Na_2HPO_4 \bullet 7H_20$	--	2.16
KH_2PO_4	--	0.2
Glucose	1.0	--

- Buffer II (5% BSA): Buffer for resuspension of beads and sperm pellets, 200uL for each specimen. Add 250 mg BSA to 5 mL stock buffer. 2 mL Buffer II is needed for 6 samples, 2 controls and 2 sets of beads.
- Filter all solutions through 0.22 µm or 0.45 µm filters and warm to 25 to 35°C before use.

Quality control for SpermMAR™ and Immunobead™ Tests

All methods are dependent on motile spermatozoa. At least 200 motile spermatozoa should be assessed for each test.

A positive control and a negative control should be included in each run. A positive control means serum from a donor with high titres of anti-sperm antibodies as detected by the indirect SpermMAR™ or Immunobead™ tests. This serum is prepared and assayed in each run.

Limitations

Results are based on the analysis of motile spermatozoa. Consequently, in samples with poor motility, false negative results may be obtained.

Results from SpermMAR™ and Immunobead™ tests are not fully concordant. A positive finding of more than 50% of motile spermatozoa with attached beads is regarded to be of clinical significance.

Equipment and materials

- Microscope with 40X phase contrast objective
- Microscope slides (standard size) and coverslips (22 x 22 mm # 1.5 thickness)

Quality Control and Quality Assurance

The importance of a distinct policy for Quality Assurance (QA) has become more evident during the last decade. This chapter will focus on some aspects of Quality Assurance and important procedures of the underlying technical Quality Control (QC).

QC is only one part of QA. It is beyond the aims of this manual to cover all details of QA, and we will focus only on some basic aspects of QC in relation to the WHO manual.

The fourth edition of the WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction (1999) has a new, comprehensive chapter dealing mainly with technical Quality Control issues.

Quality Control

Two main parts of QC can be distinguished: Internal QC (IQC) and external QC (EQC). All laboratories must have IQC to be able to provide reliable results. EQC is necessary to allow comparisons of results from different laboratories. However, EQC is only valid and useful if the laboratory also runs an effective IQC programme. If basic, internal control does not work properly, the results of the EQC will be random. **Thus, participation in an EQC program cannot replace the IQC component in an individual laboratory.**

Internal Quality Control IQC

IQC has many different levels:

- Control at individual analysis-level
- A basic IQC program
- Monthly averages of results
- Control of equipment (calibration of pipettes, regular maintenance of instruments and other equipment such as counting chambers)

Control at individual analysis-level

One basic aspect of IQC is **duplicate assessments** with verification that the duplicates do not differ too much. A large difference increases the risk that a random error has occurred somewhere in the process. With the recommended method for comparison, the most important factor is the *number of cells assessed*. In Graph 2 (Appendix II), the 95% Confidence Interval for proportions is shown in relation to number of assessed spermatozoa. It is obvious that the interval – and the uncertainty of results – is large when less than 200-400 spermatozoa have been assessed. In Graph 1 (Appendix I), a relative (%) 95% Confidence Interval is shown for sperm concentration. It is obvious that the uncertainty of assessments is large, when less than 200-400 spermatozoa have been assessed.

Another, equally important, aspect of IQC at analysis-level, is the knowledge of the technologist – awareness of sources of errors, weaknesses of the methods and equipment used, and also understanding of the biological and clinical context of the analysis.

Basic IQC programme

A basic programme for running IQC should include replicate assessments of the main semen variables by different technologists. This could be organized in different ways, depending on the structure of the particular laboratory. It is important to comply with the following points:

- Sperm concentration, motility, morphology and vitality must be included.
- All technologists participating in analysis work must be included.
- All IQC assessments must be done with the same – or equivalent – methods and equipment as used for routine analyses. For motility assessments this will require that (1) IQC samples are recorded, and that (2) assessments of the routine samples are done with the same equipment as used to view the IQC recordings (i.e. on a video monitor, but note that this does *not* require that the routine samples be videotaped).

Monthly averages of results

This measurement is widely used in other clinical laboratory disciplines, but needs further evaluation in Clinical Laboratory Andrology due to expected seasonal variations in sperm production (cf. Vierula *et. al.: High and unchanged sperm counts of Finnish men*. Int. J. Androl. 1996, 19: 11-17). With properly applied IQC the monthly averages should not show other changes than those due to seasonal

or other true changes in the population examined.

Control of equipment and instruments

The documentation of methods must also include specifications for calibration and maintenance. There should also be a record for each instrument to document calibration results and all repairs and modifications that have occurred.

External Quality Control EQC

The extent of the EQC is limited mainly by practical reasons – expense (time and cost) for the production of high quality material and their transportation to the various laboratories. It must be based on good IQC because with a few assessments, the EQC can focus on accuracy (i.e. how correct the result is). A laboratory with high precision (low variation) controlled by good IQC will also provide stable results to an EQC programme, while a laboratory with low precision is more likely to produce variable results to an EQC programme.

Also the material used in an EQC program should be assessed with methods and equipment equivalent to those used for routine analysis to be valid for the clinical analyses. This calls for a much more stringent international standardization of both basic methods and QC programmes than are available today. Therefore, the aims of ESHRE (courses on Basic Semen Analysis, and development and initiation of EQC programs) and NAFA (with the joint courses on Basic Semen Analysis and the Nordic Network for EQC) and, of course, this manual are of the utmost importance for the qualitative development of semen analysis.

With the full operation of an EQC, including remedial action for laboratories that submit poor EQC results, a true External Quality Assurance programme (EQAP) will develop.

Quality Assurance

The modern concept of QA encompasses all efforts to ensure a high quality of the service or product provided. This includes many different aspects:

- The overall organization with clearly identifiable responsibilities for all individual staff members.
- Requirements for competence of all staff members.
- Continuous education of all staff members.
- Documentation of procedures for all services provided (including technical methods for analyses and equipment, routines for contacts with patients and referring physicians)
- Documented routines for handling of errors in all procedures: actions to correct the occurred error, and actions to prevent recurrence of the error.
- Internal and external Quality Control to decrease errors in the results due to method variations and low accuracy, and allow comparisons with other laboratories

Appendix I

SUM	VALUE	SUM	VALUE
969-1000	61	376-395	38
938-968	60	357-375	37
907-937	59	338-356	36
876-906	58	319-337	35
846-875	57	301-318	34
817-845	56	284-300	33
788-816	55	267-283	32
760-787	54	251-266	31
732-759	53	235-250	30
704-731	52	219-234	29
678-703	51	206-218	28
651-677	50	190-205	27
625-650	49	176-189	26
600-624	48	163-175	25
576-599	47	150-162	24
551-575	46	138-149	23
528-550	45	126-137	22
504-527	44	115-125	21
482-503	43	105-114	20
460-481	42	94-104	19
438-459	41	85-93	18
417-437	40	76-84	17
396-416	39	67-75	16
		59-66	15
		52-58	14
		44-51	13
		38-43	12
		32-37	11
		27-31	10
		22-26	9
		17-21	8
		13-16	7
		10-12	6
		7-9	5
		5-6	4
		3-4	3
		2	2
		1	1
		0	0

This appendix is a table to help finding out if two duplicate assessments of sperm concentration are close enough to be accepted.

Search the **total number of spermatozoa** counted in the *left* column (SUM). If the **difference** between the two assessments is *less or equal to the value* in the *right column* (VALUE), the assessments can be accepted

If the difference between the duplicates is *above* the VALUE, the assessment must be *discarded* and two new chambers prepared and assessed. The shadowed part of the table is where too few spermatozoa have been counted, meaning that the confidence interval in the final result (millions/mL) becomes larger than the ± 10% interval (cf Table 5, Chapter 2).

This table is calculated from the formula given in the WHO manual, page 37, and complies with Figure 2.4, on page 117 of the WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction (1999).

Confidence in the result due to number of counted spermatozoa

How certain a result of sperm concentration is, depends to a large extent on the number of spermatozoa actually counted. The table to the right gives the confidence intervals for results obtained by assessing different numbers of spermatozoa.

If you have counted 10 spermatozoa to get the result 1 million/mL in a Makler chamber, the confidence of your result is between 0.4 and 1.6 million/mL (1 million ± 62% according to the table). Thus, your result may vary up to 4 times. If you instead have counted 100 spermatozoa in an improved Neubauer haemocytometer, the confidence of your result is between 0.8 and 1.2 million/mL (1 million ± 20%). To reach the confidence ± 10% (0.9-1.1 million) you would need to assess 4 improved Neubauer haemocytometers or 40 Makler chambers.

Counted	± %
1	196
5	88
10	62
20	44
30	36
40	31
50	28
60	25
70	23
80	22
90	21
100	20
150	16
200	14
250	12
300	11
400	10
500	9
600	8
700	7
1000	6
1300	5
1900	4
3200	3

Appendix II

Average %	Limit difference
0	1
1	2
02-03	3
04-06	4
07-09	5
10-13	6
14-19	7
20-27	8
28-44	9
45-55	10
56-72	9
73-80	8
81-86	7
87-90	6
91-93	5
94-96	4
97-98	3
99	2
100	1

This appendix is a help to finding out when duplicate assessments of **percentages** (e.g. % motile spermatozoa, % normal spermatozoa, or (optional) % live spermatozoa) can be accepted.

Since the recommendation is that 200 spermatozoa should be counted in duplicate (2 x 200) values are only given for this sample size. This means that the table below is not valid for duplicate counts of 100 spermatozoa (i.e. 2 x 100).

Calculate the **average** (rounded off to an integer) for the two percentages and the **difference** between them. Look up the row corresponding to the **average** percentage (*left column*). The *difference* between the two assessments must be *less than or equal to* the **limit difference** given in the *right column*. If the difference is greater than the limit, the assessments must be discarded and two new be done.

This table is calculated from a formula[1] based on the binomial distribution needed to determine asymmetrical confidence intervals for proportions (which have absolute minimum and maximum, here 0% and 100%). The formula given in the WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction (1999), page 40, is not adequate, especially not for proportions close to the endpoints.

On next page, a table is given to show the confidence in your result with respect to the number of spermatozoa you have assessed to obtain the result.

[1] Cf. Motulsky H: Intuitive Biostatistics, Oxford University Press, 1996.

The confidence in your results due to number of assessed spermatozoa

Average percentage found	Total number of spermatozoa assessed		
	400 (2x200)	*100*	*200*
	confidence interval		
0	0-1	0-4	0-2
1	0-3	0-5	0-4
2	1-4	0-7	1-5
3	2-5	1-9	1-6
4	2-6	1-10	2-8
5	3-8	2-11	2-9
6	4-9	2-13	3-10
7	5-10	3-14	4-11
8	6-11	4-15	5-13
9	6-12	4-16	5-14
10	7-13	5-18	6-15
11	8-14	6-19	7-16
12	9-16	6-20	8-17
13	10-17	7-21	9-18
14	11-18	8-22	10-20
15	12-19	9-24	10-21
20	16-24	13-29	15-26
25	21-30	17-35	19-32
30	26-35	21-40	24-37
35	30-40	26-45	28-42
40	35-45	30-50	33-47
45	40-50	35-55	38-52
50	45-55	40-60	43-57
55	50-60	45-65	48-62
60	55-65	50-70	53-67
65	60-70	55-74	58-72
70	65-74	60-79	63-76
75	70-79	65-83	68-81
80	76-84	71-87	74-85
85	81-88	76-91	79-90
90	87-93	82-95	85-94
91	88-94	84-96	86-95
92	89-94	85-96	87-95
93	90-95	86-97	89-96
94	91-96	87-98	90-97
95	92-97	89-98	91-98
96	94-98	90-99	92-98
97	95-98	91-99	94-99
98	96-99	93-100	95-99
99	97-100	95-100	96-100
100	99-100	96-100	98-100

When you assess a proportion (as % Motile or % Normal spermatozoa) the total number of spermatozoa examined will influence how certain the proportion you obtain is. The higher number of spermatozoa that have been examined, the more certain the result will be. This can be described as the ”95% Confidence Interval” of the obtained proportion[2]. The following table will give an idea of how certain different proportions are in relation to the total number of spermatozoa examined.

The recommendation is that at least 400 spermatozoa are assessed (2x200 when assessment in duplicate). In the table data is given also for 100 and 200 assessed spermatozoa for comparisons and for cases where less than 400 spermatozoa are available for assessments.

Example

We obtain the result 6% normal spermatozoa after examining the recommended 2 x 200 spermatozoa. The true proportion is most likely in the range 4-9%. If we had examined only 100 spermatozoa the range would be 2-13%. Thus, the certainty of the result is higher if we count 400 spermatozoa instead of 100.

Correspondingly, the result 50% progressively motile spermatozoa has a confidence range of 45-55% if 400 spermatozoa have been assessed, and 40-60% if only 100 have been assessed.

[2] The 95% Confidence Interval of a proportion means that there is 95% probability that the true value of the proportion is within the interval. This interval cannot reach below 0% or above 100%.