The sixth edition of the *WHO Laboratory Manual for the Examination and Processing of Human Semen:* ensuring quality and standardization in basic examination of human ejaculates

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A basic semen investigation has established principles that are necessary for ascertaining reliable and internationally comparable results. Although these principles have been present in the WHO manual since its inception, the baseline issue across most published studies and practice in reproductive medicine (in which the male is considered) is repetitive failure to adhere to these principles, thereby leading to relevant comparable data and accuracy.

To address this failure, the sixth edition of the WHO manual includes revised basic methods, and a complementary formal standard of the International Standards Organization (ISO23162:2021) for basic semen examination has been published. Perhaps the most significant change in the sixth edition is the reintroduction of the four-category distinction of sperm motility, which causes additional work for laboratories in changing reporting parameters but is clinically important.

Another essential change is the widened focus from mainly a prognostic tool for medically assisted reproduction to additionally raising awareness of semen examination as a measure of male reproductive functions and general male health. (Fertil Steril® 2022;117:246–51. ©2021 by American Society for Reproductive Medicine.)

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INTRODUCTION

In the sixth edition of the *WHO Laboratory Manual for the Examination and Processing of Human Semen* (1), the basic methods have been revised. These revisions were made to improve the logical order of assessment procedures to make them easier to follow, to explain why certain specific actions are necessary, and thereby eliminate the multitude of inaccurate methods incorrectly cited as following the WHO laboratory manual. In addition, evidence-based adjustments have been made to reduce unnecessary workload. The basic semen examination now focuses on obtaining accurate sperm concentration, motility (including the reintroduction of the rapid-progressive category), vitality, and morphology, which are essential if the results are to be interpreted alongside reference limits.

Concurrent with the launch of the WHO manual, a formal standard of the International Standards Organization (ISO23162:2021) for Basic Semen Examination was published (2), which is based on the same principles as the WHO manual and was created to support laboratories seeking accreditation for basic semen examination. It is difficult to appreciate how long a comprehensive basic diagnostic semen examination would take. However, the basic examination is now focused on sperm number, motility, morphology, and—only if motility is poor—vitality. Furthermore, based on available evidence, a comparison of replicate assessments is only required for assessing sperm concentration and motility. Therefore, the contention is that a basic semen examination will provide reliable results with less time spent on assessing questionable values.

Only through strict compliance and standardization of methods, together with good practice, quality control, and assurance, can the true diagnostic power of semen examination be further improved and explored.

CURRENT PRACTICE IN SEMEN EXAMINATION

Globally, true compliance with WHO recommendations for semen examination has been poor (3–7), resulting in reduced accuracy and limited comparability of results between centers. The problem with a lack of standardization has been known for a long time (8, 9) despite the publication of WHO manuals from 1980–2010 (10–14). The introduction of in vitro fertilization has certainly revolutionized the possibilities for infertile couples to have children. However, the knowledge surrounding male factors for infertility has not been developed to the same extent as the practical experience of searching and finding sperm to produce a viable, transferable embryo to the uterus. The main interest, which was limited to establishing pregnancies, has now shifted to a focus on understanding the causes. As such, many centers have chosen to use equipment and procedures that may be sufficient for exclusive daily medically assisted reproduction (MAR) work, although high variability in such results may also cause the less appropriate choice of in vitro treatment modalities (7). With the increasing evidence of semen examination as a tool to identify men with possible health issues (15), the issue with unreliable laboratory results has become even more important.

LABORATORY PRINCIPLES TO FOLLOW FOR PRODUCING RELIABLE RESULTS

There are several principles that must be observed to achieve reliable results from semen examination. The updated step-by-step procedures given in the sixth edition have been designed to reduce inaccuracy and imprecision in measurements, resulting in comparable semen parameter results among individual technicians and across laboratories worldwide. These are to be read specifically in the context of an initial diagnostic semen analysis because preparation of semen for therapeutic use may have different requirements for individualized goals.

Sample Production

We have now highlighted within the manual that the ejaculatory frequency before sample collection is important in understanding the ejaculate sample. For practical reasons, the recommended 2–7 days of ejaculatory abstinence is primarily maintained to reduce interference with the life and habits of the individual. Studies on human sperm output indicate that after 2–3 days of daily ejaculations, it is possible to determine the sperm output by investigating one single ejaculate (16, 17). With a longer abstinence duration, the sperm numbers can be expected to increase, whereas the proportion of motile spermatozoa is likely to decrease.

Initial Handling and Assessment of a Sample

The entire ejaculate should be collected in one specimen container because different parts of the ejaculate are usually entangled in a gel-like substance. This is then liquefied by enzymatic activity, which breaks down larger molecules into smaller particles, resulting in an increased osmolality that can affect sperm motility (18). This natural property of the ejaculate, as a series of fractions from different zones of the male reproductive tract, underlies the lack of homogeneity in the ejaculate. It is also worth noting that this extended mixing of the ejaculate is unlikely to occur in vivo, as the fertilizing sperm rapidly colonize cervical mucus within minutes or even faster. As sperm motility in the ejaculate decreases with time after ejaculation, it is essential to initiate examination within 30 minutes of ejaculation when normal liquefaction is complete.

To assess the ejaculate volume, the recommendation is to first determine the weight of the empty sample container and then the weight of the container with the specimen, because using pipettes or measuring cylinders will cause volume loss (19). The variation in this loss is larger than the possible error by assuming the specific weight of semen to be 1.0 g/ml (19–22). With modern digital balances of the relevant accuracy, there is no reason that every laboratory worldwide cannot move to this practice.

Although macroscopic examination may not always be a standard category in reports, the initial macroscopic (visual by eye) examination of the semen sample is a crucial part of the analysis. This may provide key information, such as the initial indication that a sample may be a pre-ejaculate and not a full semen sample, or if it contains blood or urine.

Obtaining a Representative Sperm Count (Subsampling the Ejaculate)

Although appearing well-mixed macroscopically, a liquefied ejaculate will usually have many small compartments with varying contents of spermatozoa. In samples with less-uniform liquefaction, this will increasingly be the case. Having an inadequate sample volume underlies most of the variability and error in alternative methods.

To reliably assess the sperm number, a subsample of 50 μl taken with a positive displacement pipette (those with a piston within the tip that contacts the semen), is required (23). Low volumes, such as 15 μl, are likely to be less representative, and slight variations in volume (e.g., if ordinary air-displacement pipettes are used for semen) may also cause large errors in assessments.

To ensure counting accuracy, the semen aliquot must be diluted. Dilution ensures that the counting chambers fill correctly to avoid problems that may occur when filling with viscous semen (24, 25). Furthermore, a solvent that immobilizes spermatozoa makes it possible to count easily, which helps to prevent duplicate-counting or other issues found with assessing motile sperm.

Compared to the fifth edition, the sixth edition features a return to recommending fewer variants of dilution. The reason is that if there are many alternatives, there are more "limits" to consider and prepare with an increased risk for confusion and mistakes. For many samples, the 1:20 dilution will work well, and the total number of spermatozoa to be counted can be more easily controlled by using up to all nine large fields in the hemocytometer with improved Neubauer ruling.

A hemocytometer of 100-μm depth is recommended because the volume under the properly fitted coverslip will be sufficient. There exist shallower chambers where the filling is likely to be compromised because of the viscosity of undiluted semen (24, 25). Furthermore, the total volume examined will be much smaller with a depth of 10 μm, and if the coverslip position is wrong by 1 μm, the volume error will be 10%.

It can be summarized from the above that a representative sperm count involves the following:

1. 50 μl sample of semen taken by positive displacement pipette
2. Sperm being fixed/killed by a diluent, followed by thorough mixing
3. Counting on an improved Neubauer hemocytometer (or one meeting these specifications) (1)

To validate any other sperm count method, a comparison to the precise procedure is necessary and must include the use of the full range of fresh semen samples at the correct timing (in which the subsample volume has the largest effect because of differences in liquefaction). Unfortunately, small-volume chamber measurements, such as those used in computer-assisted sperm analysis (CASA), can never deliver this reproducible accuracy in everyday use. Equally, many simple systems of observing raw semen, such as "Makler" or "Ruby" chambers, also do not accurately deliver this count because of volume and subsample effects (26–28); this does not make their use invalid for other purposes (primarily counting and assessing sperm removed from semen), but they should not be quoted or used as an equivalent for accuracy of spermatozoa count in ejaculates. When disposable chambers are used, they should be of comparable depth and marking to the improved Neubauer hemocytometer for this accuracy (29). Chambers of lesser depth thereby visualize a smaller volume (as used for CASA) and will not provide an accurate result.

As in the case of dilutions, it is important to have as few variants of calculations as possible. Therefore, in the new manual, calculations for basic semen examinations have been revised to achieve simplicity without sacrificing reliability. By increasing the areas to count in case of low numbers of spermatozoa in the chambers, the laboratory work and calculations will be less complicated and less prone to error.

Assessment of Sperm Motility

Perhaps the most significant change in the sixth edition is regarding the reintroduction of the distinction of rapid-progressive from slow-progressive spermatozoa, which was previously omitted in the fifth edition compared with earlier editions. This was unfortunate because the information on the presence or absence of rapid-progressive sperm is of clinical importance (30–41). The four-part categorization that was the main recommendation in the third and fourth editions has therefore been reinstated.

We are aware of some debate around the ability to "distinguish" a rapidly progressing sperm. It is important to keep in mind that to make the distinction between slow and rapid spermatozoa, the exact velocity of each individual spermatozoon does not need to be assessed—this is only possible by CASA. Put simply, a rapidly progressive spermatozoon is one that moves >5 head-lengths per second; as with all assessments, the training of staff (discussed in detail later) is essential to enable the accuracy of this measure. Options such as metronome beats and eyepiece graticules (a grid of horizontal and vertical lines, sometimes also known as a reticle or reticule) may aid the initial training, and data support replicable results being achievable (17).

For motility assessments, a 10-μl aliquot (under a 22 × 22-mm coverslip) is the most practical volume. To reduce the possible risk of poor representativity of a 10-μL aliquot, assessment of motility in a second, replicate aliquot is necessary (23). Because sperm velocity is highly dependent on the temperature, standardization of the assessment temperature is essential. Therefore, a standard temperature of 37°C for sperm motility assessment is also required, which is most easily achieved with a heated microscope stage. As sperm hyperactivate as temperatures approach 40°C, a key property is the stability of this stage temperature, which should therefore form part of any validation.

Assessment of Sperm Morphology

The Tygerberg Strict Criteria are based on observations of the morphology of spermatozoa that have penetrated through cervical mucus (42) and are able to bind to human zona

pellucida (43). It should be noted that the change in reference ranges for morphology in different editions of the manual has caused some confusion by teams seeing this as an increase in severity of scoring what was "normal." In fact, the evidence-based 4% using the Tygerberg criteria in the fifth edition of the manual onwards represents an entirely different classification system, which is stable and consistent because of its origin as described. It is common that the only reported parameter is the proportion of "normal" spermatozoa and that the distribution of the defects is ignored. Although morphology classification in recent years has predominantly focused on predicting fertility success, it is often forgotten that this assessment can provide information to better understand the reduced functional capacity of spermatozoa from certain men (e.g., those with partial or complete immotility because of tail or midpiece abnormalities, or a lack of fertilizing capacity because of the increased occurrence of acrosome defects). Taking a longer view, better assessments are likely to provide information on spermatogenesis and other functions of the male reproductive organs, thereby disclosing factors that affect male reproductive health.

The approach in the sixth edition emphasizes that all major parts of spermatozoa should be assessed, with all staff who perform morphology assessments being trained to detect defects in all sperm regions.

The choice of sperm stain is essential for consistent results as different stains enhance different parts of the sperm morphology. The sperm-modified Papanicolaou stain gives the overall best staining of the entire spermatozoon, has the most extensive validations, and is, therefore, the main recommendation in the WHO manual. Internationally, there are an array of other stains in use, often chosen for their simplicity (such as the Shorr and Diff-Quick stains), but this is problematic for standardization and diagnostic interpretation. In particular, there is global agreement that each stain provides quite different results down to the level of sperm sizes and fixation effects.

Training, internal quality control (IQC), and external quality assessment (EQA) (discussion to follow below) should also consider all parts of the spermatozoa (i.e., agreement on the number of head/midpiece/tail/cytoplasmic droplet defects within each category—not across an overall single percentage of normal spermatozoa). This stricter requirement will directly facilitate an improvement in the consensus and standard scoring of morphology results. To support this, a structured approach to sperm morphology is suggested in the sixth edition based on a model originally presented by Rothmann et al. (44). The structured approach, starting with training, makes routine morphology assessments more consistent and especially avoids the underreporting of abnormalities in other parts of sperm than the head.

TRAINING, QUALITY CONTROL, AND ASSESSMENT ARE ALL ESSENTIAL FOR RELIABLE, CONSISTENT, AND COMPARABLE BASIC SEMEN ASSESSMENT

An ongoing and significant issue with basic semen examinations is the interoperator and intraoperator variabilities that are seen within and between laboratories worldwide. To address this variability and ensure the consistency of results regardless of where or who performs the assessment, it is essential that all laboratories have robust protocols for training, IQC, and participation in EQA.

All staff needs to be trained in-house for basic semen examination, which is best implemented by examining archive material for comparison (45). Access to externally standardized courses (such as the ESHRE Basic Semen Analysis Courses [15]) can provide a good introduction to semen examination, but for continuous and long-term maintained outcomes, they must be combined with in-house training.

A key component of ensuring within-laboratory reliability of results is the implementation of robust IQC procedures. These may involve repeated tests of performance against well-characterized samples and should provide the opportunity for discrepancies in results to be addressed. Even for fully trained and experienced laboratory staff members, these repeated tests of performance by IQC should be mandatory to maintain the quality and reliability of results.

In addition to IQC, it is essential to participate in an EQA program. Without external validation, it is impossible to conclude that the laboratory's results comply with the recommendations laid out in the sixth edition and properly reflect the descriptions in scientific publications from other external centers. External quality assessment can be challenging to organize, particularly for assessments where performance is likely to deteriorate in time (such as with sperm motility analysis). To combat this, schemes often use online examinations of filmed samples. As pointed out by UK NEQAS (46), this may not be ideal as the use of recordings to assess sperm motility is not as set out in the WHO manual; however, the aim of EQA is to ensure standardization across laboratories, which can only be achieved through the assessment of the same well-characterized data.

Although the sixth edition of the WHO manual aims to set out all the necessary procedures for conducting a comprehensive basic semen analysis, in many countries, the WHO manual is not directly applicable for laboratory accreditation; historically, this has usually been through the ISO15189 standard for medical laboratories (47). The lack of a standard specifically for basic semen examination has provided a barrier to laboratories that wish to accredit their procedures, and might have contributed to the current poor compliance with the WHO recommendations for semen examination (48).

Because the existing standard did not provide specific requirements for basic semen examination, a specific and formalized standard (ISO23162:2021) was drafted in the summer of 2020 and later published in the summer of 2021 (2). It is based on the same principles as the WHO manual and is therefore expected to help laboratories that specifically seek accreditation for basic semen examination. With this formal standard, the individual laboratory is not required to prove the reliability and usefulness of the assessment techniques as long as they are being correctly followed in all detail.

THE SIXTH EDITION IS BUILT ON MANY VOICES

The content of the sixth edition of the manual addresses the feedback from a worldwide community in semen analysis.

The feedback was cohesively assembled into the current form by the editorial team. In this, we have tried to listen to all the comments and provide clarity on why these specific methods to be followed are provided.

CONCLUSION

In the new revision, the *WHO Laboratory Manual for the Examination and Processing of Human Semen* aims at facilitating precise compliance with the recommended procedures with step-by-step instructions that are easier to follow, and fewer variants in dilutions and calculations. The concurrent publication of a formal ISO standard based on the same evidence base facilitates accreditation according to the WHO principles in this field of laboratory science.

Semen analysis parameters have long-suffered from being something where "men don't die from an inaccurate result" or it is "accurate enough for MAR." Laboratories worldwide need to achieve improved diagnostic compliance, particularly if they seek to publish findings or are performing clinical trials. Editorial and reviewing teams should refuse publication of known substandard methodology to help enforce some improvement (7), and so complacency can no longer be an excuse.

There are many present and emerging technologies for semen analysis which for commercial and/or simplicity reasons wish to assert equivalence to these core methods. As described in the manual, it may be better to focus on the potential of those technologies as separate advanced diagnostics, rather than the pretense that they are a universal, accurate, and appropriate answer to automate this core diagnostic.

Basic semen examination and the entire WHO manual widens the perspective of semen and sperm examination to not only be a matter for MAR but also to be used as a tool to understand functions and disorders of the male reproductive organs and general sexual and reproductive health. Thus, the man can be viewed as a person and not only a sperm supplier. Recognizing this, we would also make a plea that clinical teams carefully consider psychological interpretations of their use of "abnormal" results to patients. When quoting against reference limits, results may be better expressed as "not typical" for a highly fertile man, which would also reflect current data for the accuracy of the diagnostic.

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