Difference between the WHO 6th ed. and WHO 5th ed. manuals

Applies to the following: MES & ALL SQA/QwikCheck/YO system customers

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In 2021, the WHO 6th edition manual for semen analysis was published. Below is a table that compares the most significant WHO 6th ed. to WHO 5th ed. manual guidelines.

<table>
<tr><th>Update</th><th>WHO 5th</th><th>WHO 6th</th></tr>
<tr>
<td>Definitions of reference ranges</td>
<td>
Measurements made on semen samples need to be compared with reference values to allow decisions to be made about patient management and thresholds for clinical trials or investigations. The reference values given here have been generated from the results of several prospective, cross-sectional studies of semen quality and fertility. They were obtained by direct, retrospective selection of fertile men, defined as men whose partner conceived within 12 months after stopping use of contraception (Cooper et al., 2009; WHO 5th ed. manual, p. 223).

Statistical tradition is to take the 2.5th centile from a two-sided reference interval as the threshold below which values may be considered to come from a different population. However, a one-sided reference interval was considered to be more appropriate for semen parameters, since high values are unlikely to be detrimental to fertility. The 5th centile lower reference limits are given in Table A1.1 (WHO 5th ed. manual, p. 223):

Table A1.1 Lower reference limits (5th centiles and their 95% confidence intervals) for semen characteristics
<table>
<tr><th>Parameter</th><th>Lower reference limit</th></tr>
<tr><td>Semen volume (ml)</td><td>1.5 (1.4–1.7)</td></tr>
<tr><td>Total sperm number (10^6 per ejaculate)</td><td>39 (33–46)</td></tr>
<tr><td>Sperm concentration (10^6 per ml)</td><td>15 (12–16)</td></tr>
<tr><td>Total motility (PR + NP, %)</td><td>40 (38–42)</td></tr>
<tr><td>Progressive motility (PR, %)</td><td>32 (31–34)</td></tr>
<tr><td>Vitality (live spermatozoa, %)</td><td>58 (55–63)</td></tr>
<tr><td>Sperm morphology (normal forms, %)</td><td>4 (3.0–4.0)</td></tr>
<tr><td colspan="2">Other consensus threshold values</td></tr>
<tr><td>pH</td><td>≥7.2</td></tr>
<tr><td>Peroxidase-positive leukocytes (10^6 per ml)</td><td><1.0</td></tr>
<tr><td>MAR test (motile spermatozoa with bound particles, %)</td><td><50</td></tr>
<tr><td>Immunobead test (motile spermatozoa with bound beads, %)</td><td><50</td></tr>
<tr><td>Seminal zinc (µmol/ejaculate)</td><td>≥2.4</td></tr>
<tr><td>Seminal fructose (µmol/ejaculate)</td><td>≥13</td></tr>
<tr><td>Seminal neutral glucosidase (mU/ejaculate)</td><td>≥20</td></tr>
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<td>
The data presented in the fifth edition have been further evaluated and complemented with data from around 3500 men in 12 countries (WHO 6th manual, p. 211).

WHO 6th, p. 213:

Table 8.3 Distribution of semen examination results from men in couples starting a pregnancy within one year of unprotected sexual intercourse leading to a natural conception. From Campbell et al. (5); fifth percentile given with variability (95% confidence interval)
<table>
<tr><th></th><th></th><th colspan="10">Centiles</th></tr>
<tr><th></th><th>N</th><th>2.5th</th><th>5th</th><th>(95% CI)</th><th>10th</th><th>25th</th><th>50th</th><th>75th</th><th>90th</th><th>95th</th><th>97.5th</th></tr>
<tr><td>Semen volume (ml)</td><td>3586</td><td>1.0</td><td>1.4</td><td>(1.3–1.5)</td><td>1.8</td><td>2.3</td><td>3.0</td><td>4.2</td><td>5.5</td><td>6.2</td><td>6.9</td></tr>
<tr><td>Sperm concentration (10^6 per ml)</td><td>3587</td><td>11</td><td>16</td><td>(15–18)</td><td>22</td><td>36</td><td>66</td><td>110</td><td>166</td><td>208</td><td>254</td></tr>
<tr><td>Total sperm number (10^6 per ejaculate)</td><td>3584</td><td>29</td><td>39</td><td>(35–40)</td><td>58</td><td>108</td><td>210</td><td>363</td><td>561</td><td>701</td><td>865</td></tr>
<tr><td>Total motility (PR + NP, %)</td><td>3488</td><td>35</td><td>42</td><td>(40–43)</td><td>47</td><td>55</td><td>64</td><td>73</td><td>83</td><td>90</td><td>92</td></tr>
<tr><td>Progressive motility (PR, %)</td><td>3389</td><td>24</td><td>30</td><td>(29–31)</td><td>36</td><td>45</td><td>55</td><td>63</td><td>71</td><td>77</td><td>81</td></tr>
<tr><td>Non-progressive motility (NP, %)</td><td>3387</td><td>1</td><td>1</td><td>(1–1)</td><td>2</td><td>4</td><td>8</td><td>15</td><td>26</td><td>32</td><td>38</td></tr>
<tr><td>Immotile spermatozoa (IM, %)</td><td>2800</td><td>15</td><td>20</td><td>(19–20)</td><td>23</td><td>30</td><td>37</td><td>45</td><td>53</td><td>58</td><td>65</td></tr>
<tr><td>Vitality (%)</td><td>1337</td><td>45</td><td>54</td><td>(50–56)</td><td>60</td><td>69</td><td>78</td><td>88</td><td>95</td><td>97</td><td>98</td></tr>
<tr><td>Normal forms (%)</td><td>3335</td><td>3</td><td>4</td><td>(3.9–4.0)</td><td>5</td><td>8</td><td>14</td><td>23</td><td>32</td><td>39</td><td>45</td></tr>
</table>

The lower fifth percentile of data from men in the reference population (Table 8.3) does not represent a limit between fertile and infertile men (WHO 6th manual, p. 214).

Distribution of results from presumed fertile men is not sufficient to establish clinically useful decision limits (WHO 6th manual, p. 4).

A decision limit is based on clinical and statistical considerations that point to a need for a certain diagnostic or therapeutic intervention (WHO 6th manual, p. 213).
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<tr>
<td>Preparation for microscopic investigation</td>
<td>The nature of the liquefied ejaculate makes taking a representative sample of semen for analysis problematical. If the sample is not well mixed, analysis of two separate aliquots may show marked differences in sperm motility, vitality, concentration and morphology. To be certain of obtaining reproducible data, the sample should be thoroughly mixed before aliquots are taken for assessment.</td>
<td>For reliable results of microscopic investigation, it is essential that the aliquots examined are representative of the entire ejaculate. The nature of the liquefied ejaculate, which is still more viscous than water, makes taking a representative sample of semen for analysis highly problematic. If the sample is not well mixed, analysis of two separate aliquots is unlikely to be representative of the entire ejaculate and can show marked differences in sperm concentration, motility, vitality and morphology. Even if the liquefied ejaculate is macroscopically homogeneous, small aliquots can have very different composition (WHO 6th, p. 18).</td>
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<table>
<tr><td>Semen volume</td><td>The volume is best measured by weighing the sample in the vessel in which it is collected. Calculate the volume from the sample weight, assuming the density of semen to be 1 g/ml (Auger et al., 1995).</td><td>The volume is best measured by weighing the sample in the container in which it has been collected (WHO 6th manual, p. 15).
In the first 5 minutes – Initial sample handling:
For practical reasons it is convenient to determine sample volume by weighing during the pre-examination phase.
• Measuring semen volume by weight can preferably be done at the time the sample is received, and before liquefaction
(WHO 6th manual, p. 12).</td></tr>
<tr><td>Liquefaction</td><td>In the first 5 minutes:
• Placing the specimen container on the bench or in an incubator (37 °C) for liquefaction.
The complete sample usually liquefies within 15 minutes at room temperature, although rarely it may take up to 60 minutes or more.</td><td>The collected ejaculate should without unnecessary delay be allowed to liquefy, preferably in an incubator at 37 °C (WHO 6th manual, p. 15).
In the first 5 minutes – Initial sample handling:
• Allow time for liquefaction to occur (usually no more than 30 minutes) (WHO 6th manual, p. 12).</td></tr>
<tr><td>Collection-to-test time</td><td>Semen analysis should begin with a simple inspection soon after liquefaction, preferably at 30 minutes, but no longer than 1 hour after ejaculation, to prevent dehydration or changes in temperature from affecting semen quality.</td><td>• The time between collection and the start of the ejaculate examination should be recorded at the start of macroscopic evaluation and presented in the final report. Preferably assessment should start within 30 minutes after collection and no more than 60 minutes after collection.
• Prolonged in vitro exposure to the liquefied ejaculate fluid will affect qualities such as motility and morphology (WHO 6th manual, p. 15).</td></tr>
<tr><td>pH</td><td>The pH should be measured after liquefaction at a uniform time, preferably after 30 minutes, but in any case within 1 hour of ejaculation since it is influenced by the loss of CO_2 that occurs after production.
For normal samples, pH paper in the range 6.0 to 10.0 should be used.</td><td>The clinical interest of ejaculate pH is a low value. If pH is to be assessed, it should be done at a uniform time, preferably 30 minutes after collection, but in any case, within one hour of ejaculation.
For normal samples, pH test strips in the range 6.0 to 10.0 should be used (WHO 6th manual, p. 17).</td></tr>
<tr><td>Sample dilution</td><td>Table 2.3 Semen dilutions required, how to make them, which chambers to use and potential areas to assess
<table><tr><th>Spermatozoa per ×400 field</th><th>Spermatozoa per ×200 field</th><th>Dilution required</th><th>Semen (µl)</th><th>Fixative (µl)</th><th>Chamber</th><th>Area to be assessed</th></tr><tr><td>>101</td><td>>404</td><td>1:20 (1+19)</td><td>50</td><td>950</td><td>Improved Neubauer</td><td>Grids 5, 4, 6</td></tr><tr><td>16–100</td><td>64–400</td><td>1:5 (1+4)</td><td>50</td><td>200</td><td>Improved Neubauer</td><td>Grids 5, 4, 6</td></tr><tr><td>2–15</td><td>8–60</td><td>1:2 (1+1)</td><td>50</td><td>50</td><td>Improved Neubauer</td><td>Grids 5, 4, 6</td></tr><tr><td><2</td><td><8</td><td>1:2 (1+1)</td><td>50</td><td>50</td><td>Improved Neubauer or large-volume</td><td>All 9 grids
Entire slide</td></tr></table></td><td>Table 2.1 Sufficient volumes of ejaculates - final volumes of diluted sperm suspensions for adequate handling (WHO 6th manual, p. 20).
Table 2.1 Sufficient volumes of ejaculates – final volumes of diluted sperm suspensions for adequate handling
<table><tr><th>Spermatozoa per ×400 field</th><th>Spermatozoa per ×200 field</th><th>Dilution</th><th>Ejaculate (µl)</th><th>Fixative (µl)</th></tr><tr><td>> 200</td><td>> 800</td><td>1 : 50 (1 + 49)</td><td>50</td><td>2 450</td></tr><tr><td>40–200</td><td>160–800</td><td>1 : 20 (1 + 19)</td><td>50</td><td>950</td></tr><tr><td>16–40</td><td>64–160</td><td>1 : 10 (1+ 9)</td><td>50</td><td>450</td></tr><tr><td>2–15</td><td>8–64</td><td>1 : 5 (1 + 4)</td><td>50</td><td>200</td></tr><tr><td>< 2</td><td>< 8</td><td>1 : 2 (1 + 1)</td><td>100</td><td>100</td></tr></table></td></tr>
</table>

Testing temperature	The procedure may be performed at room temperature or at 37°C with a heated microscope stage but should be standardized for each laboratory. If sperm motility is to be assessed at 37°C, the sample should be incubated at this temperature and the preparation made with prewarmed slides and coverslips.	The velocity of motile spermatozoa is temperature dependent. It is therefore essential to standardize the temperature during motility assessment. It is often easiest to control a temperature similar to body temperature, but that requires that the microscope is equipped with a temperature-controlled object stage, that microscope slides and coverslips are prewarmed, and that the sample is also warmed to 37 °C before assessment. These aspects are easily fulfilled when the sample liquefies in a 37 °C incubator. Using room temperature is more problematic, not least because room temperature is not defined and therefore can vary substantially (WHO 6th manual, p. 23).
Counting chamber	The use of 100-µm-deep haemocytometer chambers is recommended.	The use of haemocytometer chambers with improved Neubauer ruling is recommended (WHO 6th manual, p. 29).
Haemocytometer incubation	Store the haemocytometer horizontally for at least 4 minutes at room temperature in a humid chamber (e.g. on water-saturated filter paper in a covered Petri dish) to prevent drying out. The immobilized cells will sediment onto the grid during this time.	Store the haemocytometer horizontally for at least 10–15 minutes (to allow complete sedimentation of spermatozoa in the 100 µm deep chamber) at room temperature in a humid chamber (e.g. on water saturated filter paper in a covered Petri dish) to prevent drying out (WHO 6th manual, p. 31).
Number of sperm counted	200 per replicate	200 per replicate
Lower sperm concentration detection limit	2 M/ml	It is important that the laboratory does not stop assessing the number of sperm at low concentrations (2 million/ml), as suggested in the past edition, but report lower concentrations, noting that the errors associated with counting a small number of spermatozoa may be very high (WHO 6th manual, p. 3).
Calculation of sperm concentration	The concentration of spermatozoa in semen is their number (N) divided by the volume in which they were found, i.e. the volume of the total number (n) of rows examined for the replicates (20 nl each for grids 4, 5 and 6), multiplied by the dilution factor. That is, $C = (N/n) \times (1/20) \times$ dilution factor. For 1 + 4 (1:5) dilutions, using grids 4, 5 and 6, the concentration $C = (N/n) \times (1/20) \times 5$ spermatozoa per nl = $(N/n) \times (1/4)$ spermatozoa/nl (or 10^6 per ml of semen). For 1 + 19 (1:20) dilutions, using grids 4, 5 and 6, the concentration $C = (N/n) \times (1/20) \times 20$ spermatozoa per nl = (N/n) spermatozoa/nl (or 10^6 per ml of semen). For 1:50 (1 + 49) dilutions, using grids 4, 5 and 6, the concentration $C = (N/n) \times (1/20) \times 50$ spermatozoa per nl = $(N/n) \times 2.5$ spermatozoa/nl (or 10^6 per ml of semen).	The sum of the two accepted replicate counts is divided by a factor that is determined by the dilution and number of large squares or grids assessed in both counting chambers (if three attempts have been done without reaching sufficient agreement between replicate counts, the average of the three sums is used) (WHO 6th manual, p. 34). WHO 6th manual, p. 35: Table 2.4 Calculation of sperm concentration from sperm count Dilution / Number of *large squares* counted in each chamber (5, 10, 25) / Number of *grids* counted in each chamber (2, 3, 4, 5, 6, 7, 8, 9) / Correction factor values 1 : 2 — *20* *40* *100* — 200 300 400 500 600 700 800 900 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) grids of equal size. The central grid consists of 25 large squares, each surrounded by a triplet line, while the 8 peripheral fields each consist of 16–20 rectangles.

Total Sperm Numbers	The total number of spermatozoa per ejaculate and the sperm concentration are related to both time to pregnancy (Slama et al., 2002) and pregnancy rates (WHO, 1996; Zinaman et al., 2000) and are predictors of conception (Bonde et al., 1998; Larsen et al., 2000).	It is essential to calculate and report the total number of spermatozoa per ejaculate, as this parameter provides a much better measure of testicular sperm production and of the number of spermatozoa transferred to the female during coitus. This is obtained by multiplying the sperm concentration by the volume of the whole ejaculate (WHO 6th manual, p. 35).
Sperm Motility grades	Total motility (PR+NP) Progressive motility (PR) Non-progressive motility (NP) Immotility (IM)	The recommended categories are (with approximate velocity limits) (WHO 6th manual, p. 24): • rapidly progressive (25 µm/s) – spermatozoa moving actively, either linearly or in a large circle, covering a distance, from the starting point to the end point, of at least 25 µm (or ½ tail length) in one second; • slowly progressive (5 to < 25 µm/s) – spermatozoa moving actively, either linearly or in a large circle, covering a distance, from the starting point to the end point, of 5 to < 25 µm (or at least one head length to less than ½ tail length) in one second; • non-progressive (< 5 µm/s) – all other patterns of active tail movements with an absence of progression – i.e. swimming in small circles, the flagellar force displacing the head less than 5 µm (one head length), from the starting point to the end point; and • immotile – no active tail movements.
Post vasectomy sample processing	In a PV sample, high speed centrifugation must be avoided.	When detection of motile spermatozoa is essential, it is important to determine whether centrifugation procedures used can damage sperm and impair sperm motility and therefore cause false results. If centrifuged samples are used, the laboratory must ensure that their procedures do not harm sperm motility or fertilizing ability (WHO 6th manual, p. 36).

Morphology criteria and range	Strict (Kruger et al., 1986; Menkveld et al., 1990; Coetzee et al., 1998). All borderline forms should be considered abnormal. Using these guidelines, the range of percentage normal values for both fertile and infertile men is likely to be 0–30%, with few samples exceeding 25% normal spermatozoa (Menkveld et al., 2001).	For the evaluation of the male reproductive organs, it is not sufficient to only determine the proportion of "normal" spermatozoa. It is important to evaluate the specific morphology of head, neck/midpiece and tail, and the possible presence of abnormal cytoplasmic residues (WHO 6th manual, p. 41). The criteria presented here were developed from investigations of the morphology of spermatozoa able to penetrate cervical mucus and bind to the zona pellucida. By the strict application of certain criteria of sperm morphology, relationships between the percentage of "normal" forms and various fertility endpoints (time to pregnancy, pregnancy rates in vivo and in vitro) have been established (WHO 6th manual, p. 42). All borderline forms should be considered abnormal (WHO 6th manual, p. 49). The range of percentage normal forms for both fertile and infertile men is likely to be well under 30%. This will inevitably produce low thresholds discriminating between fertile and infertile populations; indeed reference limits and thresholds of 3–5% normal forms have been found in studies of in vitro fertilization (WHO 6th manual, p. 42).
DNA Fragmentation	-	The halos of the spermatozoa in the samples can be classified according to the criteria of Fernández et al. (25): • Large: Halo width is similar to or larger than the minor diameter of the core • Medium: Halo size is between those with large and with small halo • Small: Halo width is similar to or smaller than one third of the minor diameter of the core • Without halo • Without halo-degraded: Those that show no halo and present a core irregularly or weakly stained. This category is associated with severe damage affecting both DNA and protein compound. The results should be represented as a percentage of each category. The percentage of spermatozoa with fragmented DNA is the sum of those with small halo, without halo and without halo-degraded (WHO 6th manual, p. 96).

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