
Basic semen examination — Specification and test methods

Analyse de base du sperme — Spécifications et méthodologie analytique

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

This document was developed in response to global demand for standards for reliable examination of human semen. The five editions of a laboratory manual for human semen analysis published by the WHO between 1980 and 2010 have provided general recommendations for suitable laboratory procedures, but even the latest edition (World Health Organization 2010 [16]) does not constitute a Technical Standard adequate for use under ISO 15189.

A Technical Standard based on best available evidence and global consensus regarding laboratory procedures most likely to give reliable results will facilitate any laboratory seeking accreditation for human semen examination. Subjects, and biomedical science in general, would benefit from fewer random factors affecting the accuracy of results. Clinically this would support improved diagnoses as well as provide more objective grounds for choosing between possible management strategies or alternative treatment modalities. Furthermore, to support the evaluation and validation of new methods to improve the diagnosis and treatment of infertility, these standardized techniques can serve as reference methods.

The pre-examination preparation of human semen is important not only in manual basic semen examination, but also for Computer-Aided Sperm Analysis (CASA). Standardized handling and preparation of semen samples is essential to the quality of the data obtained.

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Basic semen examination — Specification and test methods

1 Scope

This document specifies the minimum requirements for equipment and critical aspects of the test methods for best practice in laboratories performing basic examination of human semen collected by ejaculation.

This document is applicable to the entire process of basic manual semen examination and also to sample preparation for Computer-Aided Sperm Analysis (CASA).

This document does not apply to the post-vasectomy assessments.

NOTE Given the medico-legal ramifications surrounding the evaluation of post-vasectomy ejaculates, the methodology in this document is in all likelihood inadequate to establish an ejaculate as being completely “clear” (i.e. no spermatozoa in the ejaculate).

2 Normative References

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 15189, *Medical laboratories — Requirements for quality and competence*

ISO/TS 20914, *Medical laboratories — Practical guidance for the estimation of measurement uncertainty*

3 Terms and Definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

air displacement pipette

common laboratory pipette with disposable tips where the volume aspirated is controlled by the displacement of an equivalent volume of air inside an enclosed chamber inside the pipette handle

Note 1 to entry: An air displacement pipette can only give accurate volumes for liquids with viscosity close to that of water.

3.2

azoospermia

complete absence of spermatozoa in the *ejaculate* (3.4)

Note 1 to entry: The term azoospermia is not a clinical diagnosis but a description of a laboratory finding. Complete lack of spermatozoa is difficult to determine in absolute terms. Since only parts of an *ejaculate* (3.4) can be examined, the modern definition is based on probability calculations derived from data obtained from investigations of random aliquots from an *ejaculate* (3.4) (See [Annex A](#)).

3.3

CASA

computer-aided sperm analysis

automated examination of *ejaculates* (3.4) with equipment using imaging technology

Note 1 to entry: Examination based on image analysis of video sequences to obtain information on *sperm concentration* (3.18) and motility, more seldom sperm morphology.

Note 2 to entry: There are CASA systems commercially available, but no common standard for validation, evaluation, reliability in analyses or contents of reports. The scope of this document is not to provide a standard for CASA, although the pre-examination aspects can be useful also to developers, manufacturers, and users of CASA equipment.

3.4

ejaculate

semen sample, which is a mixture of spermatozoa and secretions, mainly from the seminal vesicles, the prostate and the epididymides

Note 1 to entry: The ejaculate can be obtained by various methods including masturbation, intercourse, vibratory stimulation or electro-ejaculation.

3.5

ejaculate viscosity

property of an *ejaculate* (3.4) describing its resistance to flow like water after *liquefaction* (3.10)

Note 1 to entry: Incompletely liquefied semen is not a homogenous liquid due to the contents of gelatinous structures in the ejaculate fluid.

3.6

high power field

area of a slide which is visible in the microscope under high power magnification ($\times 400$)

Note 1 to entry: This is not a standard field area as the size varies according to the type of oculars used (e.g. standard or wide field) (see [Annex B](#)).

3.7

immotile

total lack of active tail movements

3.8

interlaboratory comparison

organization, performance and evaluation of measurements or tests on the same or similar items by two or more laboratories in accordance with predetermined conditions

[SOURCE: ISO/IEC 17043:2010, 3.4]

3.9

ideal spermatozoon

spermatozoon with the morphology typical of spermatozoa able to penetrate into and migrate within cervical mucus and reach the site of fertilization

[SOURCE: Menkveld, et al., 1991,^[9] Menkveld and Kruger, 1995^[10]]

3.10

liquefaction

process of change in the consistency of the *ejaculate* (3.4) from gel-like or coagulum-like into a liquid phase

Note 1 to entry: Liquefaction occurs due to degradation of the gel-like or coagulum-like property, by enzymatic action on macromolecules.

3.11**non-progressive sperm motility**

active tail movements leading to a sperm propagation of less than approximately 5 $\mu\text{m/s}$

Note 1 to entry: A normal head length is approximately 5 μm .

3.12**positive displacement pipette**

common laboratory pipette working by piston-driven displacement within a capillary, not the displacement of air within an enclosed chamber

Note 1 to entry: The piston in the pipette tip is in direct contact with the liquid specimen.

Note 2 to entry: Use to avoid major volume errors with viscous liquids like semen.

3.13**progressive sperm motility**

forward motility of a spermatozoon of at least 5 $\mu\text{m/s}$

Note 1 to entry: See also *slow progressive sperm motility* (3.16) and *rapid progressive sperm motility* (3.14).

Note 2 to entry: Spermatozoa moving in circular paths are considered progressive based on space gain.

3.14**rapid progressive sperm motility**

forward motility of a spermatozoon of at least 25 $\mu\text{m/s}$

3.15**sexual abstinence**

time between the collection of *ejaculate* (3.4) for analysis and the most recent previous ejaculation

Note 1 to entry: Expressed in days or hours as appropriate for the intended use.

3.16**slow progressive sperm motility**

forward motility of a spermatozoon of at least 5 $\mu\text{m/s}$ but less than 25 $\mu\text{m/s}$

3.17**specimen collection container**

receptacle used to collect primary samples

Note 1 to entry: Specimen collection container shall be not toxic to spermatozoa.

Note 2 to entry: If an *ejaculate* (3.4) can only be collected at sexual intercourse, a non-toxic, Silastic™ condom can be used. The *ejaculate* (3.4) shall be transferred to an ejaculate sample container upon receipt by the laboratory; this shall be noted in the report form.

3.18**sperm concentration**

number of spermatozoa per unit volume

Note 1 to entry: Sperm concentration is expressed in millions or thousands/millilitre.

Note 2 to entry: It shall not be confused with sperm density (mass/volume).

3.19**sperm vitality**

percentage of vital spermatozoa, independent of their ability to move

3.20

total sperm number

calculated total number of spermatozoa in the *ejaculate* (3.4)

Note 1 to entry: Total sperm number is the *sperm concentration* (3.18) multiplied by the *ejaculate* (3.4) volume.

Note 2 to entry: Total sperm number is not the same as *sperm concentration* (3.18).

3.21

Tygerberg strict criteria

sperm morphology criteria based on the morphology of spermatozoa able to penetrate into and migrate within cervical mucus

3.22

Teratozoospermia Index

TZI

average number of defective regions (head, neck/midpiece, tail, and/or cytoplasmic droplet) in abnormal spermatozoa

Note 1 to entry: This index is, by definition, never outside the interval of [1.00;4.00].

4 Staff Training and Competence

4.1 General Aspects

General requirements for staff training and competence are covered in ISO 15189. How these requirements are applied to human semen analysis is covered here.

4.2 Training

4.2.1 General

Semen examination involves many analytical steps that require operator training to minimize subjectivity in order to provide accurate reliable results^{[7][12][1]}.

4.2.2 Training for quantitative assessments

All assessors performing assessments of sperm motility, sperm concentration, sperm vitality and/or sperm morphology shall receive training using either commercial, in-house or EQA-derived validated reference materials to ensure that their results conform to the laboratory's pre-determined measurement error limits. Without such training staff cannot be expected to be able to provide accurate or reliable results for these assessments, and participation in EQA schemes is pointless.

NOTE Effective goal-oriented reiterative training procedures for these assessments have been published^{[12][14]}; a $\pm 10\%$ range of measurement error is expected between novices upon completion of their training and the laboratory's experienced staff (see also [Annex C](#)).

4.2.3 Training for qualitative assessments

Competency training for qualitative assessments, such as viscosity and round cells, shall achieve agreement between trainee and expert in at least 90 % of cases.

4.2.4 Training for pH assessment

The ability of assessors to read test strips against the comparator scale shall be verified.

4.3 Maintenance of Competence

Ongoing verification of competence shall be demonstrated by all personnel performing these assessments at regular intervals as defined in the laboratory's quality framework.

NOTE According to 4.2, the same $\pm 10\%$ range of measurement error is expected for ongoing verification of competence by all trained staff performing these assessments.

5 Semen Characteristics, Sampling and Pre-Examination Handling

5.1 General Characteristics

Examination of the ejaculate is in some important aspects different from investigations of other human bodily fluids. The subject is expected to accomplish the collection of the ejaculate. Results are dependent on ejaculation frequency before collection, as well as on the time and temperature before initiation of investigations. In case of infertility diagnosis, clear reference limits are missing due to the fact that the desired outcome is dependent on the particular clinical situation of each couple trying to achieve a pregnancy.

5.2 Physical and Chemical Characteristics

There is no internal homeostatic control in an ejaculate collected in a device for laboratory investigations. Initially the entire ejaculate is incorporated into a gel-like coagulum that is gradually degraded (liquefaction) into a still viscous but more water-like liquid. During this process carbon dioxide evaporates causing a change in pH. Enzymatic degradation of gel components causes a significant increase in osmotic properties of the liquid surrounding the spermatozoa, which in turn affects sperm performance.

5.3 Sample Collection and Initial Handling

Sample collection shall, except for some men with, for example, disabled limbs, spinal cord injury or paraplegia, always be done by the subject. If necessary, the subject's partner can help with sample collection. For subjects with ethical or religious objections to masturbation a non-spermotoxic (Silastic™¹⁾) condom can be used to collect an ejaculate during intercourse. However, this collection method will result in some loss of the overall sample as it is recovered from the condom. Collection of ejaculates by coitus interruptus ("withdrawal") is not recommended as the first, sperm-rich, fraction of the ejaculate is often lost. Use of lubricants can be necessary by some subjects; such products shall be validated as non-toxic to spermatozoa^[13].

After ejaculation, the sample shall be kept as close as possible to 37 °C and never higher; cooling or warming can cause artefacts and sperm dysfunction. Due to all the changes occurring after ejaculation, investigations shall start as soon as possible after liquefaction, that typically is completed within 30 min after ejaculation. Incomplete liquefaction at 60 min after ejaculation indicates an abnormality. Initiation of assessments after completion of liquefaction is best achieved if the ejaculate is collected near the laboratory. Since the duration and level of sexual arousal experienced by the subject will affect the ejaculation, sample collection could be best performed in a place chosen by the subject in case of major difficulty. When an ejaculate is collected outside the lab environment it shall be delivered to the laboratory, preferably within 30 min, but at least within 60 min (circumstance for ejaculate collection and transport shall be noted in the report). Nonetheless, considerations of temperature and time to investigation remain important for the quality and robustness of the examination.

1) Silastic™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5.4 Subject Information and Data Collection

5.4.1 Information to be Provided to Subjects

The following information shall be provided to the subject in writing in a language understandable by the subject and shall include the following:

- a) General information:
 - Contact information for the laboratory;
 - The reason for the investigation if made available from the requester;
 - An outline of what will be investigated;
 - How results of the laboratory investigations will be communicated to the subject.
- b) Ejaculate collection, handling and transportation:
 - How to collect the ejaculate;
 - Effect of delay between sample collection and initiation of assessments;
 - Importance of avoiding cooling down or warming up of the ejaculate;
 - Importance of reporting correct sexual abstinence time;
 - Importance of reporting any incompleteness of sample collection.

5.4.2 Data Collection from the Subject

a) Required information

Each subject shall be asked to provide the following information to be recorded by the laboratory:

- Reliable personal identification (at least two unique identifiers attributable to the patient and specified by the organization);
- Duration of sexual abstinence;
- Time of sample collection;
- Transport of ejaculates should be avoided but if not collected at the premises of the laboratory: confirmation that during transport to the laboratory the specimen was protected from extremes of temperature;
- Completeness of sample collection; in case of incomplete collection, with information of which parts in the sequence of ejaculation that have been missed in collection.

b) Additional information

Information that is of importance to the clinical interpretation and that can be practical to obtain when the subject visits the laboratory. The collection of this information is, however, not part of the laboratory work:

- Medical history, which can include:
 - Any episode of severe inflammatory process the last three months;
 - Any previous surgery (inguinal hernia, varicocele, cryptorchidism or other problems related to the urogenital sphere) or treatment with chemotherapy, cytostatics or radiation of the urogenital organs;

- Any use of pharmaceutical drugs except short term use of non-prescription drugs (e.g. pain killers, and anti-allergy drugs).
- Any use of recreational drugs, anabolic steroids or other performance enhancing dietary additions (like protein powders).

5.5 Initial Sample Handling

- Every ejaculate should be considered potentially infectious and handled accordingly (see ISO 15190:2020, Annex B).
- Information provided by the subject shall be recorded.
- Specimen collection container shall be clean, non-toxic and for single use.
- Specimen collection container should preferably be weighed before sample collection and its weight recorded in grams with two decimal places.
- Ejaculate volume should preferably be determined by weight. In this case the specimen collection container is weighed (recorded in grams with two decimal places) before and after specimen collection and the weight difference used as the volume, assuming 1,0 g of ejaculate equals 1,0 ml of ejaculate [3]. If a calibrated serological pipette is used, some semen will always be lost in the specimen collection container and inside the pipette after making the measurement. The lab should be aware of the differences of the two methods. The ejaculate volume shall be reported in ml to one decimal place.
- All documents and the specimen collection container shall be labelled with at least two unique identifiers.
- As soon as possible after collection the specimen collection container shall be kept at a temperature between 35 °C and 37 °C to facilitate liquefaction and prepare for motility assessment at standardized temperature, preferably on a moving tray to enhance mixing during liquefaction (frequent manual agitation is required when moving tray is not available).

Spermatozoa are affected by the earth gravity and sediment to the bottom of any container (“geotaxis”) even if they are motile. Consequently, when sampling an ejaculate, it shall be well-mixed to evenly distribute the spermatozoa and other elements of the ejaculate. Even sitting for a short period of time will result in an uneven distribution of the cellular elements of the ejaculate. It is therefore important to gently mix the ejaculate thoroughly before any aliquot is taken for examination, noting that a vortex mixer shall not be used.

5.6 Sperm Toxicity Testing

To ensure that materials in contact with ejaculates (specimen collection container, pipette tips) are not toxic to spermatozoa, a basic toxicity test shall be performed on every new batch of material. The principle is based on comparison of motility of spermatozoa exposed to present material and the new material [6]. The time of exposure shall be at least twice the expected time of exposure of sperm to the material – seconds for pipette tips and 30 min to 60 min for sample collection containers.

6 Examinations

6.1 Required Equipment

Sperm motility is largely influenced by the ambient temperature, especially regarding velocity. The use of temperature-controlled equipment reduces the influence of variable room temperature.

The following equipment is required:

- Laboratory balance, range 0,00 g to 50,00 g (reading to two decimal places);

- An incubator or warm plate that can maintain the ejaculate at human body temperature, preferably including a moving tray (orbital mixer);
- Upright light microscope with phase contrast (10×, 20× and 40× objectives recommended) and bright field (100× oil immersion, high resolution objective) optics, and 10× oculars and an ocular micrometer (or an ocular reticle or grid calibrated with a stage micrometer scale), and means of keeping wet preparations at body temperature (e.g. heated stage);
- Positive displacement pipette for the assessment of sperm concentration, 0 µl to 50 µl or 0 µl to 100 µl capacity;
- Air displacement pipettes (1 µl to 20 µl, 20 µl to 200 µl and 200 µl to 1 000 µl sizes) for concentration diluent, wet preparations and other preparations;
- Equipment to stain and mount morphology and vitality slides (slide holders, staining jars, disposable pipettes for mounting medium);
- Centrifuge (e.g. for 15 ml conical centrifuge tubes) – a swing-out bucket is preferred to produce more discrete pellets – and either sealed buckets or a sealed rotor to protect operators from possible aerosol contamination should a tube break during centrifugation;
- Haemocytometers with Improved Neubauer ruling (100 µm depth).

NOTE 1 The majority of international experts recommend using haemocytometers with Improved Neubauer ruling [12][14][16]. Other patterns of haemocytometers can be used so long as the correct calculation factors are employed

NOTE 2 Non-disposable haemocytometers need to be checked regularly that wear and tear does not change the depth of chamber. Disposable haemocytometers can be used provided they are properly validated and evaluated[5].

NOTE 3 Makler chambers have lower accuracy than haemocytometers[17].

NOTE 4 Some disposable counting chambers intended for urine analysis have insufficient accuracy for this purpose[17].

NOTE 5 Fixed depth chambers using capillary action are subject to the Segré-Silberberg effect and hence will have a variable error[4].

- Humid chamber for sedimentation of spermatozoa in haemocytometers
- Vortex mixer (for agitating fixed sperm suspensions for assessment of concentration)

6.2 In-house Prepared Reagents

A diluent for sperm concentration assessment is essential and can be prepared in-house (See [Annex D](#)). The purpose is to immobilize (kill) spermatozoa to make counting more reliable. For the ease and reliability of assessment it is also an advantage if growth of micro-organisms can be prevented.

6.3 Assessments

6.3.1 Initiation of Assessments

For a reliable assessment of ejaculate characteristics liquefaction shall be completed. For most ejaculates this is achieved within 30 min if kept at 37 °C after collection. If liquefaction is not completed, the sample can be left in the incubator for a further period of time (maximum 30 min, so that motility can be assessed within 60 min after collection), after which assessments shall be initiated. Incomplete liquefaction at the start of examination shall be noted in the report as well as the time between sample collection and initiation of wet preparation assessment.

6.3.2 Macroscopic Assessment

In the absence of metrological standards for physical characteristics of human semen it is not possible to achieve proper standardization of methods or establish an uncertainty of measurement. However, some observations regarding colour or perception of its odour can have clinical relevance concerning the provenance of the specimen or the subject's medical status. Consequently, any such comments included in the report are considered as having been reported by exception, i.e. as attempts to describe observations of characteristics that are outside the expected.

a) Visual appearance:

- 1) Colour (Normal: a wide range of opalescent, greyish-white, sometimes slightly or even bright yellowish. Abnormal: brownish or red, transparent; strong yellow);
- 2) Liquefaction (should be complete within 30 min after ejaculation at 37 °C; abnormal: remaining gel clumps).

b) Other physical observations:

- 1) Viscosity – measured by allowing semen to slowly drop by gravity from a wide bore pipette (e.g. a 5 ml serological pipette or a non-toxic glass or plastic Pasteur pipette). Normal: discrete droplets with < 2 cm “threads”;
- 2) Odour (there is no “normal” smell to semen, and its odour is highly subjective. However, a strong, putrescent smell is often indicative of an active infection; a mild putrescent smell might indicate prolonged abstinence time or a strong urine smell could indicate contamination of the semen with urine);
- 3) Semen pH – measured by placing an aliquot of liquified semen on a validated pH test strip within 30 min after ejaculate collection at least in case of absence of spermatozoa and low semen volume.

6.3.3 Direct Microscopy of the Wet Preparation

A wet preparation shall be made by placing a 10 µl aliquot of well-mixed semen on a labelled pre-warmed microscope slide and covering it with a pre-warmed 22 mm × 22 mm cover slip (thickness #1½ or #2, to allow full spreading of the droplet) to obtain a preparation depth of about 20 µm (for 18 mm × 18 mm cover slips only 6,5 µl is required for the same depth).

- Observe the presence of spermatozoa, other cells, debris, crystals.
- Observe the presence of sperm agglutinates and aggregates.
- Estimate the suitable dilution for the assessment of sperm concentration (See [Annex E](#)).

Observations of other cellular and non-cellular elements that could be present in a human ejaculate all lack metrological standards, and cannot therefore be assessed objectively, even using qualitative descriptions because no metrological standards are available. However, some observations can have clinical relevance concerning the subject's medical status. Any such comments included in the report are therefore to be considered as having been reported by exception, i.e. as attempts to describe observations that are outside the expected. The laboratory shall ensure it can demonstrate minimal difference in subjectivity between practitioners.

6.3.4 Sperm Motility Assessment

- Shall be performed on two independent wet preparations from a well-mixed sample at 35 °C to 37 °C.
- At least four different fields shall be assessed in each replicate.
- At least 200 spermatozoa shall be classified in each replicate.

Classify each spermatozoon as being either rapid progressive, slow progressive, non-progressive or immotile (see [Table 1](#)).

Table 1 — Definitions of sperm motility classes

Class	Motility type
Rapid progressive (a)	Active tail movements, progression at least 25 $\mu\text{m/s}$
Slow progressive (b)	Active tail movements, progression at least 5 $\mu\text{m/s}$ but less than 25 $\mu\text{m/s}$
Non-progressive (c)	Active tail movements, progression 0 $\mu\text{m/s}$ to 4 $\mu\text{m/s}$
Immotile (d)	No active tail movements

NOTE 1 In each field first count the rapid and slow progressive spermatozoa. Then count the non-progressive and immotile spermatozoa within the same field. If the concentration of spermatozoa is very high, it is advisable to only count the spermatozoa seen in a smaller field area, e.g. in the central four squares of an eyepiece grid or reticle.

NOTE 2 Motile and immotile spermatozoa that are bound in agglutinates or aggregates are not included in the motility evaluation. If more than 20 % to 25 % of spermatozoa are estimated to be trapped, in such clumps, and therefore not included in the motility assessment it would be noted in the report.

- Concordance of the two replicate assessments (see [Annex F](#)) is required in order to calculate a result with the expected accuracy and uncertainty of measurement.

6.3.5 Sperm Concentration Assessment

- One aliquot of well-mixed semen sample shall be taken and diluted. Volume of 50 μl or 100 μl (exact volume by positive displacement pipette) depending on estimated concentration (see [Annex E](#)).
- The aliquot is diluted (see [Annex E](#); volume dependent on the observation made on the wet preparation) to immobilize the spermatozoa and to achieve a concentration that can be counted with confidence in the haemocytometer.
- A well-mixed aliquot of the sperm suspension is loaded into one side of a counting chamber. A second well-mixed aliquot from the same sperm suspension is loaded into the other side of the counting chamber. Then the counting chamber is left to rest horizontally for at least 10 min in a humid chamber to allow sedimentation of the spermatozoa onto the counting grid. If beyond 20 min, the humidity of the chamber shall be checked.

Replicate assessment shall be performed with comparison of at least 200 spermatozoa in each replicate, unless the count is such that is not possible e.g. a concentration of less than 1 million spermatozoa per ml.

The recommendation of at least 200 spermatozoa in duplicate is to reduce the statistical error (95 % Confidence Interval) to $\leq \pm 10\%$. If at least 100 spermatozoa in duplicate are assessed, the corresponding error is $\leq \pm 14\%$. Any result based on less than 200 observed spermatozoa shall be commented upon in the final report.

Concordance of the two replicate assessments (see [Annex G](#)) is required in order to calculate a result with the expected accuracy and uncertainty of measurement.

6.3.6 Assessment of Absence of Spermatozoa

Thorough, systematic visual inspection [(200 $\times$ to 400 $\times$ magnification; scanning the entire area under a 22 mm $\times$ 22 mm coverslip)] of two independent 10 μl aliquots without finding any spermatozoon is the first part of assessment. The second part is the inspection of the entire area under a 22 mm $\times$ 22 mm coverslip of 10 μl aliquot of a centrifugation pellet (1 000 $\times$ g, 15 min) of the entire ejaculate. If no spermatozoa have been detected in either of these visual inspections, it is considered sufficient for a

clinical laboratory to state that an ejaculate is not likely to contain spermatozoa, although presence of occasional sperm cannot be excluded (see [Annex A](#)).

6.3.7 Sperm Vitality Assessment

Sperm vitality is not usually assessed routinely, only if there is a low percentage of motile spermatozoa seen in the wet preparation, e.g. <40 % total motility.

Use an evaluated and validated method to distinguish between live and dead spermatozoa, e.g. well-mixed semen mixed with a combined supravital stain with a background stain such as Eosin Y and Nigrosin^[2] (see [Annex H](#)).

NOTE Vitality test with Eosin only has only been validated for negative phase contrast microscopy.

6.3.8 Sperm Morphology Evaluation

Smears for morphology should preferably be made within 60 min after collection of the ejaculate (see [Annex I](#)). Prolonged exposure to the liquefied ejaculate could increase the presence of coiled sperm tails due to a continuous increase in osmolality of the ejaculate.

Every spermatozoon shall be assessed with respect to abnormalities of the head, the neck and midpiece, the tail, and to the presence of a residual cytoplasmic mass (see [Annex I](#)). Only complete spermatozoa (with head and tail) shall be evaluated. If more than 20 loose heads per 100 complete spermatozoa, the number of loose head shall be reported separately as number of loose heads per 100 complete spermatozoa.

Extensive assessor training on validated reference materials (for example, smears stained with Papanicolaou stain or other validated methods, with validated reference results) is required in order to perform reliable human sperm morphology assessments. Without this training, as well as ongoing competency verification, results are likely to have limited or no scientific or clinical value.

7 Post-Examination Handling and Test Report

7.1 General

The post-examination requirements include calculations of results and presentation of results.

Each laboratory shall determine measurement uncertainty according to ISO/TS 20914.

7.2 Results Calculations and Presentation

7.2.1 Total Amount in the Ejaculate

Concentration of cells in the ejaculate are difficult to interpret correctly since the final volume is dependent of contributions from several different accessory glands that can vary from time to time within a subject, as well as between ejaculates from different subjects. It is therefore of great importance also to calculate the total numbers per ejaculate.

— Total sperm number = ejaculate volume × sperm concentration

7.2.2 Other Calculations

a) Motility

Motility percentages shall be calculated from the average value of two accepted replicate assessments and presented as percentages (based on the number of spermatozoa counted, only use integer values, i.e. no decimal places):

- Rapid progressive;
- Slow progressive;
- Non-progressive;
- Immotile.

Besides the recorded percentages, the following can be calculated from the recorded results and presented as integer percentages without decimal places:

- Percentage Progressive (rapid + slow progressive);
- Percentage Motile (rapid + slow progressive + non-progressive).

NOTE A four category motility assessment, including the distinction between rapid and slow progressive spermatozoa, gives important information for making clinical decisions^[4].

b) Morphology

Based on the number of spermatozoa counted, only use integer values, i.e. no decimal places, when presenting morphology assessments, with only the TZI as an exception where two decimal places shall be used:

- Percentage of “ideal” spermatozoa^[15];
- Percentages of the recorded abnormalities (head, neck/mid piece, tail or cytoplasmic residue) as their respective percentages of all spermatozoa assessed (i.e. not just their prevalence among the abnormal spermatozoa);
- Specific abnormalities (e.g. round-headed spermatozoa [i.e. globozoospermia], pyriform spermatozoa, loose sperm heads) shall be reported separately if their presence exceeds 20 per 100 spermatozoa;
- Teratozoospermia Index (TZI) – is optional and calculated as the total number of defective regions (head, neck-midpiece, tail and cytoplasmic residue) seen divided by the number of abnormal spermatozoa in which they were counted (by convention reported with 2 decimal places).

7.3 Presentation of Results

7.3.1 General

- Percentages are given as percentages, integer numbers (no decimal places);
- If reported: TZI – always given with two decimal places;
- Sperm concentration and number: integer values without decimal places for results ≥ 10 million; for values between 0,05 and 9,95 million, one decimal place can be justified for the sake of visible clarity. For lower values, e.g. 0,05 million, it could be expressed in thousands instead of millions to clarify the approximate nature of the obtained number (e.g. 40 thousand instead of 0,04 million).

7.3.2 Contents of the Semen Examination Report

Besides the formal requirements defined in ISO 15189, the following data specific to ejaculated semen examination shall be included:

- Duration of sexual abstinence in days;

- Information whether the ejaculate was collected at the laboratory or elsewhere and transported to the laboratory. In the latter case, the time for arrival at the laboratory should also be included in the final report;
- Viscosity, liquefaction and other macroscopic observations;
- Ejaculate volume in ml;
- Sperm concentration in millions/ml;
- Total sperm number in millions/ejaculate;
 - For ejaculates with no observed spermatozoa in the wet preparation (10 µl) a report on any observed spermatozoa in a centrifugation pellet is required
- Motility percentages;
- Morphology percentages and TZI (when measured);
- Percentage live spermatozoa if measured;
- pH when measured;
- Any other significant or unusual findings seen at microscopic investigation of the wet preparation, e.g. aggregates and agglutinates, presence of round cells, debris and crystals;

NOTE 1 The differentiation between different types of round cells in the ejaculate is often difficult. Tests for active inflammatory cells (leukocytes) can sometimes give an indication and smears stained by Papanicolaou (or other validated methods) reveal presence of immature germ cells (spermatids, spermatocytes or in rare cases spermatogonia) but clear distinction is often not possible. However, observations of more than 1 million peroxidase positive cells per ml is recommended in the WHO manual to be included in a report (World Health Organization, 2010^[16]).

- Observations of unusual physical characteristics of the ejaculate, e.g. colour, odour, viscosity (see [6.3.1](#) and [6.3.2](#)).

NOTE 2 Interpretation and Advice on the Semen Examination Report is the responsibility of the requestor.

7.4 Practical Aspects of Quality Assurance

7.4.1 Internal Quality Control

The laboratory shall implement internal quality control (IQC) to evaluate the performance of the whole analytical process and to detect any event occurring due to a defect of reagents or equipment used, in the operating procedure application or from an inappropriate training of the assessors.

Control materials for sperm motility (video recordings), and permanently mounted stained slides for morphology and vitality, respectively, can be produced and saved for repeated use, or obtained from participation in inter-laboratory comparison schemes.

Pre-diluted and fixed semen samples can be very poorly stable and techniques for preparation shall be validated and evaluated before use for long-time for monitoring sperm concentration. Furthermore, to some extent alternative techniques can be used to validate the accuracy of sperm counting using products like latex spheres. Although video recordings of motile sperm cannot evaluate all aspects of sperm motility assessments, the use of video recordings is essential to evaluate each assessor's ability to assess the most important part of motility examination: to distinguish between the different classes of sperm motility properly.

7.4.2 Intralaboratory Comparisons

The same sperm sample shall be assessed by each assessor to evaluate the inter-assessor variability. For the time consistency of evaluation, repeat evaluations shall be run on the same material.

- Sperm concentration – After fresh sperm dilution with fixing diluent the actual counting should not be time dependent within a reasonable time interval of hours up to a few days (depending on the airtightness of the dilution vial and whether spermatozoa stick to its walls). The main problem is a general increase in sperm aggregates that reduces the concentration of free spermatozoa.
- Sperm motility – reliable evaluation of comparisons is difficult to obtain due to the changes in sperm motility in the ejaculate. Video recordings of sperm motility are more consistent material for inter-individual evaluation of variability and for time consistency of assessments. For the validity of quality controls via video recordings, it is essential that the observed video sequences are presented to the assessor in the same way as ejaculates are examined in routine. Routine evaluation using the same video or computer screen as for QC evaluation is therefore recommended.

NOTE This document does not consider motility evaluation by CASA.

- Sperm morphology – evaluation of inter-individual variability can be performed with fixed, stained and mounted smears.
- Sperm vitality – evaluation of inter-individual variability can be performed on stained and mounted smears.

The results obtained by the different assessors shall be monitored in order to establish a long-term performance evaluation. The laboratory shall define the frequency of these intra-laboratory comparisons considering risk assessment for each sperm characteristic as well as the goals to be achieved for the different parameters measured.

7.4.3 Interlaboratory Comparisons

Every laboratory performing manual/visual semen examination should also participate in Interlaboratory Comparisons concerning at least the basic ejaculate characteristics (sperm concentration, motility, morphology, and vitality) examined and reported by the laboratory.

NOTE Programs ideally provide target values, and do not rely only on mean values for participating laboratories that constitute a population with variable experience and competence.

Annex A

(informative)

The statistical basis for determination of absence of spermatozoa

The statistical basis for determination of absence of spermatozoa is illustrated in the [Table A.1](#) below that considers the probabilities of spermatozoa being present although no spermatozoa were observed in the entire 20 µl aliquot of an ejaculate (Confidence Interval, CI; Poisson distribution).

NOTE GraphPad QuickCalcs, tested September 15, 2018 <https://www.graphpad.com/quickcalcs>. The meaning of the confidence interval is that even if no spermatozoa have been observed in 20 µl, the true number – with 99,5% probability – is up to 1 325 spermatozoa in an ejaculate of 5 ml. It is only 0,5% probability that the true number is above the number 1 325.

Table A.1 — Number of possible spermatozoa despite negative observations – 20 µl aliquots examined from ejaculates of various volumes

Confidence interval	Ejaculate volume				
	1 ml	2 ml	3 ml	4 ml	5 ml
95 % CI	50	100	150	200	250
97,5 % CI	185	369	554	738	923
99,5 % CI	265	530	795	1060	1325

Annex B (informative)

High power field

The area of a “high power field” (×40 objective and ×10 ocular) varies depending on the type of ocular used. In older microscopes the field is significantly smaller than in more modern, “wide field” oculars. For the proper estimation of suitable dilution for assessment of sperm concentration it is important to establish the area of the high-power field of the microscope used for the initial assessment. The following [Table B.1](#) given as example of the difference in field area (field diameter as measured with a calibrated microscope slide) and in the relation between the number of observed spermatozoa and the ejaculate concentration.

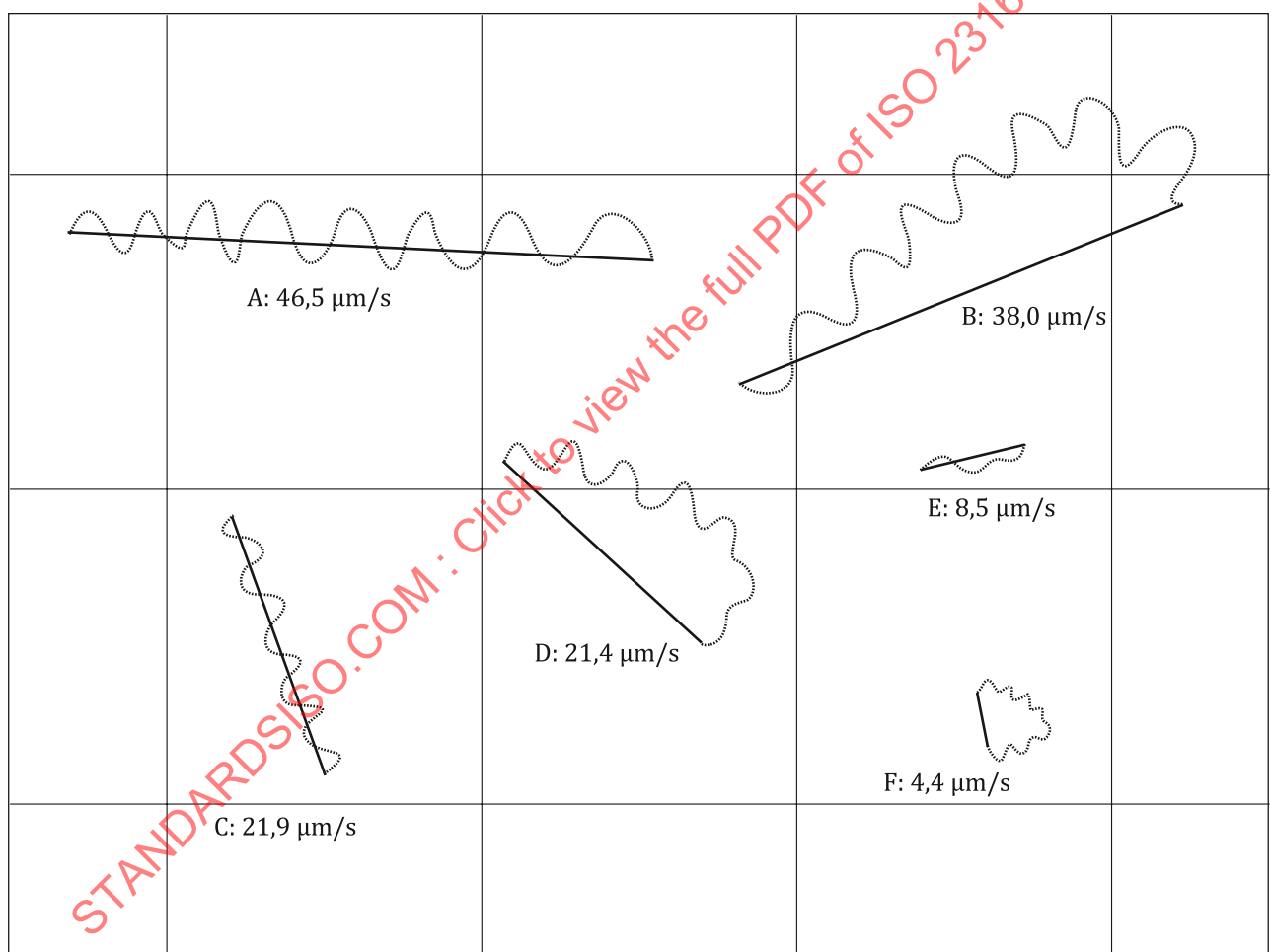
Table B.1 — Examples of relation between high power field, examined volume and approximate sperm concentration

Type of ocular	Field diameter (mm)	Field Area $\pi \times r^2$ (mm ²)	Field volume (assuming 20 µm depth) (nl)	Spermatozoa seen			
				1	15	40	200
				Corresponding to ejaculate concentration (10 ⁶ /ml)			
Wide	0,53	0,22	4,4	0,2	3	9	45
Older	0,38	0,11	2,3	0,4	7	18	88

Annex C (informative)

Motility assessment training

Training of Motility Assessment is a prerequisite for the proper examination of human semen. Theoretical understanding of sperm motility categories shall be combined with ample practical training in the correct categorization of spermatozoa with different classes of motility (see [Figure C.1](#)). It is essential for the diagnosis of the man and the treatment of the couple to recognize whether an ejaculate has sufficient spermatozoa with adequate progression to pass through the cervical mucus and reach the site of fertilization.



Key

A & B = rapid progressive.

C, D & E = slow progressive.

F = circular swimming non-progressive.

NOTE The square-ruled grid has a spacing of 25 μm to facilitate visual classification of rapid progression.

Figure C.1 — Illustrative tracks of motile spermatozoa showing how tracks are classified based on their net space gain (sometimes referred to as their "progression velocity")

Aim of Training

- Categorization of each spermatozoon into one of four groups
 - (a) Rapid progressive (active tail movements, progression at least 25 $\mu\text{m/s}$)
 - (b) Slow progressive (active tail movements, progression 5 $\mu\text{m/s}$ to 24 $\mu\text{m/s}$)
 - (c) Non-progressive (active tail movements, progression 0 $\mu\text{m/s}$ to 4 $\mu\text{m/s}$)
 - (d) Immotile (no active tail movements)

NOTE It does not mean that the exact velocity of each spermatozoon is assessed, only that the motility category of each spermatozoon is identified.

Training Materials

- Live semen samples, microscope with video camera and display
 - For initial introduction, preferably side-by-side with an experienced assessor
 - Recommended to have a grid on the display with squares calibrated to correspond to 25 $\mu\text{m} \times 25 \mu\text{m}$ to help the distinction between rapid and slow progressive spermatozoa (see. [Figure C.2](#)).
- Video recordings
 - of ejaculates with a wide range of sperm concentrations and motility distributions
 - with at least 5 different microscope fields allowing the assessment of in total at least 200 spermatozoa
 - Recommended to have a grid with squares either on the display (e.g. [Figure C.2](#)) or overlaid in the video recording and calibrated to correspond to 25 $\mu\text{m} \times 25 \mu\text{m}$ to help the distinction between rapid and slow progressive spermatozoa.

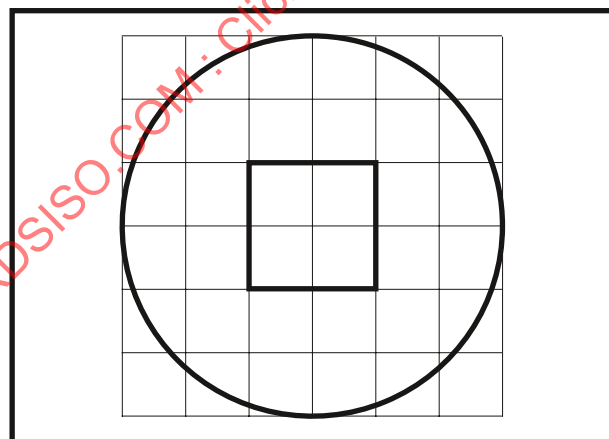


Figure C.2 — An example overlay for a video monitor to be used for human sperm motility assessment (adapted from Mortimer et al., 2021^[14])

The circle depicts a field of view comparable to looking down the microscope, and the central group of 4 boxes defined by the thicker line is used to define a smaller field area to facilitate counting when the sperm concentration is very high. The grid lines are spaced 25 μm apart following calibration of the microscope field magnification (using a stage micrometer), so if a spermatozoon swims across a box in 1 s it can be classified as having a progression velocity of at least 25 $\mu\text{m/s}$, i.e. it is "rapid progressive".

Note that an overlay is specific for a particular combination of optical elements (microscope manufacturer and model, objective, intermediate magnification, tube factor if the optics are not infinity

corrected, camera adapter, camera ocular if used, and video camera model). If multiple combinations are used within a lab then each combination require its own correctly calibrated overlay.

Training Process

- Basic introduction to principles of sperm motility
 - Theory: explanation of motility categories
 - Practical: watching some video recordings of ejaculates together with an experienced assessor to identify spermatozoa from the four categories
 - Goal oriented training^{[14][12]}
 - Give the “Novice” a series of recorded semen samples (can initially be a lower number, e.g. 8 samples, later a set of up to 20 samples) with known target values obtained by experienced assessors (“Expert”) who participate regularly in an EQC programme that is able to establish their concordance with motility assessments on material with established reference values
 - When the assessment of a set is completed, the results from each sample are compared with the Expert values for % Motile (a+b+c), % Progressive (a+b) and % Rapid progressive (a), respectively
 - Difference calculated as novice result subtracted by expert result
- NOTE A negative difference indicates the Novice underestimates the category/ies
- Calculate the mean of the Differences, the Standard Deviation and the 95 % Confidence Interval of the Mean
 - After each set of samples feedback is given to the Novice with indications of under- and overestimations
 - Training is performed by repeating sets of samples until the Novice approaches a Mean Difference of 0 % (± 1 %) and a 95 % Confidence interval $< \pm 10$ % and approaching ± 5 % for the proportions of motile, progressive, and rapid progressive spermatozoa

Re-training

Staff members returning to laboratory work after an extended non-attendance (e.g. more than 6 months), or after showing IQC results that are substandard according to the definitions of the laboratory, are strongly recommended to undergo re-training by examining series of 20 sets to 30 sets of recorded samples (archive material with known target values) to achieve the same goal as for the initial training process.

Annex D **(informative)**

Diluent for sperm concentration assessment

Make an aqueous solution containing 0,595 M sodium bicarbonate and approximately 0,14 M formaldehyde. For example, to prepare 1 l:

1. Dissolve 50,0 g NaHCO_3 in about 500 ml of distilled water, add 10,0 ml of 36 % to 40 % formaldehyde solution (approximately 13,3 mol/l) and add water to 1 000 ml.
2. Store at +2 °C to +8 °C for up to 12 months.
3. If crystals are formed, filter the solution.

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Annex E (informative)

Estimation of suitable dilution for the assessment of sperm concentration

Estimation of suitable dilution for the assessment of sperm concentration using the Improved Neubauer haemocytometer in 6.3.5: on average per microscope field using a 40× objective.

For dilution never use less than 50 µl ejaculate. Instead vary the volume of diluent to obtain required dilution. For a 1:5 dilution 100 µl ejaculate + 400 µl diluent is recommended to provide sufficient volume for mixing and assessing (see Table E.1).

Table E.1 — Relation between number of spermatozoa in microscope field and suitable dilution

Spermatozoa per microscope field, 40× objective	Dilution	Ejaculate (µl)	Diluent (µl)
less than 15	1:5 (1 + 4)	100	400
15 to 40	1:10 (1 + 9)	50	450
41 to 200	1:20 (1 + 19)	50	950
more than 200	1:50 (1 + 49)	50	2450

Annex F (informative)

Comparison of concordance between two replicate assessments that report percentages

This annex refers primarily to sperm motility. Each replicate assessment should, if possible, be based on the categorization of the motility of at least 200 spermatozoa.

1. For each replicate assessment, the percentage motile and the percentage immotile are calculated (integer percentages without decimal places).
2. Acceptable concordance is when the probability that the difference depends on random factors is < 5 %, calculated from the probabilities of binomial distributions using asymmetrical confidence intervals^[14].
3. Calculate the difference between the replicates, using the greater of either the % motile or % immotile.
4. Compare the difference with the limit shown in [Table F.1](#)
 - a. Find the row where the average of replicate results is included in the range of averages.
 - b. If the difference is less than or equal to the limit, the two replicate assessments can be accepted as being concordant.
5. Actions:
 - a) If the replicate assessments are accepted, the mean values for the different motility categories are reported as results for the sample.
 - b) If two replicates are not sufficiently close, then repeat using two new replicate assessments.
 - c) If replicate comparison fails to show sufficient concordance over three attempts, then the average is calculated using all six assessments and a comment is included in the report that the result is based on an average of non-concordant assessments.

Table F.1 — Limits for accepting replicate counts of percentages

Average %	Limit for difference
1	2
02 to 03	3
04 to 06	4
07 to 09	5
10 to 13	6
14 to 19	7
20 to 27	8
28 to 44	9
45 to 55	10
56 to 72	9
73 to 80	8
81 to 86	7

Table F.1 *(continued)*

Average %	Limit for difference
87 to 90	6
91 to 93	5
94 to 96	4
97 to 98	3
99	2

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Annex G **(informative)**

Comparison of concordance between two replicate counts of sperm concentration

Each replicate should, if possible, be based on counting at least 200 spermatozoa in a haemocytometer with Improved Neubauer ruling (see [Figure G.1](#)).

For both replicates, the same volume of sperm suspension shall be assessed.

1. Acceptable concordance is when the probability that the difference between counts depends on random factors is $<5\%$, calculated from the probabilities of binomial distributions^[14].
2. For each replicate assessment, the total number of counted spermatozoa is noted.
3. Calculate the sum of the two replicate counts and the difference between them
4. Compare the difference with the limit (see [Table G.1](#))
 - a. Find the row where the sum of the two counts is included in the range of counts.
 - b. If the difference is less than or equal to the limit, the two replicate assessments can be accepted

Table G.1 — Limits for accepting replicate sperm counts

Range of sums	Limit for difference	Range of sums	Limit for difference
969 to 1000	61	376 to 395	38
938 to 968	60	357 to 375	37
907 to 937	59	338 to 356	36
876 to 906	58	319 to 337	35
846 to 875	57	301 to 318	34
817 to 845	56	284 to 300	33
788 to 816	55	267 to 283	32
760 to 787	54	251 to 266	31
732 to 759	53	235 to 250	30
704 to 731	52	219 to 234	29
678 to 703	51	206 to 218	28
651 to 677	50	190 to 205	27
625 to 650	49	176 to 189	26
600 to 624	48	163 to 175	25
576 to 599	47	150 to 162	24
551 to 575	46	138 to 149	23
528 to 550	45	126 to 137	22
504 to 527	44	115 to 125	21
482 to 503	43	105 to 114	20
460 to 481	42	94 to 104	19
438 to 459	41		
417 to 437	40		
396 to 416	39		

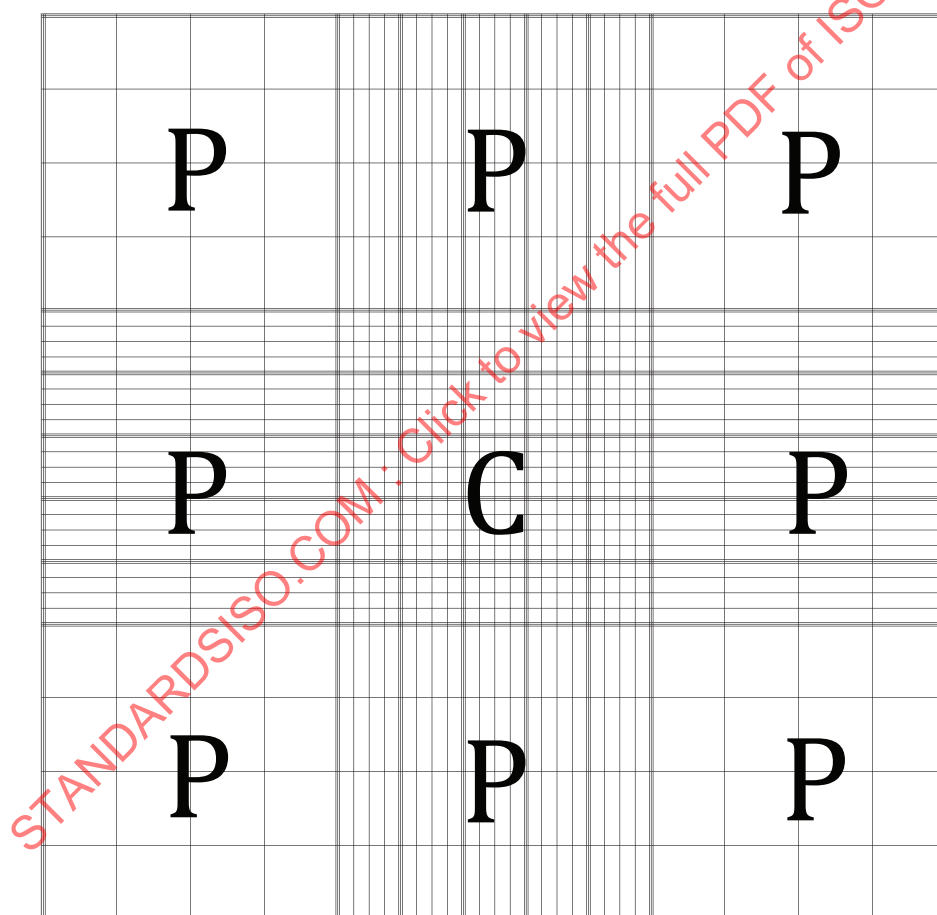
5. Actions:

- a) If the replicate assessments are accepted, the mean values for each concentration are reported as results for the sample (see [Table G.2](#)).
- b) If two replicates are not sufficiently close, then repeat using two new replicate assessments.
- c) If replicate comparison fails to show sufficient concordance over three attempts, then the average is calculated using all six assessments and a comment is included in the report that the result is based on an average of non-concordant assessments.
- d) If less than 200 spermatozoa have been counted in each replicate it shall be noted in the report that the sperm concentration result is based on low number of observed spermatozoa.

Table G.2 — Factors used to divide sum of two replicate sperm counts, depending on assessed volume and dilution

	Number of large squares counted in each chamber			Number of areas counted in each chamber							
				2	3	4	5	6	7	8	9
Dilution	5	10	25								
	Correction factor values										
1:5	8	16	40	80	120	160	200	240	280	320	360
1:10	4	8	20	40	60	80	100	120	140	160	180
1:20	2	4	10	20	30	40	50	60*	70*	80*	90*
1:50	0.8	1.6	4	8	12*	16*	20*	24*	28*	32*	36*

NOTE A haemocytometer with Improved Neubauer ruling has two counting chambers. Each counting chamber consists of nine (3×3) areas of equal size (see [Figure G.1](#)). The central area consists of 25 large squares, each surrounded by a triplet line, while the eight peripheral fields each consist of 20 to 16 squares and rectangles. *) indicates that dilution is not appropriate

**Figure G.1 — Appearance of the entire counting chamber with Improved Neubauer ruling 9 areas of equal size: C=central area, 25 large squares; P=peripheral area, each with 16-20 large squares of different sizes (adapted from Mortimer et al 2021^[14])**

The recommended counting chamber is a commonly available device (haemocytometer with Improved Neubauer ruling) with a high degree of accuracy. Other validated and verified counting chambers can be used but the dilution tables and calculation factors shall be elaborated to achieve equivalent accuracy and precision during use. Currently the Improved Neubauer chamber is by far the most commonly used, to the extent that it is effectively a de facto standard.