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Evolution of the WHO "Semen" Processing Manual from the First (1980) to the Sixth Edition (2021)

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Short Narrative Abstract

Stated clearly in all editions of the *WHO laboratory manual for the examination and processing of human semen,* the goal of the manual is to meet the growing needs for standardization of procedures for semen analysis. With the constant advances in andrology and reproductive medicine, and the advent of sophisticated assisted reproductive technologies for treatment of infertility, the manual has been continuously updated, to meet the need for new evidence-based

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and validated tests, not only to measure semen and sperm variables, but also to provide functional assessment of spermatozoa. The sixth edition of the WHO manual, launched in 2021, can be freely downloaded from the WHO website with the hope to gain wide acceptance and utilization as the essential source of latest evidence-based information for laboratory procedures required for the assessment of male reproductive function and health.

Capsule:

This review describes the evolution of the *WHO laboratory manual for the examination and processing of human semen* over six editions providing procedures to allow global standardization of semen analysis.

Keywords

Semen Analysis Standardization; Basic and Extended Methods; Advanced Methods; Quality Control; References Ranges

Goals of the WHO laboratory Manual for Examination of Human Semen

The World Health Organization (WHO) “Laboratory manual for the Examination and processing of human semen”, was first published in 1980, to standardize the procedures for the examination of human semen. This WHO “semen” manual has undergone five revisions (Fig. 1), and translated into multiple languages, in response to growing global needs in andrology and reproductive medicine, to standardize procedures for the examination of human semen. The WHO manual provides standard laboratory methods for semen analysis that are used extensively by clinical and reference laboratories for the understanding of reproductive function in man, diagnosis and planning treatment of sub-fertile couples, assessment of male contraceptive methods, and large-scale population studies and research activities, such as effects of viral, environmental, and other toxicants on male reproductive function.

Development of each edition of the manual was coordinated by the UNDP/UNFPA/ UNICEF/ WHO/ World Bank Special Programme of Research, Development and Research Training in Human Reproduction (HRP) within the WHO Department of Reproductive Health and Research, that worked with the respective Task Forces or later, Research Group for the Regulation of Male Fertility. The latter, including the editorial board, was drawn from global experts working in male reproductive function. HRP has played very vital roles in convening the development of the manual, including overall coordination, technical input, supporting evidence generation, document production, dissemination, and promotion, ensuring continuity across editions, and providing the necessary financial support.

The Beginning

Between 1976 and 1977, the *WHO Task Force on Methods for the Regulation of Male Fertility* (led by C.A. Paulsen, M.D., University of Washington, Seattle, USA, and M.R.R Prasad, Ph.D., Manager of the Task Force) recognized the importance of standardization

of semen analysis to improve the quality of the results allowing exchange and combining data across laboratories. A series of consultations in Barcelona, Geneva, Berlin, and Hong Kong were held to develop a laboratory manual for semen analysis. These consultations/ workgroups were conducted in collaboration with two other WHO Task Forces on the *Diagnosis and Treatment of Male Infertility* and *Vaginal and Cervical Devices for Fertility Regulation)* within HRP From the first to the current (6^{th}) Edition, the support of the respective HRP directors and scientists has been critical in the development and publication of the manuals. The first edition called the *Laboratory Manual for the Examination of Human Semen and Semen Cervical Mucus Interaction* that was based on these consultations, with 33 participants from Asia, Australia, Europe, North and South America. The focus of the manual was on semen analysis as the first laboratory test to study the physio-pathological testicular function in adults, establishing male fertility status in infertile couples as well as monitoring spermatogenesis during and following male fertility regulation. The main objective of the first edition was to provide laboratory procedures for semen analysis that are standardized, precise, reproducible, sensitive, and validated. The manual consisted of only 43 pages with sections on collection of the semen sample, initial examination, and assessment of sperm motility, concentration, morphology (including plates showing normal and abnormal spermatozoa) and viability. It included an appendix with morphological classification and Papanicolaou staining for human spermatozoa with a standardized example for a semen analysis report form. It also included a section on Sperm-Cervical Mucus Interaction as a surrogate of sperm function, assessing sperm penetration of the cervical mucus using *in vitro* (capillary tube test) and *in vivo* (post-coital test) tests (1) (Table 2).

Second Edition

The second, third and fourth editions of the WHO semen manual were led by G.M.H. Waites, Ph.D., then as area Research Manager within HRP, in response to the developments in andrology, with increasing awareness of the importance of objective assessment of the quality and functional characteristics of human spermatozoa (Table 1). More importantly, semen analysis was established as an important tool in the assessment of the male partner, with the rapid development of assisted reproductive techniques. A working group or experts in semen analysis, andrology and reproductive medicine was constituted to revise the semen manual. The second edition was published in 1987 (2). Among the changes from the first edition included, was division of semen analysis into standard (all tests described in the 1980 manual plus anti-sperm antibody tests) and optional tests, including semen culture, seminal fluid biochemical tests, zona-free hamster oocyte penetration test, and sperm migration test (“swim-up”). The section on semen-cervical mucus interaction was not changed. Importantly, the second edition introduced the “normal” values for sperm variables based mainly on the early studies of Macleod and Gold in 1951(3–5) and updated in 1979 (6) with consensus -based modifications. The manual suggested these values from such large scale studies could be used by laboratories to guide interpretation of semen analysis results. However, it was noted that though the data of Macleod et al were obtained from large numbers of fertile and infertile men, the time taken to achieve a pregnancy in the female partner was not defined. The manual suggested that “normal values” should be evaluated

from men who had achieved a pregnancy with their partner within 12 months (Table 2). Appendices were expanded to provide technical details of some of the procedures. This 1987 version translated into seven languages was used in the WHO Andrology Workshops that were held in Africa, Asia, and Eastern Europe with hands on demonstration of semen analysis techniques.

The third edition

Recognizing the rapid progress in assisted reproductive techniques used for the treatment of the infertile couple, the proliferation of many andrology laboratories in infertility treatment centers, the increasing concern about the putative effects or environmental pollutants on male reproductive function, and the need for new and better standard methods of semen analysis, the WHO laboratory manual was revised by a group of experts in 1992 (7). The semen analysis section was divided into three sections: Standard Procedures, Optional tests, and Research tests. The research tests included the zona free hamster oocyte penetration test, human zona pellucida binding tests, acrosome reaction, and computer assisted sperm analysis (CASA). The section on sperm-cervical mucus interaction was not significantly altered but the title of this edition was changed to *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction*. This edition contains two additional chapters relevant to the needs of laboratories: sperm preparation techniques and quality control of laboratory performance of semen analyses. In addition, in the appendices, this manual introduced basic safety guidelines and minimal equipment needed for any laboratory performing semen analysis. These additions were timely and responsive to the needs of many laboratories.

In the few years preceding the revision of the third edition of the manual, the classification of sperm morphology was changed, based on the morphology of spermatozoa of spermatozoa able to reach the oocyte and bind to the zona pellucida. Emerging evidence related to outcomes in *in vitro* fertilization (IVF) suggested that pregnancy rates in the female partner were more frequent when normal sperm morphology was above 14% (8, 9). In Appendix 1, the reference limits of semen variables were provided based on consensus limits in the first and second edition. It was noted there were no population based clinical studies on semen variables completed at that time, and an empirical reference value for sperm morphology in men was quoted as 30% without supportive data (7) (Table 2). This empirical cut-point of 30% normal forms was reported not to be clinically useful as approximately half of fertile men had sperm morphology below this value (10). It is important to keep in mind that the criteria for characterization of human sperm morphology have changed substantially from those used by MacLeod (5) to the strict Tygerberg criteria (11).

The fourth edition

In 1999, the WHO/HRP fourth edition of the semen manual was published (12). This revision was in response to the advances in the genetics of male infertility (y chromosome microdeletions)(13); the success of intracytoplasmic sperm injection (14); the demonstration of contraceptive efficacy of hormonally induced suppression of spermatogenesis (15, 16);

and reports of declining sperm counts (17) and increasing incidence of testicular cancer (18). These factors added urgency to the search of new methods to achieve better standardization and improvement of procedures for semen analysis. The WHO sought advice from experts throughout the world via letters, journals and national andrology societies and formed a working group to revise the manual. The fourth edition of the WHO semen manual was a companion to the *WHO Manual for the Standardized Investigation and Diagnosis of the Infertile Couple* (19). In this fourth edition, the multiple sperm defects index and the hypo-osmotic swelling test were added to the optional tests and measurement of reactive oxygen species was added to research test. Importantly, the quality control section was expanded to include statistical consideration of counting errors. Again, in Appendix 1, "Reference Values for Semen Variables" it was clearly stated that these reference values are not to be used as the minimum/lowest semen values compatible with fertility *in vitro* or *in vivo*. Taking into the consideration comments from the prior edition, this edition left the morphology variable blank with a note suggesting that a percent normal morphology < 15 % may be associated with a lower *in vitro* fertilization rate (Table 2). This fourth edition of the WHO Semen Manual was considered a significant improvement in the description and standardization of techniques and the demand for the manual was high (20, 21).

The expanded fifth edition

After reviewing prior editions, WHO/HRP, in consultation with its Research Group for Male Fertility Regulation, the fifth edition of the laboratory manual's name was changed to "WHO laboratory manual for the examination and processing of human semen" and the publisher changed from Cambridge University Press to the World Health Organization (22). The manual thus became free, available in print and as an electronic version downloadable from the WHO website (https://apps.who.int/iris/handle/10665/44261), in an attempt to establish the manual as a global guidance that will set standards across various laboratory settings and resources. This increased the demand even further, with an external evaluation of HRP noting that the manual was amongst its global publications with the highest citations (4271) and downloads (53,863 in the past 6 months) and it received further requests for translations. The fifth edition was the most extensive and comprehensive edition of the manual with 271 pages.

Ten years after the fourth edition, the WHO considered revisions to the manual necessary because of advances in andrology technology and new tests that may be useful in the management of the infertile couple with an up to date supporting evidence. After consultations with experts in the field and professional associations, the WHO/HRP, led by then Research Manager and later its Director, M. Mbizvo, Ph. D, established an editorial committee with 20 members from Africa, Asia, Australia, Europe, and North America. The committee nominated as editor-in-chief T. G. Cooper, Ph.D. from the Institute of Reproductive Medicine at the University of Munster, Munster, Germany, a WHO-collaboration center, with support from the WHO Secretariat. After receiving comments from laboratory technicians and scientists, this edition improved on the prior editions and provided for each procedure detailed description with a rationale of alternate methods, examples, notes and comments. Dr. Cooper and the editorial committee decided to edit the 5th edition in accordance with the principles of "Evidence Based Medicine ". This led

the editors to collection and critical appraisal of original clinical studies in the field of spermatology and the definition of evidence-based thresholds and reference values. Semen analyses procedures remained the same, but emphasis was placed on counting errors when inadequate number of spermatozoa are assessed. Sperm preparation techniques extended beyond the ejaculate and included collection of spermatozoa from the testis and epididymis. A chapter on cryopreservation of spermatozoa was added. Quality control chapter was rewritten with suggestions on how to improve laboratory performance. Most importantly, the fifth edition for the first time provided reference ranges based on *in vivo* data derived from studies in fertile men whose partner conceived within 12 months (23). The lower fifth centile was used as the lower reference range with a clear statement indicating that this lower limit does not distinguish sub-fertile and fertile men, and that clinical information must be used together with semen analyses data (Table 2).

While this edition of the WHO semen manual became one of the most frequently downloaded publications of the WHO/HRP, the reviews of the edition continued to be very favorable. Comments were received from all over the world on the usefulness of this edition in clinical and research settings (24–33). Of note, some scientists commented that the wide use of assisted reproductive technology using intracytoplasmic sperm injection often by-passed in-depth evaluation of the male partner. In some men, understanding of the qualitative and functional deficiency of spermatozoa including poor motility (e.g. primary ciliary dyskinesia), low viability (e.g. damage by reactive oxygen species), gross morphological abnormality (e.g. globozoospermia, macrozoospermia, different abnormalities of sperm flagella), allowed couples to consider further sperm functional assessment and genetic tests before making informed decisions on the optimal method of treatment (34, 35). Importantly, traditional semen parameters in at least a single ejaculate are considered necessary for the appropriate investigation and treatment pathway for couples with male infertility (36). Comments also suggested that this laboratory manual should follow the standardization in describing tests in clinical laboratories according to International Federation of Clinical Chemistry and Laboratory Medicine and the International Union of Pure and Applied Chemistry (37) as well as the International Organization for Standardization.

Using the WHO laboratory Manual to disseminate information and improve standardization

In the years between 1987 and 1999, the WHO/HRP sponsored many Andrology workshops in Africa, Asia, and Eastern Europe to introduce the standard techniques of semen analyses to many countries. Many of the centers were participating in studies sponsored by the Task Force on Methods for the Regulation of Male Fertility, which later became the Research Group on Male Fertility Regulation. The WHO manuals continued to be translated into many languages by individuals from national andrology societies (Arabic, Chinese, French, German, Greek, Indonesian, Italian, Japanese, Portuguese, Turkish, Russian). This enabled Laboratory Technicians who were not proficient in English, to be able to use the manual.

Most importantly national and international societies such as the American Society of Andrology and The European Society for Human Reproduction and Embryology (ESHRE) held many basic semen analysis courses (38) using the various versions of the WHO laboratory manual as the teaching guide. In the African region, semen analysis and andrology workshops were funded by HRP, using their Research Capacity Strengthening grants, and hosted by T. Kruger, M.D., and his group in Cape Town, South Africa. Based on 20 ESHRE Basic Semen Analysis courses, these structured training courses led to substantial reduction in between observer variability in semen analyses (39). A recent publication showed that the participation of external quality control program reduced inter-observer variability of sperm concentration but participation of the ESHRE Courses reduced variability not only for sperm concentration but for sperm motility, and morphology (40).

The effectiveness of the WHO laboratory manual in improving global standardization is exemplified in large population-based studies of healthy men where five of the 32 studies used only the WHO laboratory manual recommended hemocytometer for assessing sperm concentration (23). In the follow up data, four of the five additional studies used the same hemocytometer for assessing sperm concentration (41). These data were derived from laboratories in five continents that reported compliance with the WHO laboratory manual. Racial-ethnic difference in semen variables were not reported by these analyses but there is paucity of studies from Asia and Africa and none from South America. Regional geographic differences in semen quality have been reported in men from Europe (42) and in the United States (43). The authors suggest that the regional difference in semen quality may be related to environmental exposures, different lifestyles and agricultural versus urban areas.

Methodology for preparation of the sixth edition

The sixth edition of the manual includes a section that describes the methodology used by WHO to prepare this sixth edition, providing transparency and assuring equity of representation as well as technical accuracy according to the WHO requirements. The WHO/HRP secretariat used the list of members and contributors from the prior editorial team, consulted with collaborating institutions, and scanned the peer-reviewed global literature on andrology and semen analyses to develop the final list. This final list of editorial committee members selected for the editorial committee had a record of accomplishment of research and practice on semen analysis, balancing laboratory experience and clinical practice. The editorial committee consisted of nine international members, two members from the WHO secretariat, and assisted by three early career scientists. This manual retained its purpose as a laboratory procedure document and would not be considered as a guideline, as the objectives and procedures for the latter are very different. This editorial team reviewed the topics, assigned members to review the literature on special topics that may be more controversial, and the reviews were discussed, and decision made based on the evidence available whenever possible.

The procedure undertaken took about 3 1/2 years, with partial interruptions to timelines from the effect of COVID-19 pandemic. The process entailed: developing outline of the chapters; preparation of two major rounds of document drafts that were reviewed internally by the editorial core group; approval and review by the editor-in -chief, Lars Björndahl,

M.D., Ph.D., Centre for Andrology and Sexual Medicine, Karolinska Institutet, Stockholm, Sweden; internal review by WHO; external review by other technical experts on andrology and semen analysis and public review (March to June 2021), with comments from 43 countries. These steps are part of the standard procedures for WHO documents development and finalization after collation and synthesis of all reviews and comments that were carefully implemented within the development of the 6th Edition. The procedures for the final launch of the manual followed the WHO best practice in this area.

The launch of the sixth edition in 2021

On July 20, 2021, the WHO through a webinar, launched the Sixth Edition of the *WHO laboratory manual for the examination and processing of human semen* (https://www.who.int/publications/i/item/9789240030787). In the press release, the WHO states “High-quality laboratory services are crucial for ensuring the value of research on health. Effective analysis and comparison of results across laboratories helps to ensure that healthcare workers receive high quality information, which in turn helps them to provide the best quality care. The sixth edition of the *WHO laboratory manual for the examination and processing of human semen* replaces the 2010 version. It provides important information on semen examination and preparation for clinical evaluation, assessment, cryopreservation, quality control in the semen analysis laboratory, and laboratory examination in the investigation of male sexual and reproductive health.” (https://www.who.int/news/item/27-07-2021-who-launches-updated-manual-to-ensure-high-quality-testing-of-human-semen-in-clinical-and-research-settings).

Retaining the stated goals of the manual articulated with its first launch in 1980, this newest edition provides step-by-step, easy to follow, procedures for technicians and scientists performing semen analyses. Consistent with previous editions, the 6th edition includes basic examination: semen volume, sperm motility, concentration, morphology, and vitality assessments as well as estimation of sperm numbers in samples with very low sperm concentration. Extended semen examination includes procedures to detect leucocytes, immature germ cells, sperm antibodies, indices of multiple sperm defects, biochemical assessment and introduces methods to detect sperm aneuploidy, sperm genetics and DNA fragmentation. Advanced examination replaced the prior research tests with extensive revisions and includes assessment of reactive oxygen species, membrane ion channels (new), acrosome reactions and CASA. Emerging new methods were briefly described including tracking individual sperm cell motility and flagellar wave forms that may provide more information of the energetics of sperm movement. Continued improvement in digital camera technology in smart phones raises the possibility of initial semen assessment without a microscope. The zona free hamster oocyte penetration test and human zona pellucida binding test are removed as these tests are rarely utilized in the laboratory because of difficulty in obtaining the test materials, lack of standardization, labor intensive and high cost. In addition, the availability of intracytoplasmic injection of sperm directly into oocyte eliminated the necessity of these tests.. Chapters on sperm preparation and cryopreservation, internal and external quality control methods have been updated. The chapter on sperm-interaction with cervical mucus has been eliminated, as the procedure is no longer being routinely performed as part of infertility investigation.

In the 2nd, 3rd and 4th editions of the WHO manual, the consensus-based lower limits of semen variables were presented. At that time population-based studies from men who have achieved a conception within the prior 12 months were not available. The 5th edition reported WHO reference ranges in 2010 based on men whose partners achieved a pregnancy within12 months of trying to conceive (23). In the 6th edition data from new publications from more diverse populations were amalgamated to the 2010 data, the results from about 3500 fertile men from 12 countries in five continents, validated and expanded the distribution of semen variables in the fifth edition (41). It was emphasized again that this distribution data with fifth centile as the lower reference range do not define limits between fertile and subfertile men. There is a substantial overlap of semen evaluation between fertile and subfertile men (3) and there is no distinct boundaries between fertile versus infertile. The clinician may explain to the infertile couple that using the distribution of semen variables in fertile men, for instance, that the male's sperm concentration/total number in the ejaculate is lower than 5% of fertile men (5th centile sperm concentration <16 million/ml or total sperm number <39 million/ejaculate) (41) and then based on clinical assessment discuss the options of further investigation or treatment. The manual in the final form can be downloaded using this URL https://bit.ly/WHOSM6. It is anticipated that this edition of the manual will be a widely used teaching guide to semen analyses workshops and andrology training programs, both basic and more advanced methods, adapting to the virtual meeting format that gained wide acceptance in the past year. Continued improvement in virtual meeting systems with hands on demonstration by the trainer followed by each course participant showing step by step procedures back to the trainer to allow guidance and further instructions. This may allow training of clinicians, scientists, laboratory staff and students to learn and adopt the standardized methods, that is the goal of the semen manual. However, it is essential to provide in-house performance training following the virtual sessions (44). Unlike the prior manuals, the 6th edition described only one recommended method of assessment of semen volume, sperm concentration, motility, and morphology which simplifies the recommended procedures for laboratories. The challenge is to implement external quality control to ascertain that method recommended by WHO laboratory manual is implemented by laboratories to achieve standardization and reproducibility of results across laboratories around the globe (45). A further support to the global standardization is the recently published ISO standard (International Standards Organization. ISO 23162:2021 Basic semen examination — Specification and test methods, 2021. ISO, Geneva.) based on the same principles and evidence base as the 6th edition.

Conclusion

The *WHO laboratory manual for the examination and processing of semen analyses* has undergone five revisions (Table 1) to meet the needs of laboratories performing semen analyses. Continuous improvement/updating of the manual occurs every 8 to 10 years to introduce new tests into practice and emerging tests for research and clinical validation of significance. The goals of this laboratory manual remain the same. It is designed for everyone who utilizes semen analyses for the understanding the function of the male reproductive function and dysfunction, diagnosis and planning treatment of the infertile couple, assessment of sperm output and other variables following male contraception, and

for epidemiological and other research studies of, among others, potential environmental pollutants and other toxicants on male reproductive function.

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Fig. 1.
The six editions of the WHO “Semen” Manual.

Table 1.

The major changes from the 1st to the 6th edition of the *WHO laboratory manual for the examination and processing of human semen*.

Edition	Year	Pages	Major Changes
1st	1980	43	Semen: sample collection, initial examination, sperm motility, sperm density, sperm morphology (plates and stians0 Sperm cervical mucus interaction: collection of mucus, in vitro test, post-coital test
2nd	1987	67	Semen: Standard tests- includes all in the 1st edition + sperm antibody tests Optional tests – semen culture, seminal fluid biochemistry, zona free hamster oocyte penetration test, sperm migration test Criteria of normality of semen samples
3rd	1992	107	Standard test – use “strict criteria for assessment of sperm morphology Research tests -zona free hamster oocyte penetration test, human zona pellucida binding test, acrosome reaction, computer assisted sperm analysis (CASA) Sperm preparation Quality control of semen analysis
4th	1999	128	Optional tests – added hypoosmotic swelling test, multiple sperm defects index Research Test – reactive oxygen species Quality control- statistical analyses of counting errors
5th	2010	271	Most extensive and comprehensive revision of the semen manual Detailed description of each procedure Added total sperm output per ejaculate as a semen variable Sperm motility combined rapid and slow into one grade of progressive motility Sperm preparations include spermatozoa from testis and epididymis Using quality control to improve laboratory performance Added chapter on cryopreservation of spermatozoa
6th	2021	276	Step by step easy to follow procedure Basic examination = standard tests, reintroduce slow progressive motility Extended examination =optional tests included leucocyte, immature germ cells, added sperm aneuploidy, sperm genetics and DNA fragmentation Advanced examination = research tests, added membrane ion channels Emerging methods of semen analyses without a microscope Eliminated hamster zona free penetration test, human zona binding test and section on sperm-cervical interaction

Table 2.

Changes in Reference Values from the 2nd to the 6th edition of the *WHO laboratory manual for the examination and processing of human semen.*

Edition	Nomenclature	Semen Volume (ml)	Sperm concentration (10^6/ml)/*Total number (10^6/ ejaculate)*	Total/ *Progressive Sperm motility (%)*	Normal forms (%)	Reference
2nd	Normal Values	≥ 2	≥20/*≥40*	≥ 50/*≥25*	≥50	Modified from (3–5), data from 1000 men with known fertility (female partner currently pregnant) and 1000 men in infertile marriage
3rd	Normal Values	≥ 2	≥20/*≥40*	≥ 50/*≥ 25*	≥30	Same as 2nd Edition Empirical value used for normal sperm forms
4th	Reference values	≥ 2	≥20/*≥40*	≥ 50/*≥ 25*	*	Same as 3rd Edition. * Normal forms - population-based studies of normal forms in progress, data from ART suggest <15 normal forms may be associated with decreased *in vitro* fertilization rate
5th	Reference values of fertile men (5th centile lower reference limits)	1.5	15/*39*	40/*32*	4	Population based studies of about 1900 fertile men (defined as men whose partner conceived within 12 months after stopping contraception) (23). Morphology using “strict” criteria (8, 11).
6th	Distribution of semen variables from fertile men (5th centile)	1.4	16/*39*	42/*30*	4	Population based studies of about 3500 fertile men (defined as men whose partner had a natural conception with a confirmed time to pregnancy of ≤ 12 months)(41).. Morphology using “strict” criteria (8, 11).