

<b>Document Title: PROCEDURE FOR PERFORMING MANUAL SPERM COUNTS AND MOTILITY</b>
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**REVISION HISTORY**

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SH.CP.AU.hem.0020.0005	11/16/2015	Update content and format

## **TITLE: Procedure for Performing Manual Sperm Counts and Motility**

### **I. PURPOSE**

Occasionally Computer Assisted Sperm Analyzer (CASA) will yield questionable results due to presence of debris, clumping, or when the sperm count or motility is severely low, or when all sperm are non-motile. In order to report the most accurate data, a manual sperm count and motility should be done whenever the repeated IVOS sperm count/ml is less than 10 million/ml, or the motility is less than 10%, or the IVOS results differ greatly from what is visually observed.

### **II. SCOPE**

To be used by URMCA Automated Lab staff for manual semen analysis.

### **III. RESPONSIBILITIES**

Department and functional responsibilities are defined below:

<b>Group/Person</b>	<b>Responsibility</b>
Quality Assurance	<ul style="list-style-type: none"><li>• Supports the development of this document</li><li>• Review and approval of this document</li></ul>
Laboratory Director Medical Director	<ul style="list-style-type: none"><li>• Ensures that the procedure is followed</li><li>• Review and approval of this document</li></ul>
Supervisor	<ul style="list-style-type: none"><li>• Ensures that the procedure is followed</li><li>• Review and approval of this document</li></ul>
End User	<ul style="list-style-type: none"><li>• Follows the procedure</li></ul>

### **IV. SPECIMENS**

An aliquot of 4.0 µl of liquefied semen is needed. See Automated Determination of Sperm Count and Motility (SH.CP.AU.Hem.0021) for specific details about patient registration, specimen collection, and initial semen analysis by the IVOS. The same microcell slide prepared for initial Semen Analysis by the IVOS can be utilized.

### **V. QUALITY CONTROL**

**A. SEE THE QUALITY CONTROL SECTION OF Automated Determination of Sperm Count and Motility (SH.CP.AU.HEM.0021) FOR THE procedure for counting Accubeads at the Ivos. The same microcell slide prepared for the Ivos can be utilized for manual quality control.**

**B. NEW LOT WORKUP**

Prior to using Accubeads for clinical use, a crossover study must be performed and results must be within the laboratory acceptable range.

### **C. MORPHOLOGY STAIN QC**

A stained slide must be assessed each day to assure staining solutions are working. Stain should be checked daily for intensity of color. If acrosomes and tails are easily identified, stain should be marked as good. If stain starts to lighten, but sperm components are still identifiable, chart should be marked as such. If stain quality is poor, stain must be discarded and new stain used. If precipitate appears on slide or in solutions, change solutions. Staining solutions should be changed at least every week or as needed.

## **VI. MATERIALS**

### **A. EQUIPMENT**

1. Manual/Hand Counter.
2. Nikon eclipse 50i microscope with 10x10 eyepiece reticle.
3. 10X and 40X objective lenses.
4. Bench centrifuge (e.g. Eppendorf or any other model with swinging bucket rotor).
5. Specimen Warmer (37°C).
6. Coplin jars (for morphology slides)

### **B. SUPPLIES**

1. Eppendorf Pipette(s) able to measure 4.0 and 100 µl.
2. Pipette tips.
3. Microcell slides (20 mm).
4. Kimwipes or Lint free lens paper.
5. Glass slides with frosted ends for labeling
6. Coverslips
7. Immersion oil
8. Transfer pipets
9. Glass slide and coverslip, or loaded MicroCell slide from IVOS/manual counts
10. STAT III STAIN (Consists of Fixative, Eosin, and Azure solution)

### **C. REAGENTS**

1. HTF Media

2. Eosin Y Certified Biological Stain – Sigma Aldrich

To make stock solution of Eosin Y:

- a. Sodium Chloride Solution: 180 mg NaCL in 20ml dH<sub>2</sub>O
- b. Eosin Solution: 100 mg Eosin Y in 20 ml sodium chloride solution

3. Distilled water

## VII. SPECIAL SAFETY PRECAUTIONS

- A. All patient specimens should be considered potentially infectious and must be handled with precautions as described in CDC (Center for Disease Control) recommendations and in compliance with the Federal OSHA.
- B. Clorox Ultra: Avoid acidification or contact with ammonia containing products which can generate hazardous chlorine gas.
- C. Clorox Ultra contains a strong oxidizing agent that could cause substantial but temporary eye injury, may irritate skin and may cause nausea and vomiting if ingested. Exposure to vapor or mist may irritate nose, throat and lungs.
- D. If the instrument centrifuge or microscope emits an abnormal odor or smoke, turn off the power switch immediately and disconnect the power plug from the wall socket.

## VIII. PROCEDURE

### A. Count/Motility

1. Slowly place 4.0  $\mu$ l of semen into each chamber of the microcell slide. Label slide with the patient's name. *Microscope oculars and microcells should be clean and free of scratches. Be sure there are no air bubbles in microcell chamber.*
2. Using the 40X objective lens, select a field for analysis from the center of the microcell slides that appears to be uniform and representative. One eyepiece contains a 10 X 10 grid. All sperm cells within one box of the grid must be counted. Depending upon the number of sperm present, count the sperm in at least 3 grids, keeping track of the number of grids counted. Increasing the number of grids counted will increase the accuracy.
3. All sperm cells within a grid (100 boxes) must be counted before continuing to the next grid. Keep track of the number of grids counted and divide the number of sperm counted by the number of grids counted to obtain average sperm per grid (N). Multiply N by the factor of 0.87 (this factor may change for your microscope or magnification) for the Nikon 50i microscope to obtain the sperm concentration (C). **See Calculations section for further details.**

4. The percent motility of the sample may be determined at the same time as the count, or can be counted separately using fields from the center of the microcell slide that appear to be uniform and representative. A minimum of 200 sperm, both motile and nonmotile, must be counted. In the case of extremely low sperm count, counting this number of sperm cells may be limiting. Record the number of motile sperm and the total number of sperm counted (motile + nonmotile). To obtain the % motility, divide the # of motile sperm by the total # of sperm counted, and then multiply by 100%. Standardized motility tape is run daily to compare IVOS and manual counts. **See Calculations section for further details.**
5. Repeat steps 1-3 for side B of the MicroCell slide. If count and motility values for side B are within 10% of the 1st count, report the average count and motility.
6. If count and motility values vary by greater than 10%, prepare a second slide and repeat count. Repeat steps 1-4. Report the average of the two counts.
7. When reporting manual motilities, comment must be made on final report that manual motility was performed at room temperature.
8. Tech must load an additional microcell slides if the first one does not load well due to viscosity problem or air bubble.
9. It is helpful (when manual replaces CASA) to have a second technician do a manual count to confirm data, particularly when there is abundant clumping or when multiple slides yield vastly different counts or motilities.
10. The more sperm and the more boxes counted, the more accurate a reading will be obtained.
11. For **Post-Vasectomy** samples, on the fresh sample, count all the sperm seen on the entire MicroCell slide. If no motile sperm are seen, centrifuge the remainder of the sample at 1800-2000 rpm's for 10 minutes, remove the supernatant, re-suspend the pellet, load another MicroCell slide with this suspension and examine the entire slide). In the comment section of the report, note what you saw (i.e. 5 non-motile sperm seen in or pellet). If no spermatozoa are seen after examining the pellet, the sample is considered azoospermic and report the result in the comment field as "No sperm seen on fresh and centrifuged pellet".

## **B. VIABILITY STAINING BY DYE EXCLUSION**

Sperm viability is indicated by the eosin dye exclusion procedure. The dye exclusion technique is based on the principle that dead cells with damaged plasma membranes absorb certain stains and change color. These techniques make it

possible to differentiate spermatozoa that are immotile but alive from those that are dead. This technique also provides a check on the accuracy of the motility evaluation, since the percentage of viable cells should be greater than the percent motility. The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the tail (WHO manual).

Eosin dye exclusion should be performed on all semen specimens that present with a motility of less than 40% or as requested by the referring physician, or if there is any question that the motility is severely low and the sperm might still be alive. Semen is examined within 60 min of ejaculation. Any unusual transport conditions which might affect viability, such as extreme heat or cold should be noted in the comments section.

#### **VIABILITY PROCEDURE:**

1. Mix 100 µl of fresh semen with 100µl of eosin solution using pipet (can be as little as one drop each). Mix well and place a drop on a slide. Cover the slide with a coverslip.
2. After 1-2 minutes, observe under 40x magnification. Count unstained (live) and stained pink (dead) sperm. If possible, at least 100 sperm should be counted. The percentage of live sperm should be reported.
3. **See Calculations section for further details.**
4. Repeat count. Two values should agree within 15%. If they do not agree, set up a new slide and recount. If the count still does not agree within 15% have another tech set up a new slide and recount.
5. Report the % live (average of the two counts in the comments section of the semen analysis).

**NOTE:** Sperm Motility should never be higher than Sperm Viability.

#### **C. DETERMINATION OF ROUND CELLS IN SEMEN**

This procedure provides an assessment of the number of round cells present in the semen sample, but does not differentiate between immature germinal cells, cytoplasmic residue and white blood cells.

#### **ROUND CELL PROCEDURE**

1. Use the same MicroCell slide which was prepared for automated/manual count and motility, or make a wet-prep slide by transferring a small amount of semen to a glass slide. Apply coverslip avoiding air bubbles.

2. Count all round cells and sperm cells in each high powered field (40X objective). Maintain a running total for each cell type on the mechanical counter. Repeat this step until 100 sperm have been counted
3. Repeat step 2 on the second side of the MicroCell slide, or in another area of the wet-prep slide.
4. Using the CSA calculator spreadsheet, calculate the number of round cells per mL. If the duplicate counts do NOT agree within 10%, the procedure must be repeated. **See Calculations section for further details.**

**\*\*NOTE:** This computation requires the sperm concentration value obtained from the CASA-IVOS or from manual count.

**D. SLIDE PREPARATION FOR MORPHOLOGY (for complete semen analysis only)**

1. Slide Preparation procedure:
  - a. Pipette approximately 10  $\mu$ l of liquefied semen is applied to the slide.
  - b. Taking another slide or cover slip, place it in the oval semen drop at 45 degree angle, and quickly run the slide away from the frosted label. Let specimen smear across slides evenly.
  - c. Make sure smears are thin and evenly dispersed.
  - d. Label the slides with the barcode label.(Complete with patient's name and accession number).
  - e. The slides should air dry at room temperature for 5-15 minutes.
  - f. 4 Smears should be made from the fresh sample in case of problems with staining/ and or loss during transportation.

**NOTE:** If sample is too thick or viscous, try to make 5-10 slides  
If a sample is from a Post Vasectomy, there is no need to make a slide.

2. Staining Procedure
  - a. Dip and leave the slide in Coplin jar labeled "Fixative" for 15 secs. Touch edge of slide to inside of fixative chamber to allow excess solution to drain and blot edge of slide onto a paper towel or gauze.
  - b. Dip and leave the slide in Coplin jar labeled 1 (solution 1 – Eosin) for 15 secs. Touch edge of slide to inside of the jar to allow excess solution to drain and blot edge of slide onto a paper towel or gauze.
  - c. Dip and leave the slide in Coplin jar labeled 2 (Solution – Azure) for 15 secs. Touch edge of slide to inside of the jar to allow excess solution to drain and blot edge of slide onto a paper towel or gauze.
  - d. Rinse gently in distilled water



- e. Allow to air dry.
- f. Scan the slide to make sure there are sperm on the slide to do the morphology before sending it to Red Creek.

## IX. LIMITATIONS

- A. **CLUMPING OF SPERMATOZOA:** If the spermatozoa are clumped and in the clumping interferes with manual counting, note this in the comment section of the patient log-sheet and include this information when entering into the LIS.
- B. **ABUNDANT DEBRIS:** If it appears that abundant debris interferes with accurate quantification, another MicroCell slide should be set up and analyzed. If second analysis achieves similar (spurious) results, the specimen should be quantified manually due to the interfering debris
- C. If the sample was spilled and the test can still be done, Perform the test with the comment: Interpret with caution: Unknown amount of sample loss during collection.
- D. If there is a significant of sample loss and volume is <2 ml with abnormal result, .ND the test with the comment "Significant amount of Sample loss during collection. Request re-collection.
- E. Extremely low volume sample - .ND the sample and comment test not performed. Extremely low volume sample. Request re-collection.
- F. Contaminated containers can influence sperm motility. Only use tissue culture grade containers that have been QC tested.
- G. Some drugs may influence sperm motility, such as calcium channel blockers used for heart conditions.
- H. If the sample was received in the lab 1 hour after collection make a comment; "Motility may be compromised. Suggest repeat analysis in order to have optimal result".
- I. If there are insufficient sperm present in a sample to determine the number of round cells present (usually < 50 sperm), comment on the report "inadequate number of sperm present to perform round cell count."
- J. If there are insufficient sperm present in a sample to determine the viability, (usually < 50 sperm), comment on the report "inadequate number of sperm present to perform viability testing."
- K. If a sample is to be rejected for any reason, document in the Sample problem/rejection log.

## X. CALCULATIONS

- A. Formula for manual sperm count using Nikon 50i microscope with 20X magnification (factor (F) = 0.22): **C = N x F**

$$\text{Sperm count} = (\text{Avg. \# sperm}) / (\text{grid}) \times 0.22$$

*Example:*

$$\text{Number of sperm counted} = 127$$

$$\text{Number of grids counted} = 3$$

$$\text{Average number of sperm per grid (N)} = 127/3=42.3$$

$$\text{Count (C) in million/mL} = \text{N} \times \text{F}$$

$$\text{C} = 42.3 \times 0.22$$

$$\text{C} = \underline{9.31 \text{ million/mL}}$$

- B. Formula for manual sperm count using Nikon 50i microscope with 40X magnification (factor (F) = 0.87) **C = N x F**

$$\text{Sperm count} = (\text{Avg. \# sperm}) / (\text{grid}) \times 0.87$$

*Example:*

$$\text{Number of sperm counted} = 67$$

$$\text{Number of grids counted} = 6$$

$$\text{Average number of sperm per grid (N)} = 67/6=11.2$$

$$\text{Count (C) in millions/mL} = \text{N} \times \text{F}$$

$$\text{C} = 11.2 \times 0.87$$

$$\text{C} = \underline{9.7 \text{ million/mL}}$$

- C. Formula for % Motility:  $\text{Motility} = (\text{\# motile sperm} / \text{total \# sperm counted}) \times 100$

*Example:*

$$\text{Number of motile sperm} = 81$$

$$\text{Number of non-motile sperm} = 132$$

$$\% \text{ Motility} = [81 \text{ motile sperm} / (81 \text{ motile sperm} + (132 \text{ non-motile sperm}))] \times 100$$

$$\% \text{ Motility} = (81 / 213) \times 100$$

$$\% \text{ Motility} = (0.38) \times 100$$

$$\% \text{ Motility} = \underline{38\%}$$

- D. Calculation for Viability

Formula: # live sperm/total # sperm counted X 100 = % live

*Example:*

Number of unstained (live) = 23  
Number of stained (dead) = 77  
 $23 / 100 = 0.23 \times 100 = 23\%$  live

#### E. Calculation for Round Cells

Formula:

$$\frac{(\text{Total \# of sperm counted})}{(\text{Total \# Round Cells counted})} = \frac{(\text{Sperm Count/ml})}{(\text{Round Cells per ml})}$$

**OR**

(Number of Round Cells/ Number of Sperm cells) x Sperm concentration (mil/ml)

*Example:*

# of sperm counted = 200  
# of Round Cells counted = 10  
Sperm count/mL = 20.0  
Round cells/mL =  $10/200 \times 20$   
Round Cells /mL = 1/mL

## XI. INTERPRETATION

N/A

## XII. RESULT REPORTING

### A. NORMAL RESULT VALUES

Reference: WHO Laboratory Manual for the Examination of Human Semen and Cervical Mucus Interaction, WHO 2010 Fifth Edition, Cambridge University Press.

Volume:	≥ 1.5mL
Sperm Concentration:	≥ 15 million/mL
Total Sperm Count:	≥ 39 million
Motility:	40% or more with good forward progression
Viability:	≥ 58%

## B. CRITERIA FOR NORMAL/ALERT VALUES

Normal range (WHO MANUAL): 58% Live (sperm cells that exclude dye). Specimens that show very low, or no motility usually show comparable viability. That is, with low motility, viability is low and if all sperm are non-motile, they all would be expected to be dead. However, when motility is low or all sperm are non-motile and viability is >50% then a defect in the function of the sperm tail would be expected.

## C. CRITERIA FOR ABNORMAL/ALERT VALUES

1. If *motile* sperm are seen in a post-vasectomy sample, notify the physician immediately.
2. Document the presence of bacteria, yeast or parasites in the comment section and fax to requesting physician as soon as possible.
3. If NO sperm are seen in a specimen that is NOT a post-vasectomy analysis, notify the physician immediately.

## XIII. TRAINING

Personnel	Training Required
Management	Read
End User	Read Perform Skills Assessment

## XIV. REFERENCES

- A. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. WHO 1992, Fourth edition, Cambridge University Press.
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