UNIVERSITÀ DEGLI STUDI FIRENZE

CRR CRIOCONSERVAZIONE DEL LIQUIDO SEMINALE
LABORATORIO DI ANDROLOGIA

Azienda Ospedaliero Universitaria Careggi

Rivisitazione del Manuale WHO per l'analisi seminale

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Evolution of the WHO semen processing manual

Editorial Board of the 6th Edition: first meeting in Geneve

Main goals of the last edition

- standardization of the procedures for basal semen analysis
- Identification and description of extended (clinically relevant) and advanced (future? Research?) examinations
- Update for sperm selection procedures
- Update for semen cryopreservation procedures
- The need of quality control and continous practicing

Not a guideline but a guidance:
For this reason a formal standard of ISO (ISO23162:2021) has been concurrently published

Here where you can find the guidelines ISO

ISO 23162:2021

Basic semen examination — Specification and test methods

ABSTRACT

PREVIEW

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).

GENERAL INFORMATION

Status : Published

Publication date : 2021-07

Edition : 1

Number of pages : 32

Technical Committee : ISO/TC 212 Clinical laboratory testing and in vitro diagnostic test systems

ICS : 11.100.01 Laboratory medicine in general

Chapter 2: Basic semen analysis: which novelties?

> “All aspects of ejaculate collection and examination must be assessed using properly standardized procedures if the results are to provide reliable information.”

All the procedures are now step by step detailed, from collection to all evaluations

Sperm counting:

- suggested dilutions based on the number of spermatozoa/field:

Spermatozoa per ×400 field	Spermatozoa per ×200 field	Dilution	Ejaculate (µl)	Fixative (µl)
> 200	> 800	1 : 50 (1 + 49)	50	2 450
40–200	160–800	1 : 20 (1 + 19)	50	950
16–40	64–160	1 : 10 (1+ 9)	50	450
2–15	8–64	1 : 5 (1 + 4)	50	200
< 2	< 8	1 : 2 (1 + 1)	100	100

1. The use of a fixative is mandatory to immobilize spermatozoa
2. Count at least 200 spermatozoa in two different squares of the Neubauer chamber
3. Consult table 2.3 (next slide) in the manual for acceptable difference between two counts (based on the number of counted spermatozoa).

Table 2.3

Range of sums	Limit difference	Error of final result based on number of observations
301–318	34	5.8%
284–300	33	5.9%
267–283	32	6.1%
251–266	31	6.3%
235–250	30	6.5%
219–234	29	6.8%
206–218	28	7.0%
190–205	27	7.3%
176–189	26	7.5%
163–175	25	7.8%
150–162	24	8.2%
138–149	23	8.5%
126–137	22	8.9%
115–125	21	9.3%
105–114	20	9.8%
94–104	19	10.3%
85–93	18	10.8%
76–84	17	11.5%
67–75	16	12.2%
59–66	15	13.0%
52–58	14	13.9%
44–51	13	15.1%
38–43	12	16.2%
32–37	11	17.7%
27–31	10	19.2%
22–36	9	21.3%
17–21	8	24.3%
13–16	7	27.7%
10–12	6	31.6%
7–9	5	37.8%
5–6	4	44.7%
3–4	3	57.7%
2	2	70.7%
1	1	100.0%

Sperm motility

- Detailed suggestions on how to chose the microscopic field to evaluate motility
- Four cathegories of motility should be evaluated: a (rapid progressive, clinically important), b(slow progressive), c (non progressive), d (immotile)
- Consult table 2.2 for acceptable differences between two measures in two different slides.

Table 2.2 Acceptable differences (based on 95% confidence interval) between two percentages for a given average, determined from replicate counts of 200 spermatozoa (total 400 counted)

Average (%)	1	2–3	4–6	7–9	10–13	14–19	20–27	28–44	45–55	56–72	73–80	81–86	87–90	91–93	94–96	97–98	99
Acceptable difference	2	3	4	5	6	7	8	9	10	9	8	7	6	5	4	3	2

Sperm morphology

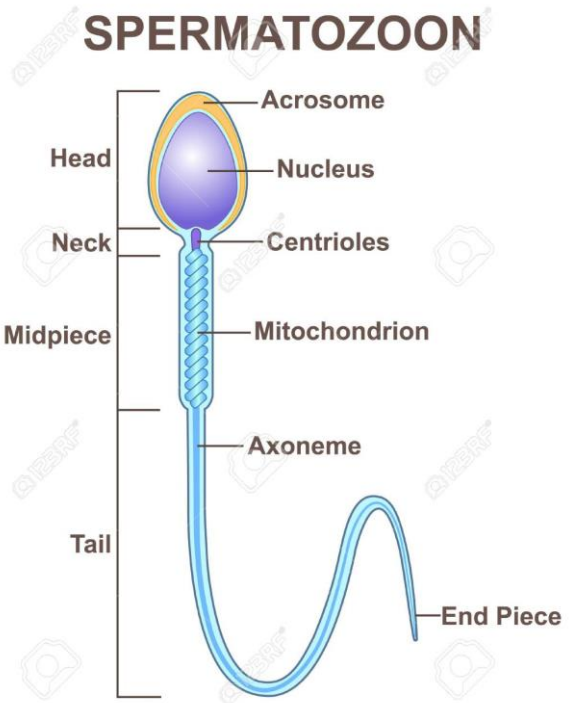
- Staining with Papanicolau is reccomended
- Classification according to Tygerberg strict criteria
- Not only «typical» and «atypical» but also evaluation of all major parts of the spermatozoon
- Consult table 2.6 for classification

There are some other common techniques described in the last part of this chapter: Shorr and rapid staining. The reason for recommending the Papanicolaou staining is that it is still the best evaluated technique. For global use, it is essential that techniques and assessment criteria are standardized. Other techniques can be used, but with proper evaluation and validation with standard techniques, especially if used for scientific studies.

Normal appearence and abnormality of sperm

Table 2.6 Classification of sperm morphology

Location	Normal (ideal/typical) appearance	Abnormal
Head	The head should be smooth, regularly contoured and generally oval in shape. There should be a well-defined acrosomal region comprising 40–70% of the head area (96). The acrosomal region should contain no large vacuoles, and not more than two small vacuoles, which should not occupy more than one fifth of the sperm head. The post-acrosomal region should not contain any vacuoles.	• acrosome less than 40% or larger than 70% of a normal head area, or • length-to-width ratio less than 1.5 (round) or larger than 2 (elongated), or • shape: pyriform (pear shaped), amorphous, asymmetrical, or non-oval shape in the apical part, or • vacuoles constitute more than one fifth of the head area or located in the post-acrosomal area, or • double heads, or • any combinations
Midpiece	The midpiece should be slender, regular and about the same length as the sperm head. The major axis of the midpiece should be aligned with the major axis of the sperm head.	• irregular shape, or • thin or thick, or • asymmetrical or angled insertion at head, or • sharply bent, or • any combinations
Tail	The principal piece should have a uniform calibre along its length, be thinner than the midpiece and be approximately 45 µm long (about 10 times the head length). It may be looped back on itself, provided there is no sharp angulation indicative of a broken flagellum.	• sharply angulated bends, or • smooth hairpin bends, or • coiled, or • short (broken), or • irregular width, or • multiple tails, or • any combinations
Cytoplasmic residue	Cytoplasmic droplets (less than one third of a normal sperm head size) are normal.	• residual cytoplasm is considered an anomaly only when it exceeds one third of normal sperm head size



Chapter 3: extended examinations

> “Clinically, there is growing awareness that chromosomal anomalies and gene mutations underlie a diverse spectrum of male infertility that underlie many of the anomalies seen in a semen analysis.”

The tests described in this chapter are not necessary for routine semen analysis but may be useful in certain circumstances for diagnostic or research purposes. Some

Which extended examination?

- Multiparametric Sperm Assessment: scored variables (e.g. sperm number, motility, and morphology) to provide evidence-based increased specificity and sensitivity of diagnosis. Ex: Teratozoospermia Index (TZI), multiple anomalies index (MAI), sperm deformity index (SDI)
- Sperm DNA quality: Fragmentation, aneuploidies
- Inflammatory indexes: leukocytes evalution, interleukins
- Anti-sperm antibodies: MAR test, IBD test
- Biochemical test: accessory gland function: fructose, zinc, α-glucosidase,
- Assessment of sequence of ejaculation

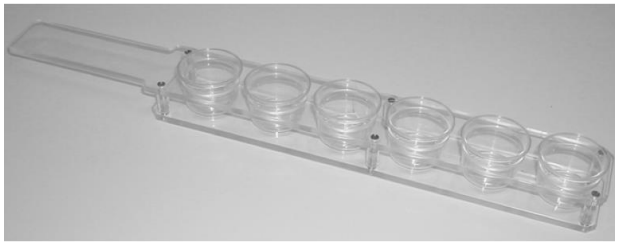
Sperm DNA quality

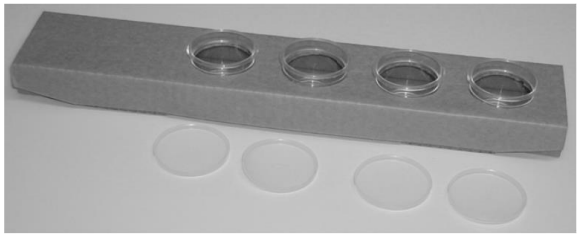
- Standardized protocols for 4 methods to evaluate DNA fragmenaiton are described: sperm chromatin structure assay with acridine orange, TUNEL, COMET and SCD
- Techniques to evaluate aneuploidies

Infammatory markers

- Evaluation of interleukins in semen (elisa methods)
- Evaluation and count of leucocytes

Evaluation of the different fractions in the sequence of ejaculation

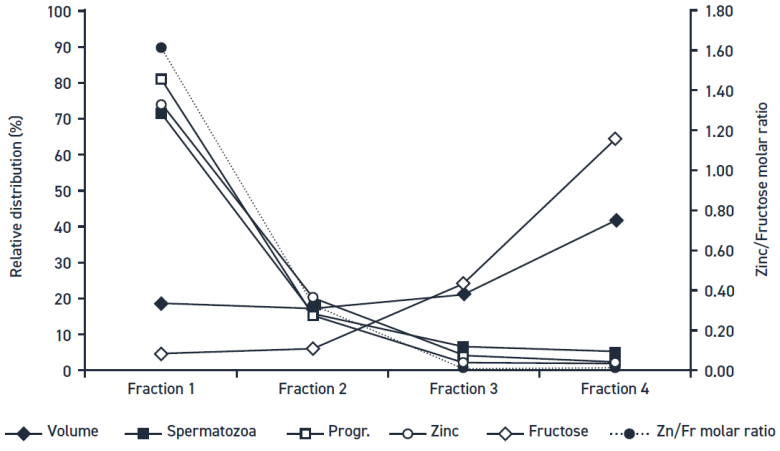




Possible collection devices
for different fractions of the ejaculate

Clear instructions to the patient must be given

Fig. 3.9 Example of graphic representation of results of a normal four-fraction split-ejaculate, showing the distribution volume, spermatozoa, progressive motility, zinc and fructose



3.9.6 Interpretation

The bulk of spermatozoa are expected to be in the first third of the ejaculate, dominated by zinc-rich prostatic secretion. A substantial contribution of seminal vesicular fluid in the sperm-rich fraction(s) indicates a non-physiological situation likely to hamper sperm functional ability.

The identification of an ejaculate fraction with good progressive motility can be useful for future selection of spermatozoa for ART – a fact described long before the era of IVF *(274)*.

Also, for laboratories unable to determine the accessory gland markers zinc and fructose, useful information can be obtained by assessing the relative distribution of spermatozoa and progressive motility, to identify possible sources of spermatozoa with a higher probability of fertilization success.

Chapter 4 Advanced Examinations: the future of diagnosis

> “To gain deeper insights into the biological basis of male factor infertility, a battery of functional tests has been developed aimed at assessing the competence of human spermatozoa to fulfil the fundamental processes essential to conception.”

- Oxidative stress and ROS tests
- Acrosome reaction
- Sperm chromatin
- Ion channels
- CASA (emphasis on hyperactivation) and other emerging technologies

Chapter 5: Sperm preparation techniques

> “An ideal sperm preparation technique should recover a highly functional sperm population that preserves DNA and does not induce dysfunction through the production of ROS by sperm and leukocytes.”

- Simple washing
- DGC
- Swim up
- MACS (Magnetic activating cell sorting- Annexin V)
- Preparing HIV infected semen
- Guide to evaluation of retrograde ejaculation

Chapter 6: Cryopreservation of spermatozoa

> Cell survival after freezing and thawing depends largely on minimization of intracellular ice crystal formation.

6.2.2 Fertility preservation

Ejaculates may be obtained and stored before a man undergoes a procedure or exposure that might impair his fertility, such as:

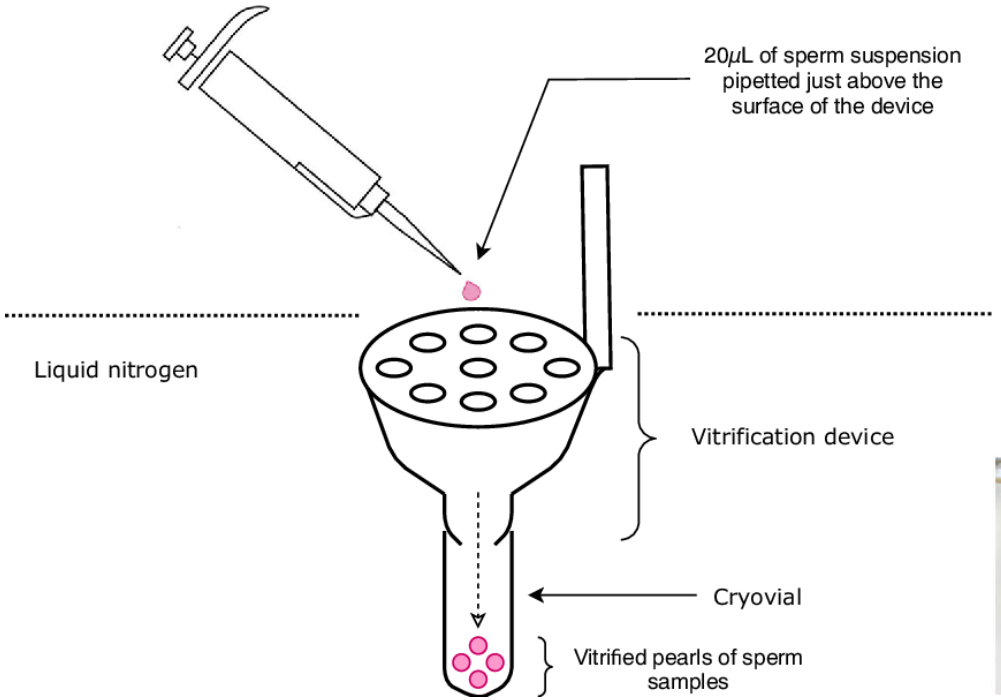
- vasectomy (in case of a future change in partner situation or desire for more children);
- treatment with cytotoxic agents or radiotherapy, which is likely to impair spermatogenesis permanently *(387)*;
- active duty in a dangerous occupation, e.g. in military forces, in countries where posthumous procreation is acceptable or genital injury may occur;
- male-to-female transgender adults and adolescents *(392)*;
- testicular trauma (in some circumstances after testicular sperm extraction, TESE) *(393)*.

Note 2: As only a single spermatozoon is needed for ICSI of each oocyte, cryopreservation of any live spermatozoa is worthwhile.

Note: Although cryoprotectants can be prepared in the laboratory, it should be noted that the performance of the solution and its safety cannot be precisely controlled. **It is usually expected that, when available, cryoprotectants commercially manufactured, certified and approved for therapeutic use are used.** This is particularly an issue for egg yolk-based cryoprotectants, as contaminants from chicken feed or the environment could be present. The procedures described below are very difficult to standardize to a suitable level for ART therapeutic use in local laboratories.

→ Important note

Vitrification has been described as an experimental technique for spermatozoa: Waiting from more studies before introducing it as SOP



Chapter 7: Quality assurance and quality control

> Semen examination is uniquely complicated and procedurally difficult to standardize, which can result in wide discrepancies in the assessments of sperm counts, motility and morphology in different laboratories.

EQC and IQC are both important, and both should be in place together, as they are complementary processes. EQC may reveal problems with accuracy that may not be apparent from IQC if control samples are not adequately masked or selected. EQC

Table 7.12 Time schedule for QC

At all times	surveillance and correlation of results within samples
Weekly/monthly	analysis of replicate measurements by different technicians
Monthly	analysis of mean results
Quarterly/biannually	participation in EQC
Biannually/annually	calibration of pipettes, counting chambers, other equipment

Chapter 8: appendix and reference values: new reference values based on the following:

Origin of study		
Country	**Continent**	**Number of subjects**
Included in WHO 2010		
Australia	Oceania	206
Norway	Europe	82
United States of America	Americas	487
Denmark, Finland, France, United Kingdom	Europe	826
Denmark	Europe	199
New since WHO 2010		
China	Asia	1200
Egypt	Africa	240
Greece	Europe	76
Islamic Republic of Iran	Asia	168
Italy	Europe	105

Campbell et al, Andrology, 2021

		Centiles									
	N	2.5th	5th	(95% CI)	10th	25th	50th	75th	90th	95th	97.5th
Semen volume (ml)	3586	1.0	1.4	(1.3–1.5)	1.8	2.3	3.0	4.2	5.5	6.2	6.9
Sperm concentration (10^6 per ml)	3587	11	16	(15–18)	22	36	66	110	166	208	254
Total sperm number (10^6 per ejaculate)	3584	29	39	(35–40)	58	108	210	363	561	701	865
Total motility (PR + NP, %)	3488	35	42	(40–43)	47	55	64	73	83	90	92
Progressive motility (PR, %)	3389	24	30	(29–31)	36	45	55	63	71	77	81
Non-progressive motility (NP, %)	3387	1	1	(1–1)	2	4	8	15	26	32	38
Immotile spermatozoa (IM, %)	2800	15	20	(19–20)	23	30	37	45	53	58	65
Vitality (%)	1337	45	54	(50–56)	60	69	78	88	95	97	98
Normal forms (%)	3335	3	4	(3.9–4.0)	5	8	14	23	32	39	45

couples having a natural conception within one year of trying (TTP ≤ 12 months). The lower fifth percentile represents the level under which only results from 5% of the men in the reference population were found. This can be of help to interpret results from an individual patient.

Table 8.1 Definition of reference population in Campbell et al. *(5)*

Inclusion criteria	Exclusion criteria

• Men whose partner had a natural conception with a confirmed TTP ≤ 12 months
• Sexual abstinence range between 2 and 7 days
• Laboratory techniques:
  • Evidence of compliance with WHO 2010 and adherence to Björndahl, Barratt, Mortimer and Jouannet *(428)*
  • Internal Quality Control
  • External Quality Assessment
	• Men who were attending an infertility clinic or undergoing fertility assessment


Thank You!