

## INSTRUCTIONS FOR MICROTOOL USE IN SEMEN ANALYSIS

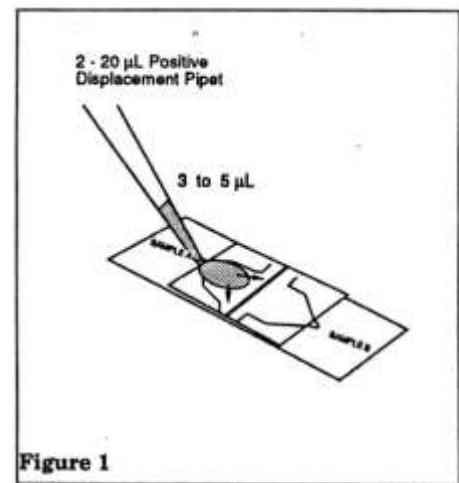
The MicroTool disposable chamber provides consistent and accurate data on sperm concentration and motion analysis (e.g., percent mobility, mean velocity, etc.). It is designed for use in both manual and automated semen analysis protocols.

### Important characteristics of the MicroTool

1. Each MicroTool contains two or four independent chambers. They can be used for duplicate sampling or for analysis of different samples.
2. The glass cover of the MicroTool is 0.5 mm thick. Therefore, it is important that the microscope objective used for analysis compatible with a 0.5 mm coverslip.

### Loading the MicroTool

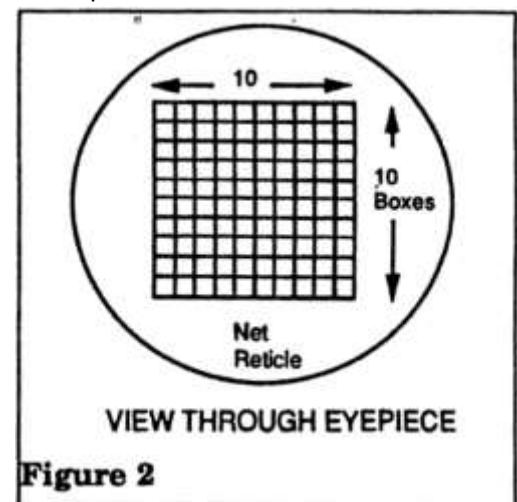
1. Allow the semen sample to completely liquefy (usually about 30 minutes at room temperature), then thoroughly mix the sample by pipetting or with a Vortex mixer.
2. Place 2-5  $\mu\text{L}$  of sample into one of the two clear zones labeled SAMPLE A and SAMPLE B in Figure 1. Allow a few seconds for the sample to load into the analysis area, and then remove any excess fluid remaining in the loading zone. *Excessive amounts of sample may cause sperm and other objects in the fluid to drift when observed.*
3. Perform the analysis near the center of the large open area of the MicroTool.



### 1. Semen Analysis by the Manual Method

1. **Microscope.** A decent quality laboratory microscope is recommended for sperm concentration determination. Phase contrast optics with an objective magnification of 10X to 40X are preferred for easy visualization of the sperm cells. The MicroTool contains no counting grid, so it is necessary to use an eye piece reticle to identify the area counted. This is best accomplished with a 10 X 10 net pattern that projects 100 boxes over the viewing field (see Fig.2). Vitrolife Corporation can provide a net reticle for virtually all major brands of microscopes. Alternatively, they can also provide a secondary replacement eyepiece with an installed reticle (please visit [www.vitrolife.com](http://www.vitrolife.com)).

2. **Calibration.** The area delineated by the grid pattern in the eye piece must be calibrated for each magnification to be used with the MicroTool. Because the actual magnification obtained from the different microscopes varies, even when identical optics and manufacturers are compared, the calibration of each microscope to be used for semen analysis should be checked. MicroTools are available in a 20  $\mu\text{m}$  depth, so that chamber



depth must be taken into account as well. Calibration will yield a factor, **F**, that be multiplied by the average number of sperm concentration in “millions of sperm per millimeter”. Once determined, **F** may be used for all samples analyzed using the same microscope magnification and MicroTool depth.

**3. Stage Micrometer.** A stage micrometer is used to determine the distance (in microns) across one box. This value and the MicroTool depth are then used to calculate the factor **F** in the following equation:

**F** = Factor determined for each microscope, magnification, and MicroTool depth

**T** = Chamber thickness (depth in microns)

**D** = Distance across a single box (in microns)

$$F = 1,000,000 \div [T \times D^2]$$

1,000,000 in the numerator of the equation converts the units from cubic microns to cubic centimeters (ml) and from single sperm to millions of sperm.

Example (from above):

Distance **D** = 47 microns

$$D^2 = 2,209$$

Chamber depth, **T** = 20 microns

To determine **F**:

$$F = 1,000,000 \div [20 \times 2,209] = 22.6$$

**4. Calculating Sperm Concentration.** Once the average number of sperm per box (**N**) are determined, simply multiply by the factor **F** to obtain the sperm concentration **C**.

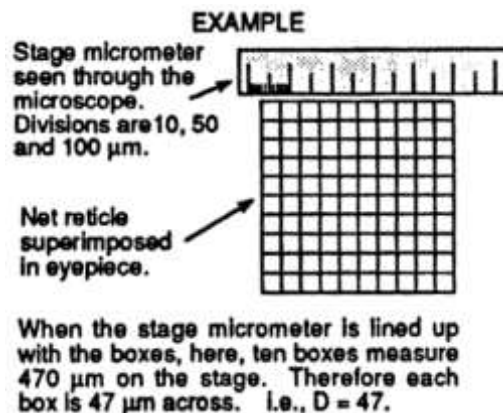
$$C = N \times F$$

**5. Performing the Sperm Count.** Select a field for analysis from the center of the MicroTool chamber. Because there is no permanent grid etched on the chamber you may select any field you wish and as many you require for your analysis. The accuracy of the concentration determination will be proportionate to the number of sperm counted. We recommend counting 100 to 200 sperm if the concentration permits. You may accomplish this by counting any number of randomly selected boxes. Keep track of the number of boxes and the total number of sperm. Divide the total number of sperm by the total number of boxes to obtain the average sperm per box, **N**, as depicted in Fig. 3. Multiply **N** by the appropriate factor (**F**=22.6 here) to obtain the sperm concentration:

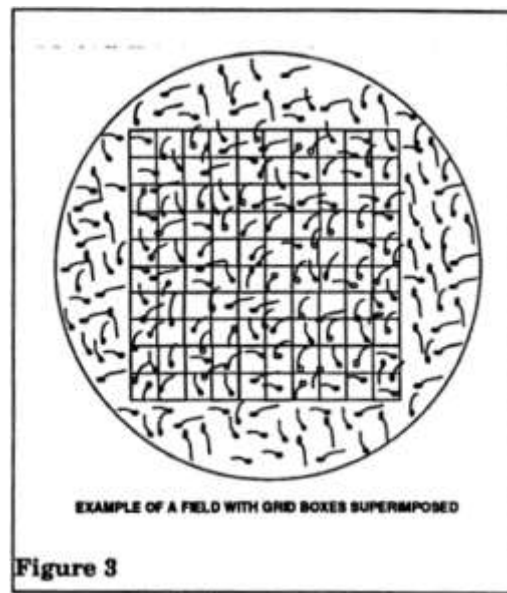
Number of boxes counted = 100

Number of sperm counted = 123

Average number of sperm per box:  $N = 123 \div 100 = 1.23$



**Note: 22.6 will be the factor used for all subsequent analysis that employ the same optical conditions and chamber depth. Only N will need to be determined for each new sample.**



Therefore:  $C = N \times F = 1.23 \times 22.6 = 27.8$  million sperm per ml

**6. Determining the Percent Mobility.** The percent mobility of the sample may be obtained along with the value of N using a slight modification of the above procedure. First count only the motile sperm in the boxes and record that number. Then recount the same boxes, only by counting the non-motile cells. To obtain N, add the number of motile and non-motile sperm counted and then divide the number of boxes. To obtain the percent mobility, divide the number of motile sperm by the sum of the motile and non-motile sperm then multiply by 100.

Example:

Factor (F) = 22.6

Number of boxes counted = 100

Number of motile sperm = 78

Number of non-motile sperm = 45

Percent mobility =  $78 \div [78+45] \times 100 = 63.4\%$  mobility

$C = N \times F = [78+45 \div 100] \times 22.6 = 27.8$  million sperm per milliliter

## 2. Computer Assisted Sperm Analysis (CASA)

The MicroTool is completely adaptable for use in most automated sperm motion analysis instruments. Because there is no counting grid etched into the chamber, you may select fields for analysis from any part of the chamber.

1. Set up the automated analysis system to accept the appropriate chamber thickness corresponding to the MicroTool selected.
2. Select 4 or more fields for analysis from the central area of the MicroTool chamber. The accuracy of your analysis will be proportional to the total number of sperm counted, just as in the manual method. This is true for motion parameters as well as concentration, so be sure that an adequate number of motile sperm have been analyzed to ensure accurate motion values.
3. Proceed with the analysis according to the instructions provided by the manufacturer of your motion analysis instrument.

## 3. Open Source Free Computer Assisted Sperm Analysis (CASA)

Commercial CASA systems can be quite expensive. Theriogenologists who are comfortable with computers can use free, open source public domain CASA software, a \$350 video camera and your microscope to assess sperm count, percent mobility, linear velocity and other sperm cells viability measures.

### Equipment Needed

MicroTool Cell Counters from [www.Cytonix.com](http://www.Cytonix.com) (<\$2 per slide)

Video camera (5.0MP) model# A3550U from Cytonix partner microscopenet (~\$350)

A good phase contrast microscope with planar objectives

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Computer running Windows XP or newer operating system.

### Programs

ImageJ (free open source) from federal government website: <http://rsb.info.nih.gov/ij/>

CASA plugin (free) from Cytonix or from <http://rsb.info.nih.gov/ij/plugins/casa.html>

Microsoft Excel

### Video

Save moving sperm cells video (30-60 seconds) as an uncompressed AVI file.

### Motility Analysis of Sperm Cells

STEP 1: Open ImageJ

STEP 2: Click: File » Open and select sperm "AVI" video file

STEP 3: In the new box "AVI reader" click "OK".

STEP 4: In ImageJ click: image » type » 8-bit

STEP 5: In ImageJ click: image » adjust » threshold

STEP 6: In threshold window, adjust the two threshold buttons back and forth to get clearest image possible and select "apply" and "OK".

STEP 7: In ImageJ click: Plugins » Compile and Run. In the next screen find and click "CASA\_.java"(HINT: After first analysis, in ImageJ click: Plugins » CASA).

STEP 8: In new window fill-in the following (experimentation needed by animal type):

min sperm size: 10

max sperm size: 40

min track length: 50

Characteristic	Description
Percent motility	Percent of sperm moving in a manner fitting motility determination parameters.
Velocity curvilinear (VCL)	Point to point velocity (total distance traveled) per second.
Velocity average path (VAP)	Point to point velocity on a path constructed using a roaming average. The number of points in the roaming average is 1/6 <sup>th</sup> of the frame rate of video used.
Velocity straight line (VSL)	Velocity measured using the first point and the average path and the point reached that is furthest from this origin during the measured time period.
Linearity (LIN)	VSL/VAP, describes path curvature.
Wobble (WOB)	VAP/VCL, describes side to side movement of the sperm head.
Progression (PROG)	The average distance of the sperm from its origin on the average path during all frames analyzed; division of this variable by VAP yields a description of the average efficiency in terms of the portion of generalized motion that results in movement away from the origin (a perfectly circular path will have a higher efficiency than an erratic path with the same linearity).
Beat cross frequency (BCF)	This value is determined in the plugin by detecting the frequency at which VCL crosses VAP. In cases where a sperm has a highly circular path, values generated are not valid (reference forthcoming). As such, we do not recommend use of this variable. Instead we suggest that directional changes per second would be a more valid measure. This measurement is present in the code of the program but is not output; we may substitute for BCF in the future depending on user demand.

STEP 9: Click "OK". Two windows with tracks and results will appear. The order of the results is: Percent Motility | VCL | VAP | VSL | LIN | WOB | PROG | BCF | Median VCL | Median VAP | Median LIN | Median WOB | Median PROG | Median BCF | Number of Sperm Tracked

### Initial Setting of Scale

For each microscope objective the pixels to microns conversion must be determined during camera installation. Please use a 0.01 mm calibration slide (stage micrometer or mm ruler for dissecting microscopes) and record a video of the standard slide with the objective used for sperm analyses and open video in ImageJ. Using the line tool (5th icon on tool bar) and draw a 100-micron line along the known length of the standardization lines.

In ImageJ click: Analyze » Set Scale...

The value in "distance in pixels" is your "Distance in Pixels" and the standardization length is the "known distance". The pixels to microns conversion is fixed for each objective, microscope and camera. Therefore, the conversion number for each objective is estimated only once during initial instrument set-up.

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## Calculations

After the scale of the video has been set, the height and width in microns of the video is displayed in the image window. Please enter height (column C) and width (column D) in microns in the Microsoft Excel spreadsheet provided by Cytonix. In column E, enter the number of mobile sperm tracked by the CASA program. In column F, the number of sperm in a milliliter of semen will be displayed.

## Counting Sperm or Other Cells from any photo

STEP 1: Start "ImageJ" Program

STEP 2: Click on: File » Open select sperm video file

STEP 3: In the new box change both "first frame" and "last frame" to "1" and click "OK".

STEP 4: In ImageJ click: image » type » 8-bit

STEP 5: In ImageJ click: image » adjust » threshold

STEP 6: In threshold window, click on "B&W" and select "Auto" (or adjust the two threshold buttons back and forth to get clearest image) and select "apply".

STEP 7: In ImageJ click: analyze » analyze particles. Next change the "size" to 15-40 (or other appropriate size). Change "show" to "outlines" and click on "display results" and "summarize". Click "OK". A new screen will appear with your sperm count results. These results can be saved as a Microsoft Excel file.

## References for Open Source CASA

Wilson-Leedy JG, Ingermann RL. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. *Theriogenology*. 2007;67:661-72.

Immune activation reduces sperm quality in the great tit. Losdat S, Richner H, Blount JD, Helfenstein F. *PLoS One*. 2011;6(7)

Subordinate male cichlids retain reproductive competence during social suppression. Kustan JM, Maruska KP, Fernald RD. *Proc Biol Sci*. 2012;279:434-43.

Social cues of sperm competition influence accessory reproductive gland size in a promiscuous mammal. Lemaître JF, Ramm SA, Hurst JL, Stockley P. *Proc Biol Sci*. 2011;278:1171-6.

## 4. Overcoming Common Problems

**A. Sperm Aggregation.** Because sperm cells are often aggregated in semen, it is best to determine sperm concentration from a diluted, killed sample that has been agitated to break up all aggregated sperm. Vitrolife and other similar companies can provide a diluent for this purpose. Prepare a 1:5 dilution of the semen sample and vigorously agitate it by repeated aspiration and expulsion with a plastic transfer pipet to break up any aggregated sperm. Killed sperm can then be loaded in one chamber for determination of concentration, while live sperm (either raw semen or semen diluted with phosphate buffered saline) are loaded into the other chamber for determination of motion parameters.

**B. Semen Viscosity Problems.** For occasional samples that do not liquify properly and remain too viscous to load correctly, it is recommended that the sample is diluted with either phosphate buffered saline for determination of motion parameters or for concentration determination. Mix the diluted sample thoroughly. Diluting the sample in this manner should adequately reduce problematic viscosity and permit proper loading

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of the MicroTool.

**C. Very Low or High Sperm Concentrations.** For very low concentration samples where it is not possible to count 100 sperm, count all the boxes in five different fields and divide the total number of sperm counted by 500 to obtain N. For low concentration samples it is best to use minimal dilution of the original semen, the lowest possible magnification, and large depth MicroTool to increase the number of sperm in each field. Remember to use the correct factor (F) in your calculations after changing the parameters. For very high concentrations, you may want to dilute the samples or use a higher magnification.

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