2nd Symposium of the Croatian Society of Clinical Embryologists and Andrology Workshop	**Workstation 1**

SEMEN ANALYSIS

Evaluation of male fertility:
Testis function and male genital tract; accessory sex glands (prostate and seminal vesicles)
Under given conditions of collection
A complete medical history and physical examination
It is impossible to characterize a man's semen quality from evaluation of a single semen sample!

Semen has two major quantifiable attributes:
- the total number of spermatozoa: this reflects sperm production by the testes and the patency of the post-testicular duct system;
- the total fluid volume contributed by the various accessory glands: this reflects the secretory activity of the glands. The nature of the spermatozoa (their vitality, motility and morphology) and the composition of seminal fluid are also important for sperm function

SEMEN ANALYSIS (WHO, 2010)
The results of laboratory measurements of semen quality will depend on :
- wheather a complete sample is collected
- the activity of the accessory sex glands
- the time since the last sexual activity (abstinence)
- the size of the testis

A minimum of 2 days and a maximum of 7 days of sexual abstinence.

Should be reported:
- Man's name, birth date and personal code number
- The period of abstinence
- The date and time of collection
- The completeness of the sample
- Any difficulties in producing the sample
- The interval between collection and start of the semen analysis

The sample should be obtained by masturbation and ejaculated into a clean container made of glas or plastic. The specimen container is placed on the bench or in an incubator (37°C) while the semen liquefies. For ART or microbiological analysis specimen containers and pipettes must be sterile. Safe handling of specimen : infectious agents (HIV, hepatitis...)

EVALUATUON
Initial macroscopic examination
- Liquefaction (15-30 minutes)
- Semen viscosity
- Apperance of the ejaculate

- Semen volume
- Semen pH

Initial microscopic examination
- Thorough mixing and representative sampling of semen
- Making a wet preparation
- Aggregation of spermatozoa
- Agglutination of spermatozoa
- Cellular elements others than spermatozoa
- Sperm motility
- Sperm vitality
- Sperm numbers
- Counting of cells other than spermatozoa
- Sperm morphology
- Leukocytes
- Immature germ cells

PREPARATION FOR SEMEN ANALYSIS
Before removing an aliquot of semen for assessment, mix the sample well in original container, without forming air bubbles (aspirate the sample 10 times into a wide-bore disposable plastic pipette (sterile when necessary).

The volume of semen analysed, and the dimensions of the coverslip must be standardized, so that the analysis are carried out on a preparation of fixed depth of about 20μm, which allows spermatozoa to swim freely. A chamber depth of less than 20 μm constrains the rotational movement of spermatozoa. If the chamber is too deep, it will be difficult to assess spermatozoa as they move in and out of focus.

SEMEN ANALYSIS – PROTOCOL
1. Place a standard volume of semen, e.g. 10μl, onto a clean glass slide.
2. Cover it with a coverslip (e.g. 22 mmx22 mm for 10μl) to provide a chamber approximately 20μm deep.
3. Take care to avoid the formation and traping of air bubbles between the coverslip and the slide
4. Asses the freshly made wet preparation as soon as the contents are no longer drifting.

ASSESING SPERM MOTILITY
Sperm motility within semen should be assesed as soon as possible after liquefaction of the sample, preferably at 30 minutes, but in any case within 1 hour, following ejaculation. The procedure may be performed at room temperature or at 37°C with a heated microscope stage. 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 lower reference limit for total motility is 40%. The lower reference limit for progressively motility is 32%.

Repeate steps from 1 to 4.
5. Examine the slide with phase-contrast optics at x200 or x400 magnification.
6. Assess approximately 200 spermatozoa per replicate for the percentage of different motile categories (avoid counting both those present initially plus those that swim into the grid section during scoring). **Do not count motile pinheads**!

7. Compare the replicate values to check if they are acceptably close. If so, proceed with calculations; if not, prepare new samples.

Categories of sperm movement:

- Progressive motility: spermatozoa moving actively, either linearly or in a large circle; regardless of speed
- Non-progressive motility: all other patterns of motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only flagellar beat can be observed
- Immotility: no movement.

SPERM NUMBERS

The total number of spermatozoa per ejaculate (N) and the sperm concentration (C) are related to both time to pregnancy and pregnancy rates and are predictors of conception. It is obtained by multiplying the sperm concentration by the semen volume (V);

N=CxV

The use of 100-µm-deep haemocytometer chambers is recommended. Other deep haemocytometer chambers may be used, but they will have different volumes and grid patterns and will require different factors for calculation.

For accurate assessment of low sperm concentrations, large volume counting chambers may be necessary.

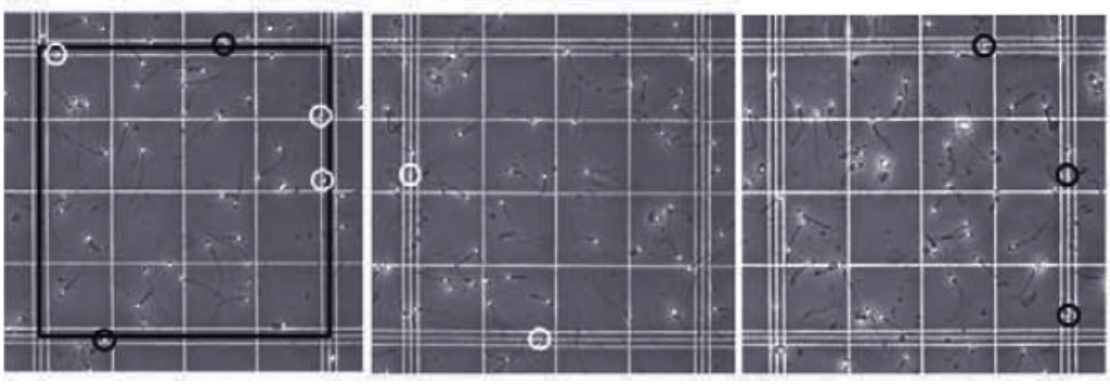
The concentration of spermatozoa (C) in semen is their number (N) divided by the volume (V) in which they were found:

$C=(N/n)x(1/V_g)$x dilution factor;

where n stands for total number of grids examined; Vg – the volume of a grid

WHICH SPERMATOZOA TO COUNT IN THE GRID SQUARES

- count only whole spermatozoa (with heads and tails). If there are many headless sperm tails (pinheads) or heads without tails, their presence should be recorded in the report.
- the decision for counting comes by the location of sperm head; the orientation of its tail is unimportant.



The middle of three lines defines the square's boundary. Thus, a spermatozoon is counted if most of its head lies between the two inner lines (white circles), but not if most of its head lies between the two outer lines (black circles).

DETERMINING THE REQUIRED SOLUTION

The dilution of semen required to allow sperm number to be measured accurately is assesed from an undiluted semen preparation.

Spermatozoa per ×400 field	Spermatozoa per ×200 field	Dilution required	Semen (µl)	Fixative (µl)	Chamber	Area to be assessed
>101	>404	1:20 (1 + 19)	50	950	Improved Neubauer	Grids 5, 4, 6
16–100	64–400	1:5 (1 + 4)	50	200	Improved Neubauer	Grids 5, 4, 6
2–15	8–60	1:2 (1 + 1)	50	50	Improved Neubauer	Grids 5, 4, 6
<2	<8	1:2 (1 + 1)	50	50	Improved Neubauer or large-volume	All 9 grids Entire slide

COMPUTER-AIDED SPERM ANALYSIS (CASA)

These machines are capable of measuring sperm motility and kinematics, and some can also be used to estimate sperm concentration.

Advantages over manual methods: - high precision
- providing of quantitative data on the kinematic parameters of spermatozoa.

The estimation of percentage motility may be unreliable, as they depend on determining the number of immotile spermatozoa, and debris may be confused with immotile spermatozoa. Motility characteristics and sperm concentration can be asseses in undiluted semen. But, in samples with high sperm concentrations (i.e. greater than $50x10^6$ per ml), collisions may occur with high frequency and are likely to induce errors. Such samples should be diluted, preferably with seminal plasma from the same men.
Disposable counting chamber, 20 µm deep, give reliable results.
12 fields scanned in total usually gives reliable results.

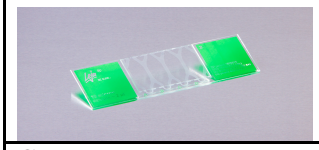
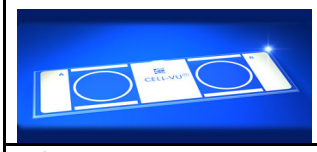
Lower reference limits for semen characteristics

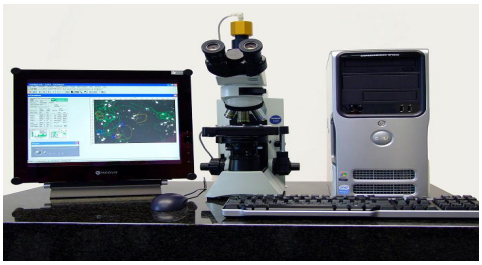
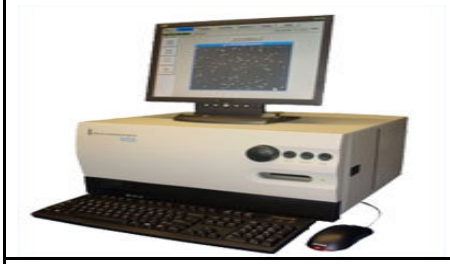
Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4–1.7)
Total sperm number (10^6 per ejaculate)	39 (33–46)
Sperm concentration (10^6 per ml)	15 (12–16)
Total motility (PR + NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)
Other consensus threshold values	
pH	≥7.2
Peroxidase-positive leukocytes (10^6 per ml)	<1.0
MAR test (motile spermatozoa with bound particles, %)	<50
Immunobead test (motile spermatozoa with bound beads, %)	<50
Seminal zinc (μmol/ejaculate)	≥2.4
Seminal fructose (μmol/ejaculate)	≥13
Seminal neutral glucosidase (mU/ejaculate)	≥20

Record form for semen analysis

Name:			
Code:			
Date (day/month/year)			
Collection (1, at laboratory; 2, at home)			
Collection time (hour : minute)			
Sample delivered (hour : minute)			
Analysis begun (hour : minute)			
Patient			
Abstinence time (days)			
Medication			
Difficulties in collection			
Semen			
Treatment (e.g. bromelain)			
Complete sample? (1, complete; 2, incomplete)			
Appearance (1, normal; 2, abnormal)			
Viscosity (1, normal; 2, abnormal)			
Liquefaction (1, normal; 2, abnormal) (minutes)			
Agglutination (1–4, A–E)			
pH [≥7.2]			
Volume (ml) [≥1.5]			
Spermatozoa			
Total number (10^6 per ejaculate) [≥39]			
Concentration (10^6 per ml) [≥15]			
Error (%) if fewer than 400 cells counted			
Vitality (% alive) [≥58]			
Total motile PR+NP (%) [≥40]			
Progressive PR (%) [≥32]			
Non-progressive NP (%)			
Immotile IM (%)			
Normal forms (%) [≥4]			
Abnormal heads (%)			
Abnormal midpieces (%)			
Abnormal principal pieces (%)			
Excess residual cytoplasm (%)			
Direct MAR-test IgG (%) (3 or 10 minute) [<50]			
Direct MAR-test IgA (%) (3 or 10 minute) [<50]			
Direct IB-test IgG (% with beads) [<50]			
Direct IB-test IgA (% with beads) [<50]			
Non-sperm cells			
Peroxidase-positive cells, concentration (10^6 per ml) [<1.0]			
Accessory gland function			
Zinc (µmol per ejaculate) [≥2.4]			
Fructose (µmol per ejaculate) [≥13]			
α-Glucosidase (neutral) (mU/ejaculate) [≥20]			
Technician:			

Types of chambers

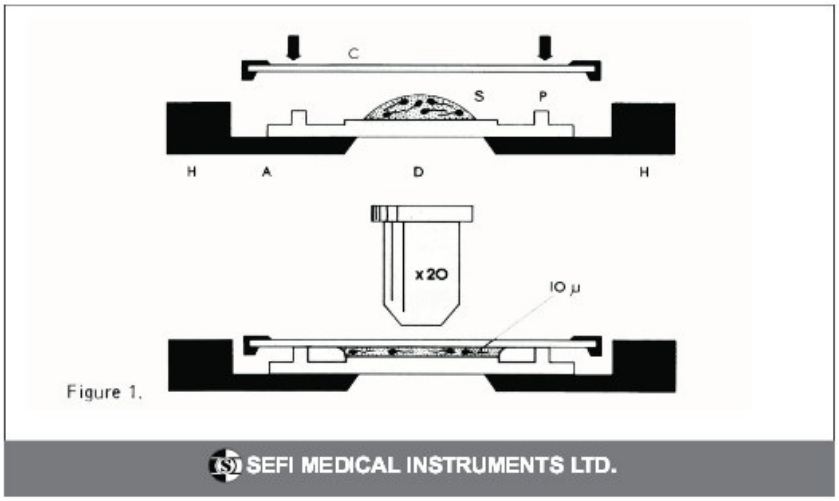
Type of counting chamber	Features	Procedure
Neubauer Improved 	Material: glass, chamber depth: 0.1 mm, counting grid located on the bottom part of the chamber, spare cover glasses available	Immobilize and dilute semen sample by using WHO diluent; dilutions 1:10, 1:20, 1:50, 1:100 depending on sperm density; count sperm number in all 25 squares of the central grid; multiply total sperm number by 0.1, 0.2, 0.5 or 1.0 (depending on dilution) to obtain sperm concentration in millions / ml
Makler 	Material: bottom part aluminium with glass insert, over glass with aluminium frame, chamber depth 0.01 mm; counting grid located on the lower surface of the cover glass; spare cover glasses available. For multiple use!	Count concentration from undiluted semen sample using a 20x objective; immobilize sperm before counting; sperm number in a strip of 10 squares indicates concentration in millions / ml
Cell Vision 	Material: glass, 2 chamber slide with chamber depth 10, 20, 100 & 200 microns; counted grid located on the bottom part of the chamber. Two identical analysis chambers are available for 2 different samples or for 1 sample in duplicate.	Dilute semen sample by using WHO diluent; dilutions 1:10, 1:20, 1:50, 1:100 depending on sperm density; count sperm number in all 16 squares of the central grid; multiply total sperm number by 0.1, 0.2, 0.5 or 1.0 (depending on dilution) to obtain sperm concentration in millions / ml.
Leja 	Material: glass with a resin containig spacers. There are slides with 2 or 4 chambers. Chamber depth of 10 or 20 microns. There are 4 separated areas for 4 different samples.	For computer aided sperm analysis. Compatible with all CASA systems. Select 4 or more fields for analysis. It can be used for manual analysis also.
Cell-VU 	Material: glass. Coverslip with the counting grid laser-etched into its surface. The slide consists of two chambers, each with depth of 20µm.	For sperm counts in undiluted specimens. Count all motile and non-motile sperm within the entire grid (100 boxes). Multiply the count by 50 000 to obtain total concentration of sperm (M/ml).
Microcell 	Two or four samples can be assesed on each Microcell. The chamber depth is 20 µm.	It is compatible with all CASA systems. It can be used for manual analysis.
etc...		

Type of CASA systems	Features
medeaLAB CASA 	Rapid analysis. Concentration&Motility is complemented by the medeaLAB Morphology Analyzer. Accurate discrimination between sperm and non-sperm cells. Video recording and storage for re-analysis. Video import and analysis for external quality control.
Sperm Class Analyzer® 	Modular automatic system for the concentration, motility, morphology, DNA fragmentation and vitality analysis. The basic hardware components of the system are an optical microscope, digital camera and a computer with a SCA software installed.With motorized stage, the analysis process is fully automatic enabling the examination of 4 slides or counting chambers consecutively. SCA® has a service of internal and external quality control to validate the results obtained and the equipment reliability.
IVOS Sperm Analyzer 	Integrated visual optical systemm with a microscope inside of the computer. Automated stage for precise temperature control and sample positioning. Optional IDENT fluorescence capability.
CEROS Sperm Analyzer 	External microscope and portable MiniTherm Stage Warmer. X-Y stage movement increases number of fields available for motility and morphology analyses. Not compatible with IDENT fluorescence capability.

MAKLER® COUNTING CHAMBER

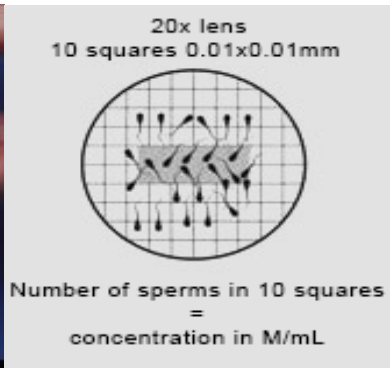
Chamber Description:

For multiple use for rapid and accurate sperm count, motility and morphology evaluation, from undiluted specimens. The Makler Counting Chamber is constructed from two pieces of optically flat glass. The lower main part has a metal base (A) and two handles (H). In the center of the base is a flat disk (D) on which the sample is placed. The upper layer serves as a cover glass (C), with a 1 sq.mm fine grid in the center subdivided into 100 squares of 0.1 x 0.1 mm each. Spacing is firmly secured by four quartz pins.



Analysis Technique:

A small, uncalibrated drop from a well mixed undiluted specimen is placed in the center of the Chamber using wooden rod or pipette and immediately covered. When the cover glass is placed on the four tips, the space bounded in a row of 10 squares is exactly one millionth of mL. A microscopic objective of x20 is required.



Motility Evaluation:

Non-motile sperm are counted within an area of nine or sixteen squares in the center of the grid. Moving sperms are then counted, and graded if desired. The procedure is repeated in several areas. Percentage of motility and its quality are then calculated.

Sperm Count:

A drop of the immobilized specimen is placed in the Chamber and counting initiated: sperm heads within a ten square area are counted in the same manner as blood cells are counted in a hemocytometer, their number represents their concentration in millions per ml.

If sperm are too dense and vivid, they should be imobilized first. In that case, a part of the original specimen is transferred to another test tube for immobilization by placing the tube in hot water (50 – 60 ^{o}C): a cup with 2/3 boiling water and 1/3 tap water is suggested.

In cases of oligospermic semen, sperms in the entire grid area are to be counted, representing their concentration in hundreds of thousands.

Cleaning and preparation for reuse: The Chamber is easily rinsed with water for reuse. Contact surfaces are wiped with special lens paper after washing. The Chamber is quickly and easily available for reuse. In a busy laboratory a large number of tests per hour can be made by a single technician with minimal technical and material requirements

For **Computer Aided Sperm Analysis** (**CASA**), the 10 micron depth of the Makler Chamber is ideal for still or movie camera photomicrography, as it approximately matches the field depth of the objective used in semen analysis.

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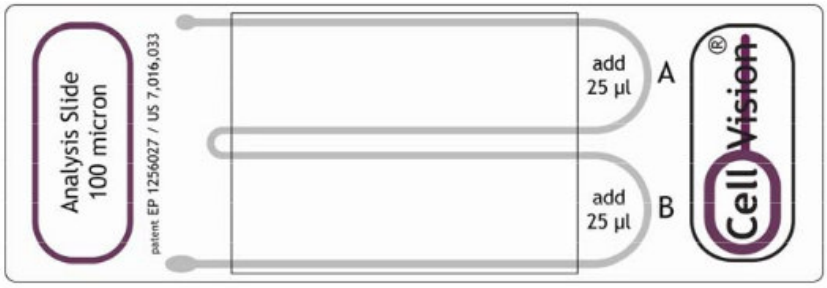
CellVision



General Information:
The CellVision, Analysis Slide, 100 micron (2 chamber Slide) is specially designed for the automatic assessment of sperm parameters with CASA systems.
This Analysis Slide can also be used for manual assessment of sperm parameters using an eye piece reticle.
Two identical analysis chambers are available for 2 different samples or for 1 sample in duplicate.

Protocol:
1. Add 25 µl of sample material (i.e. fully liquefied and homogenised semen) to entrance area A or B
2. The chamber will fill itself by capillary action
3. After the chamber has filled, wipe away any surplus if present from the entrance area
4. Perform assessment according to the (CASA system) protocol
5. For manual assessment:
    - Determine the actual sperm count according to the eye piece reticle magnification factor
    - Determine sperm motility according to the WHO manual

LEJA SLIDE SEMEN ANALYSIS CHAMBER

Leja slides are high quality disposable counting chambers especially made for semen analysis, with a resin (smola) containing spacers. A chamber consists of defined chamber depth. There are slides with 2 or 4 chambers and with chamber depths of 10 or 20 micron. All slides are covered with a special coating to prevent air bubble formation and to prevent sticking of the semen to the chamber surface. Both resin and ink are non-toxic.

Because of high accuracy and precision of the Leja chambers, your results can be compared to other laboratories, which allows combining scientific data concerning the relationship between semen parameters.

Slide loading instructions: Proper loading of the Leja slide is very important to ensure correct sperm counts. The procedure is a simple 2-step procedure.

1. Mix the semen sample gently; be convinced that the sample has been liquefied.
2. Load the pipette with some more volume than indicated at the chamber.
3. Place the tip of the pipette within the boundaries of the loading area of the slide.
   DO NOT TOUCH the cover slip to prevent premature filling of the chamber!
   Depending on viscosity of the sample filling will take 3-20 seconds or even more!
4. Push the button of the pipette to release sample for analysis.
5. Take away excess semen using a cotton tip.
6. Let the sample rest for at least 5 seconds.
7. Start the analysis directly to prevent evaporation of the sample.

Automated analysis: The Leja slides are compatible with all CASA systems. Because there is no counting grid etched into the chamber, you may select fields for analysis from any part of the chamber. Place the loaded slide at the stage of the CASA system. Count at least 4 different fields (stay away at least 2 fields from the chamber resin track).
1. Set up your automated analysis system to accept the chamber depth that corresponds to the Standard Count (20 microns).
2. Select 4 or more fields for analysis from the central area of the Standard Count Chamber. The accuracy of your analysis will be proportional to the total number of sperm counted. This is true for motility as well as concentration evaluations.
3. Proceed with the analysis according to your laboratory's procedures.

Manual analysis: Phase contrast optics with an objective magnification of 10X to 40X are preferred for an easy visualization of the sperm cells. The Standard Count contains no counting grid, so it is necessary to use an eyepiece reticle to define the area being counted. If the concentration permits, we recommend counting at least 100-200 sperm from a field selected in the center of the Standard Count chamber.

1. Calculate (N) by dividing the total number of sperm counted by the number of boxes counted. Obviously, you must keep track of the number of boxes counted in order to perform this calculation.

$$N = \#\text{ of sperm} / \#\text{ of boxes}$$

2. The factor (F) is a calibration factor designed to compensate for the optical variation that is experienced from microscope to microscope, even those of the same model and manufacturer. Once a specific microscope is calibrated and the factor F is derived, you can use that value F for all samples analyzed with the same magnification on that specific microscope.

$$F = 1{,}000{,}000 / T \times D^2$$

where F = the calibration factor determined for each microscope, magnification, and Standard Count

chamber depth.
T = the chamber depth (in microns). For the Standard Count, you would use the number 20.
D = the distance across a single box of the reticle (in microns).

3. Calculate (D) by using a Stage Micrometer. To achieve this:

1. Fit the reticle into the microscope eyepiece, insuring that it is firmly in place and parallel to the optical plane.
2. Place the stage micrometer on the microscope stage.
3. Line up the stage micrometer so that one of the larger lines is imposed upon the right edge of the reticle matrix. The divisions on the stage micrometer are 100um (distance between the large lines), 50um (distance between the secondary lines) and 10um (distance between the smallest lines).
4. Measure the distance across all 10 boxes of the reticle.
5. To calculate the distance across a single box in the reticle matrix, divide the distance obtained in step #4 by 10.

4. Incorporate the value (D) into the formula, and derive the factor F. Remember that the value D is squared in the formula to account for width x length. Assuming that the value of D is 25 microns and that you are using a Standard Count with a 20 micron chamber depth, the factor F would then be equal to

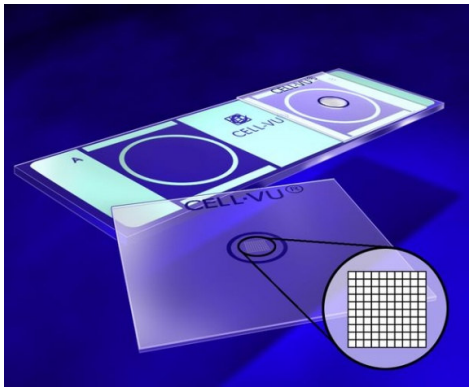
$$F = 1{,}000{,}000 / 20 \times 25^2$$
$$F = 80$$

Determining Percent Motility

Count only the motile sperm in the boxes, but record that number. Recount the same boxes and this time count only the non-motile sperm. Add the motile and non-motile sperm to obtain the total number of sperm counted Calculate percent motility as follows:

Percent Motility = # of motile sperm / total # of sperm counted

CELL-VU® Sperm Counting Chamber



For sperm counts in **undiluted specimens**. The CELL-VU® Cytometer consists of a standard specially-designed glass slide and a coverslip with the counting grid laser-etched into its surface. **Disposal** of the entire apparatus eliminates cleaning and minimizes clinician exposure to body fluids. Both the slide and coverslip are marked to ensure correct use. The coverslip's 1 x 1 mm grid is divided into 100, 0.1 mm areas and is thin enough to use under high magnification without special adapters or reticles. The slide consists of two chambers (for dual determinations), each with a depth of 20 µm. This depth is optimal for sperm cells to form in a monolayer, movement is unencumbered, motility can be assessed and counts are made easily.

Using CELL-VU® for sperm counts and motility

Isolate and view the CELL-VU® grid. This grid is divided into 100 small boxes each 0.1 x 0.1 mm. For undiluted sperm count all motile and non-motile sperm within 10 small boxes of this grid. Divide this number by 2. This result is the concentration of sperm in millions/ml.

$$\% \text{ Motility} = \frac{\text{\# Motile Sperm}}{\left[\begin{array}{c}\text{\# Motile Sperm x 100} \\ + \\ \text{\# non-Motile Sperm}\end{array}\right]}$$

For increased accuracy, count all sperm within the entire grid (100 boxes). Multiply the count by 50,000 to obtain the total concentration of sperm/ml.

	V	Motile Sperm Count	non-Motile Sperm Count	Sperm Motility
MAKLER				
NEUEBAUER				
CELL-VISION				
CELL-VU				

General chamber preparation for semen testing

Mix the sample thoroughly just before testing.

Pipette one drop (approximately **4 microliters**) of specimen. Place the specimen at the extreme edge of one of the sampling areas (Figure A). Two tests can be performed using one CELL-VU® slide.

Make sure the CELL-VU® name on the cover glass is facing the observer as the grid is etched on the reverse side.

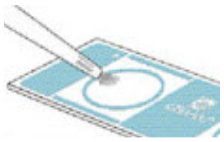


FIGURE A

Gently lower the cover glass over the specimen so that the edge of the cover glass just covers the sample (Figures B and C).

FIGURE B

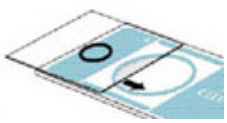


FIGURE C

Slide the cover glass into position as shown in Figure D. This will eliminate air bubbles from the counting area.

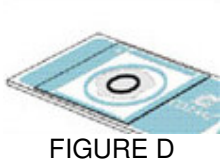


FIGURE D

Moderators:
Kristina Čavlović,
Nina Gelo,
Romina Rakoš Justament,
Sara Sivec

Work Station: Semen Analysis