EDUCATIONAL COMMENTARY – CURRENT METHODS TO DETERMINE SPERM COUNTS

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Learning Outcomes
Upon completion of this exercise, participants will be able to
- identify the available methods to perform a sperm count.
- explain how each sperm counting method works.
- evaluate the advantages and disadvantages of each method for counting sperm.
- explain how to calculate a sperm count in millions per milliliter as well as the total number of sperm per ejaculate.

In 1957 Helpler stated that the normal value for the human sperm count is 100 to 150 million/mL in Manual of Clinical Laboratory Methods. In 1965 Todd and Sandford gave the normal sperm count as 80 to 120 million/mL in Clinical Diagnosis by Laboratory Methods. In recent years this value has decreased to about 20 million/mL. Some couples have no problem conceiving with a lower level of sperm, yet others find themselves subfertile and aggressively try to achieve a pregnancy through intrauterine insemination, in vitro fertilization, or other techniques. Because the term “normal” is no indicator as to actual fertility, the WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction has changed the terminology for “normal values” in semen analysis to “reference values.” The reference values according to the 4th edition of the WHO Manual are:

<table>
<thead>
<tr>
<th>Sperm concentration (count)</th>
<th>≥20 million/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sperm number</td>
<td>≥40 million/per ejaculate</td>
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</table>

Semen analysis is the first line of testing to rule out the male factor as the basis for infertility. One of the main components of semen analysis is the sperm count. Sperm counts traditionally performed by manual methods are time-consuming, laborious, and require highly trained personnel. Due to the huge increase in demand for semen analysis in the laboratory, infertility clinics, and doctor’s offices, several companies have designed computerized systems for the performance of semen analysis. These systems accommodate the steady stream of patients who need immediate results prior to an insemination or other procedure.

Historically, sperm counts were performed manually using an improved Neubauer hemocytometer. Later, the Makler counting chamber was specifically designed to perform sperm counts and motility analysis. New techniques known as computer-assisted semen analysis (CASA) are based on image analysis.

Other systems are based on signal processing. They quickly determine results without the need for highly trained personnel. A description of each of these methods follows.
Quality Control of the Sperm Count

Quality control of the sperm count is performed daily. Solutions of latex beads such as Accu-Beads (Hamilton Thorne, Inc, Beverly, MA) or Qwik-Check Beads (Medical Electronic Systems Ltd, Caesarea Industrial Park, Israel) are available at high and low concentrations. Bead counts on these solutions are done in the same manner as directed for the sperm count for both manual methods and computerized systems.

Quality control through the bead count checks several aspects of the sperm count, including:

- Proper technique in loading the counting chamber
- Accuracy of the counting chamber (the chamber can become eroded or worn from wiping thereby affecting the depth)
- Ability of the technologist or analyzer to count objects accurately
- Ability of the technologist or analyzer to calculate final results

Editor's note: Another choice for quality control is available from Fertility Solutions, Inc., Cleveland, OH. The samples are stabilized human serum instead of latex beads.

Counting chambers for manual bead and sperm counts are checked by duplicate analysis. According to World Health Organization (WHO) guidelines, at least 200 beads or sperm should be counted in each duplicate analysis to ensure consistency and to achieve a statistically low counting error. This is achieved by:

- Counting the same area on both sides of the hemocytometer. It may be necessary to count the entire 1 sq mm square to ensure a tally of 200.
- Counting two different strips of 10 on the Makler. It may be necessary to count 50 or more small squares of the Makler to ensure a tally of 200.

Subfertile ejaculates may not display 200 sperm. This adversely affects the confidence interval of the final result. It is impractical to count multiple chambers with only 3 sperm in each to arrive at a tally of 200. Manual counting methods become laborious when statistical analysis is applied to the low sperm count. Statistical analysis is beyond the scope of this commentary. A full explanation of statistical methods for semen analysis is given the 4th edition of the WHO Manual.

Ideally, at least 200 sperm are in each duplicate, and the duplicates fall within +/-10% of the mean. For example, if 224 sperm are found on one side of the counting chamber and 244 on the other:
The sum of the 2 counts is $224 + 244 = 468$

The mean is $468/2 = 234$

The acceptable range for each duplicate count is calculated as $234 \pm 10\%$:

$10\%$ of $234 = 23$

$234 - 23 = 211 = \text{the lower acceptable count}$

$234 + 23 = 257 = \text{the upper acceptable count}$

The acceptable range = 211–257

Both counts, 224 and 244, fall within this range. The counts are both acceptable.

For CASA systems, WHO recommends performing counts of 6 different fields of the chamber on each side (12 fields total) of the counting chamber. The beads display as red dots on the monitor because they are nonmotile. At least 200 beads must be counted on each side to be valid. More fields may need to be counted to ensure a tally of 200. The counts for each side must agree within a statistically acceptable range.

Other quality control procedures for manual sperm counts consist of interdepartmental replicate analysis by different technologists, replicate counts between different laboratories, and performance of proficiency-testing surveys. Most computerized analyzers have quality control systems built into their software.

WHO recommends that the validity of a Makler counting chamber be established by comparison with the hemocytometer method.

**Semen Preparation for the Sperm Count**

Basic semen preparation is the same for all sperm-counting systems. The specimen must be kept warm or at an ambient temperature. Testing must begin within 1 hour for motility studies. The sperm count can be performed later using the hemocytometer procedure because sperm are immobilized prior to counting. Motile sperm are required to determine the cell size and density of the sperm head for computerized systems, so testing must be performed as soon as the specimen has liquefied. Upon liquefaction, mix the specimen by gentle swirling to avoid foaming and to achieve a homogeneous solution.

After achieving a homogeneous solution:

- Prepare dilutions of semen in a sperm-immobilizing diluent as required for the hemocytometer.
- Heat to immobilize the sperm at 50º-60ºC for the Makler counting chamber.
- Place undiluted semen directly on a fixed-depth counting chamber or capillary for computerized analysis.
Manual Sperm Count: Improved Neubauer Hemocytometer

The improved Neubauer hemocytometer (Hausser Scientific, Horsham, PA) is a heavy glass slide with 2 slightly recessed chambers. Each chamber has a scored-in counting grid. A moat lines each side of the grids. The inner edge of each moat supports a coverslip on the chamber that gives the chamber a depth of 0.1 mm. There are two V-shaped cutouts on each side of the chamber for easy loading by capillary action.

Looking at the grid on one side of the hemocytometer note (Image 1):

1. The grid displays 9 large squares that are 1 mm on each side thereby giving each large square an area of 1 mm².
2. The large middle square is divided into 25 small squares each with an area of 0.04 mm².
3. The 25 small squares are further subdivided into 16 smaller squares each with an area of 0.0025 mm².

mix the semen by gentle swirling. Place 5 to 10 μL on a glass slide. Coverslip. Immediately examine semen using a x40 objective. Determine the approximate number of sperm present. Are there many? Few? Rare? This can be done while performing motility studies. The WHO requires that at least 200 sperm be counted. A dilution of 1:20 or 1:10 is usually optimal for most sperm counts. A duplicate count must be performed, one on each side of the hemocytometer. Some samples, however, do not contain enough sperm to count 200 even though no dilution is made. For this reason, a thorough understanding of the formula to calculate sperm counts is key to an accurate result. More squares than usual may need to be counted to arrive at 200 sperm. An understanding of the formula allows for this flexibility.
If many sperm are present per high-power field (hpf), prepare a 1:20 dilution of the semen. Pipette 0.95 mL of diluent into a test tube. Add 0.05 mL of well-mixed semen. Mix well. Load both sides of the hemocytometer using a capillary tube or pipette tip. Gently touch the capillary tube or tip to the V-shaped cutouts on each side of the hemocytometer. Allow the diluted semen to pass into the chamber by capillary action. Place the loaded hemocytometer into a moist chamber such as a Petri dish with dampened filter paper. Allow the contents of the hemocytometer to settle for 5 minutes. Place the hemocytometer onto the microscope stage. Count the 4 small corner and center squares (0.2 mm²) located in the large center square (1 mm²) of the counting chamber.

Calculations
General formulas:

\[
\text{Area} = \text{Length} \times \text{Width} \\
\text{Volume} = \text{Length} \times \text{Width} \times \text{Depth}
\]

Formula for the hemocytometer:

\[
\text{Number of sperm per cu mm} = \frac{\text{number of sperm counted} \times \text{dilution}}{\text{number of square millimeters counted} \times \text{depth of chamber}}
\]

Calculations expressed in fractions as a visual aid:
Dilution = 1:20  Squares counted = 5 out of 25 or 5/25 mm²  Depth = 1/10 mm  256 sperm are counted

\[
\text{Number of sperm per cubic millimeter} = \frac{256 \times 20}{5/25 \times 1/10}
\]

To calculate: invert the fractions and multiply:
\[
\text{Number of sperm per cubic millimeter} = \frac{256 \times 20 \times 25 \times 10}{5 \times 1} = 256,000/\text{mm}^3
\]

Calculations expressed as decimals:

\[
\text{Number of sperm per cubic millimeter} = \frac{256 \times 20}{0.2 \times 0.1} = 256,000/\text{mm}^3
\]

Sperm counts (concentrations) are reported as sperm per milliliter. Multiply the sperm count per cubic millimeter by 1000.

\[
256,000 \times 1000 = 256,000,000/\text{mL} \text{ (sperm per milliliter)}
\]
Calculate the total sperm count per ejaculate:

Multiply the sperm per milliliter by the volume of the ejaculate to find the total sperm count per ejaculate. If total volume of ejaculate is 2.3 mL:

Total number of sperm per ejaculate = 2.3 × 256,000,000 = 588,800,000

In case of a lower sperm count, prepare a 1:10 dilution. If 256 sperm are seen in 5/25 mm² the calculation is:

Number of sperm per cubic millimeter = \frac{256 \times 10}{5/25 \times 1/10} = 128,000/mm³

or

Number of sperm per cubic millimeter = \frac{256 \times 10}{0.2 \times 0.1} = 128,000/mm³

Number of sperm per milliliter = 128,000 × 1000 = 128,000,000/mL

If only a rare sperm is seen, load the counting chamber with undiluted semen. Sperm can be immobilized by placing in a heat block or water bath at 50º to 60ºC. This is rarely necessary because there are so few sperm to keep track of when counts are this low. Count the entire large 1 mm² square.

If 5 sperm are counted in 1 mm² and the dilution factor is 1 (no dilution):

Number of sperm per cubic millimeter = \frac{5 \times 1}{1 \times 1/10} = 50/mm³

or

Number of sperm per cubic millimeter = \frac{5 \times 1}{1 \times 0.1} = 50/mm³

Number of sperm per milliliter = 50 × 1000 = 50,000/mL

Charts for the improved Neubauer hemocytometer display factors to calculate results for the more commonly used dilutions and square counts.

**Manual Sperm Count: The Makler Counting Chamber**

The Makler counting chamber (Sefi Medical Instruments Ltd, Haifa, Israel) (Image 2) is specially designed to evaluate sperm concentration and motility. It is only 10 μm deep (1/10th of the improved Neubauer hemocytometer) to ensure that all the sperm are in the same focal plane for ease and accuracy. The chamber is constructed of 2 pieces of optically flat glass. The upper layer is a cover glass with a 1-mm² grid built into the center. This grid is further divided into 100 squares that are 0.1 mm × 0.1 mm each. The cover glass has 2 dark dots on the rim that serve as markers for handling. The cover glass is secured to the chamber by 4 quartz pins.
Image 2: Makler Counting Chamber

100 square grid of the Makler

Procedure
The Makler counting chamber must be cleaned and free of dust just prior to use. Dust particles >10 μm in size may interfere with the depth of the chamber. Place the cover glass on the 4 pins, and look for color fringes on each pin (best seen against reflected fluorescent light) to check cleanliness.

Sperm must be immobilized prior to performing the sperm count. No dilution is required when using the Makler counting chamber. Transfer an aliquot of semen into a clean test tube. Place the test tube in a heating block or water bath at 50º to 60ºC for 5 minutes. If a temperature-controlled heating block or water bath is not available, place the tube in a mixture of 2/3 boiling water and 1/3 tap water.

Place a small, undiluted drop of immobilized semen in the center of the chamber using a rod or pipette tip. Apply the cover glass, grasping it at the markers, and place it on the 4 pins. Examine the chamber against a fluorescent light for color fringes to ensure proper placement. Place the chamber on the stage of a phase microscope with a ×20 objective. Note: the ×40 objective cannot be used because it may damage the cover glass. Count the number of sperm that display heads and tails in 10 squares. If very few sperm are present, count the entire 100 squares. An attempt should be made to count at least 200 sperm. The chamber and cover glass are cleaned and reused. Rinse the chamber and cover glass with a noncorrosive antiseptic solution and sterile water. Wipe dry with special lens paper or Kimwipe (Kimberly-Clark Worldwide, Inc, Roswell, GA).

Calculations
The total number of sperm counted within 10 squares is the total concentration of sperm in millions per milliliter. No further calculation is required.

65 sperm are counted in 10 squares. The total count is 65,000,000/mL.

The total count per ejaculate is the volume of the ejaculate times the concentration per milliliter.
If the volume of this ejaculate is 2.4 mL, the total number of sperm is:

\[ 2.4 \times 65,000,000 = 156,000,000 \text{ sperm per ejaculate} \]

If very few sperm are present count all 100 squares. The total number of sperm counted is reported in hundreds of thousands per milliliter with no further calculation. If 65 sperm are counted in 100 squares:

The total sperm count = \[ 65 \times 100,000 = 6,500,000/\text{mL} \]

The formula for this calculation is:

\[ \frac{\text{Sum of all squares counted}}{\text{Number of squares counted}} = \text{Average sperm count per square} \]

Average sperm count per square × 10 = number of sperm in 10 squares = total sperm count per milliliter

\[ \frac{65}{100} = 0.65 \quad 0.65 \times 10 = 6.5 = 6,500,000/\text{mL} \]

The total sperm count of this ejaculate if the volume is 2.4 mL is:

\[ 2.4 \times 6,500,000 = 15,600,000 \text{ sperm per ejaculate} \]

**CASA System Based on Image Analysis**

The Integrated Visual Optical System (IVOS) (Hamilton Thorne, Inc, Beverly, MA) is a sperm-imaging analysis system (Image 3) that contains an internal optical system thereby negating the need for an external microscope. Sperm count and motility are performed simultaneously using undiluted semen. Sperm are normally motile and this can cause blurring when tracked by a CASA system. The light source of the IVOS uses illumination strobed at 1/1000th of a second to eliminate motion blurring along the length of the sperm head. The IVOS counts sperm and performs motility analyses by tracking sperm at 60 image frames per second. Internal optics send analog signals to the imaging processor where they are digitally encoded. After encoding, each frame is analyzed by the microprocessor, which performs analyses under 3 types of internal, strobed illumination: phase contrast, bright field, and multi-wavelength fluorescence.

**Image 3: Frame displaying motile Sperm – IVOS Sperm Imaging Analyzer**
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The IVOS Windows-based Human Software determines the sperm count by analyzing the sperm cells based on several parameters such as sperm head length, width and contrast; motility tracking; and differentiation of other static (nonmotile) objects of similar dimensions. Because semen contains many different types of static objects, the IVOS has a Playback feature that displays the image frames on a monitor. Motile sperm display track lines from their movement, whereas the static objects display a red dot. The user examines the red dots on the screen to check for heads and tails denoting sperm as opposed to white blood cells, immature sperm cells, or other debris that have other identifying characteristics. This is especially important when <5 motile cells are available for analysis. Cell size and intensity are calculated from the motile cells. Less than 5 motile cells precludes the determination of a meaningful average for cell size and intensity, which is necessary to set the parameters for counting sperm heads. Playback serves as a quality control measure to ensure that blood cells and debris are not counted as immotile sperm in the final count. Sperm can also be identified by using a fluorescent stain for DNA that can be analyzed by a built-in ultraviolet light. The IVOS counts fluorescent sperm cells after proper gating using DNA parameters set by the processor for the sperm head.

The IVOS has a built-in, computer-controlled stage that maintains the temperature from ambient to 40ºC depending on desired temperature. It can be moved manually or automatically. The 20 μm 2X-CEL (Hamilton Thorne, Inc, Beverly, MA) chamber with a plain coverslip is specifically designed for the IVOS. The stage also accommodates and automatically adjusts for the fixed-depth, disposable analysis chambers most commonly used for sperm analysis such as the Leja (2153 GN Nieuw-Vennep, The Netherlands), Microcell (Conception Technologies, San Diego, CA), and Cellvu (Millennium Sciences, Inc, New York, NY).

Procedure
The counting and motility determination procedure is easy.

- Patient information and sample volume data is entered into the computer. A dilution may be necessary if the sperm count is high. If necessary, prepare the dilution and enter the final dilution into the computer.
- Load the required amount of undiluted or diluted semen (usually 10-20 μL) onto the counting chamber by capillary action.
- Immediately, place the loaded counting chamber on the stage.
- Insert the stage into the IVOS.
- Focus the image.
- Select the desired fields for counting.
- Start the analysis.

The time required for the sperm count depends on the number of sperm present. Ejaculates with fewer sperm require more fields to be counted and possible visual examination, which requires a longer period of time.
Calculations

The sperm count (concentration) and motility performance results are displayed on the monitor in minutes. No further calculation is necessary. The results are based on the number of sperm counted, the dilution of semen used (if any, and originally entered into the computer), the cells that the user approved to be counted during Playback, and the depth of the counting chamber in use. The IVOS also displays the total sperm count based on the concentration per milliliter multiplied by the total volume of the ejaculate that was initially entered into the computer.

CASA System Using CEROS

The CEROS (Hamilton Thorne, Inc, Beverly, MA) sperm analyzer is the same as the IVOS analyzer without the internal optics. An external, trinocular, negative-phase microscope (Image 4) provides images for a digital camera that is attached to the microscope. Images are sent to the processor, which analyzes the data according to the same software parameters as the IVOS. A MiniTherm (Hamilton Thorne, Inc, Beverly, MA) stage warmer maintains the counting chamber at a constant temperature of 37ºC. The MiniTherm stage accommodates the same counting chambers as the IVOS.

Procedure

The CEROS procedure is almost identical to that of the IVOS. Enter the patient and specimen data into the computer. Load the counting chamber. Place the loaded counting chamber into the MiniTherm stage warmer. Place the MiniTherm stage warmer on the microscope stage, focus the image, select the desired fields to count, and start the analysis. A playback feature allows visual examination of the fields.

Calculation

As with the IVOS, the count and motility results are quickly displayed on the monitor. No further calculation is necessary. The actual time required for the analysis depends on the number of fields that must be counted to reach a minimum count of 200 sperm.
Signal Processing Semen Analysis: SQA-V

The Sperm Quality Analyzer, SQA-V, (Medical Electronic Systems Ltd, Caesarea Industrial Park, Israel) is an instrument with Windows-compatible software (Image 5). It uses electro-optical signal processing, built-in proprietary computer algorithms, and video microscopy to perform the clinical parameters of semen analysis in as little as 75 seconds. Ejaculates with fewer sperm require a longer period of time. The SQA-V performs the sperm count using light absorption. The SQA-V emits a specific light wavelength. Sperm cells absorb more light than seminal plasma. The absorption is measured and calculated electronically by the SQA-V. Results are viewed on a personal computer screen. A visual compartment allows the user to examine the specimen visually to ensure accuracy. The optical visual system works using a standard slide or SQA-V capillary (Image 6) that is inserted directly into the instrument. The SQA-V performs a self-calibration and self-test during start-up. It also runs latex bead quality control material.

Image 5: Sperm Quality Analyzer, SQA-V

Procedure

Performing a complete semen analysis including the sperm count is straightforward. Enter the patient data information in the SQA-V. For a specimen of normal volume, using the attached syringe, fill the SQA-V capillary with 500 to 650 μL of well-mixed, undiluted semen by aspirating the semen into the capillary and cuvette sections until it just appears in the luer adaptor. Quickly wipe off excess semen.
from the outer surfaces of the capillary to prevent clogging of the SQA-V optical chamber. Confirm that no bubbles are present and that the thin chamber of the capillary is still filled with semen. Push-in the blue separating valve. Insert the capillary with the syringe attached into the measurement compartment of the SQA-V. For a low-volume specimen, fill only the thin capillary chamber with 20 μL of semen. Quickly wipe off excess semen. Examine the capillary chamber for bubbles. Remove the separating valve and syringe. Insert the capillary into the measurement compartment of the SQA-V. After the capillary is inserted, start the test.

In case of very low sperm counts, a video visualization system enables visual examination of the sperm cells. The range of the SQA-V is 2 to 400 million sperm per milliliter. Low-quality specimens are evaluated for 2 to 9 extra minutes.

**Calculation**

Results of all parameters of semen analysis are displayed on the screen in as little as 75 seconds but may take 2 to 9 minutes for low counts. No calculations are required.

In researching information on the Internet about the SQA-V, several discrepancies were found from document to document and within the same document itself. The low-volume technique values differed from 20 μL to 50 μL to fill the very thin capillary section. One source stated that 650 μL of semen were required for SQA-V analysis, whereas another stated that for specimens of normal volume, 500 μL were required to fill the capillary and cuvette section of the SQA-V capillary. The counting range for the SQA-V in one source was 0 to 400 million sperm per milliliter and 2 to 400 million per milliliter in another.

**References**


4. Agarwal, A., Sharma, R. Automation is the key to standardized semen analysis using the automated SQA-V sperm quality analyzer. Fertility and Sterility Vol. 87, No. 1, January 2007


8. NAFA (Nordic Association for Andrology) and ESHRE (European Society of Human Reproduction and Embryology) - SIGA (Special Interest Group on Andrology): Manual on Basic Semen Analysis, 2002.

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