# PURPOSE:

This procedure provides guidelines for collection of semen specimens and instructions for the preparation and examination of semen for the purpose of fertility testing.

**SCOPE:**

This procedure pertains to UPMC Hanover.

**PRINCIPLE:**

Semen analysis is part of an infertility evaluation and may include:

* Gross examination of semen (volume, pH, liquefaction, viscosity, aggregation and agglutination)
* Microscopic examination of unfixed spermatozoa (percent motility, percent progressive motility, and enumeration)
* Microscopic examination of fixed and stained spermatozoa (viability and morphology)

**SUPPLIES:**

Disposable pipette

3” x 1” glass microscope slide

Disposable cover glass

5 or 10 mL plastic syringe

12 x 75mm test tubes

MLA pipettes and tips for measuring 50, 100, 900 and 950 uL

C-Chip Disposable Hemocytometer

Petri dish with water-moistened filter paper

Microscope

37°C incubator (in Microbiology)

18-gauge needle

conical tubes with yellow caps (in Urinalysis)

Slide folder

**REAGENTS**

Diluting fluid for spermatozoa

Cytology fixative spray

PAP stain (in Cytopathology)

5% Eosin stain solution

10% Nigrosin stain solution

**CONTROLS**

1. Body fluid count quality control material is analyzed by manual hemocytometry by the same method used for spermatozoa counts. QC is performed once daily per shift. QC must be within the acceptable range before patient results are reported. For details on performing QC, refer to the Body Fluid Cell Count Quality Control SOP.
2. Stained smears of spermatozoa are available as reference for morphology differentiation. Technologists review reference slides during training to familiarize themselves with normal and abnormal forms before reading and reporting patient spermatozoa morphology. Ongoing review of spermatozoa morphology occurs during competency assessment activities. Staff assessment of spermatozoa motility is evaluated semi-annually using CAP survey material.

**SPECIMEN:**

A fresh, complete semen specimen must meet the following criteria:

* provided to the main hospital outpatient laboratory, Monday through Friday before 1400
* collected after abstinence from ejaculation for 3 days
* collected as per written patient instructions provided by the physician
* collected in a properly labeled, clean glass or rigid plastic container
* accompanied by a Patient Instruction form with patient name, type and time of collection on the form
* not exposed to temperatures of less than 25°C during transportation to the laboratory
* submitted within 1 hour of collection

**PROCEDURE:**

**Accept Specimen**

Outpatient Laboratory Staff and Central Receiving staff

1. Assure that a “Patient Instruction Sheet” accompanies the specimen and includes patient name, time of collection and collection method, and notes about deviations from recommended collection practices.
2. Record date and time of collection in the LIS.
3. Send the specimen to the hematology laboratory immediately after patient registration since analysis must begin within one hour of collection. Document deviations from this time-frame (late delivery or delayed testing) on worksheet.

Hematology staff

1. Begin examination immediately upon receipt in order to preserve spermatozoa integrity.
2. Document deviations from “one-hour to testing time” (e.g.: late delivery or delayed testing) on worksheet.
3. Attach “Patient Instruction Sheet” to worksheet.

**Evaluate and record gross (macroscopic) characteristics**

1. appearance/color
2. volume

* Measure volume using a 5 or 10 mL plastic syringe

1. pH

* Place a drop of the sample on a strip of pH paper and compare the strip to the chart on the container.

1. temperature

Semen temperature equates to room temperature.

1. liquefaction

* Within 15-60 minutes at room temperature the specimen should be watery with only small areas of coagulation remaining.

1. viscosity

* Using a pipette, draw up a portion of sample. Allow the semen to drop by gravity and observe the length of any thread. A normal sample leaves the pipette in small, discrete drops or with a thread shorter than or equal to 2 cm. An abnormally viscous specimen causes a thread longer than 2 cm.

**Evaluate microscopic characteristics**

1. When the specimen is viscous or not liquefied, gently aspirate and expel it several times through an 18- gauge needle attached to a syringe.
2. Place a drop of semen on a glass slide and cover with a coverslip, performing an assessment as soon as the contents are no longer drifting.

* If no spermatozoa are noted refer to section: **Evaluate azospermatozoaic specimens**
* If spermatozoa are noted, assess and record observations of the presence or absence of spermatozoa aggregation, agglutination; also note other cellular elements (e.g.: epithelial cells, leukocytes, bacteria).

**Aggregation**

Aggregation describes the adherence of immotile spermatozoa to each other OR motile spermatozoa to mucus strands, non-spermatozoa cells, or debris.

* Denote as present or absent.

**Agglutination**

Agglutination describes the adherence of motile spermatozoa to each other.

* Denote as present or absent.

**Examine for motility, progression**

1. Using the 40X objective, count at least 200 spermatozoa categorizing them as

* non-motile,
* motile (progressive) or
* motile (non-progressive).

1. Calculate the percentage in each of the three categories.
2. Calculate the **TOTAL %** of motile spermatozoa by adding the % motile (progressive) and % motile (non-progressive) spermatozoa.
3. When the total % of motile spermatozoa is less than 32%, perform a viability test.

**Perform spermatozoa viability test ( when % motile spermatozoa < 32%)**

1. Add 1-2 drops of well-mixed, liquefied semen specimen to 12x75 test tube.
2. Add 2 drops of 5% Eosin solution. Allow to stand at room temp for 30 seconds.
3. Add 3 drops of 10% Nigrosin solution.
4. Immediately place 1 drop of Eosin-Nigrosin-semen mixture on slide.
5. Prepare a wedge-prep smear.
6. Examine the smear using a 40X objective. The background will be dark. Categorize sperm as viable or non-viable.

* Viable spermatozoa appear unstained (white.)
* Non-viable spermatozoa appear pink or red.

1. Count a minimum of 100 spermatozoa. Determine the percent that are viable (unstained).
2. Record viability as percent of unstained spermatozoa counted.

**Dilute specimen and perform spermatozoa count**

In this section, the dilution factor is **20.**

1. Pipette 950 μL of semen diluting fluid into 12x75mm tube.
2. Pipette 50 μL of well-mixed semen into diluent tube.
3. Mix by gentle agitation.
4. Charge both chambers of a hemocytometer with 10 uL of diluted semen.
5. Let stand for at least 5 minutes in a moist petri dish, but no longer than 60 minutes.
6. Count the spermatozoa in 25 RBC (center) squares in each chamber. Each RBC square is 0.004 mm3.
7. If fewer than 80 spermatozoa are counted on one side of the chamber using this dilution, proceed with “Alternate Dilution” spermatozoa count determination in the next section.
8. If counts on sides A & B are >80 and agree +/- 10 %, perform calculations shown below. If counts do not agree, re-charge the chamber and repeat the counts.

(# spermatozoa side A + # spermatozoa side B) X dilution factor\_\_\_\_\_\_ = \_\_\_\_\_\_\_\_\_ million spermatozoa/ mm3

Total # squares counted X volume of each square X 1000

*For example:*

*# spermatozoa side A = 400*

*# spermatozoa side B = 410*

*Dilution factor = 20*

*# squares counted = 50 (25 in each chamber)*

*Volume of each square = 0.004 mm3*

*(300 + 310) X 20 \_\_ = 12200\_ = 61 million spermatozoa/ mm3*

*50 X 0.004 X 1000 200*

**Perform “alternate dilution” and spermatozoa count**

Use when less than 80 spermatozoa are counted on one side of the chamber with standard dilution. In this section, the dilution factor is **10.**

1. Pipette 900 μL of semen diluting fluid into 12x75mm tube.
2. Pipette 100 μL of well-mixed semen into diluent tube.
3. Mix by gentle agitation.
4. Charge both chambers of a hemocytometer 10 uL of diluted semen.
5. Let stand for at least 5 minutes in a moist petri dish, but no longer than 60 minutes.
6. Count the spermatozoa in the 4 corner WBC squares in each chamber. Each WBC square is 0.1 mm3.
7. If counts agree +/- 10 %, perform calculations shown in the previous section using a dilution factor of 10 and a WBC square volume 0.1 mm3. If counts do not agree, re-charge the chamber and repeat the counts.

**Evaluate azospermic specimens**

1. Pour entire specimen from collection container into a properly labeled conical tube with a yellow stopper (found in Urinalysis area) and recap.
2. Centrifuge tube at 400G for 5 minutes, 1350rpm. (Use the same setting used for routine urinalysis.)
3. Remove and discard the supernatant from the specimen without disturbing the sediment.
4. Mix well to resuspend the sediment.
5. Using a pipette, place a small drop of semen on a glass slide and coverslip the sample.
6. Using the 40X objective, examine a minimum of 20 fields, counting the number of spermatozoa in each. Average the number per field.
7. Record comments on worksheet as follows:

**# OBSERVED REPORT**

No sperm No sperm seen.

0-1/HPF Rare sperm seen.

2-3/HPF Few sperm seen.

4-10 HPF Many sperm seen.

>10/HPF TNTC

1. Take slide and worksheet to pathologist for confirmation.

**Prepare 4-6 slides for staining**

1. For **typical specimens**, place a well-mixed drop of semen about 1/3 of the way from the non-frosted end of a glass slide. When making slides for **azospermic specimens**, use sediment.
2. Place a second slide on top, allowing the semen to spread.
3. Pull the slides apart.
4. Properly label the slides.
5. Spray slides with cytology fixative while smear is still wet.
6. Place slides in slide folder.
7. Allow slides to dry overnight. Take folder to Cytology department. Slides are stained and cover-slipped with PAP stain by Cytology department.
8. Allow the coverslip medium to dry 2-4 hours before reading.

**Evaluate stain and classify/differentiate spermatozoa**

At least 2 technologists must perform morphologic differentiation.

1. Observe stained spermatozoa to assure that stain reactivity is satisfactory for evaluation of morphologic detail and that stain is uncontaminated. Record observations on worksheet. Document action taken for unsatisfactory staining.
2. Using an 100X objective, differentiate 100 spermatozoa according to the following classifications

* Normal
* Abnormal head
* Abnormal neck
* Abnormal tail
* Immature spermatozoa

NOTE: Begin observation at the head, followed by the neck and, finally the tail. If a spermatozoan has more than one abnormality, classify it according to the FIRST observation.

*Examples:*

*If a spermatozoan has an abnormal head and neck, classify it as “abnormal head.”*

*If a spermatozoan has an abnormal neck and tail, classify it as “abnormal neck.”*

1. Using the two techs’ results, calculate the average % of each form.
2. Take slide and worksheet to pathologist for confirmation.

**REFERENCE RANGES**

Appearance: opalescent, grey-white

Volume: > 1.5 mL

pH: > or = 7.2

Temperature: acceptable- 20-37 degrees Centigrade

Liquefaction: normal

Viscosity: normal

Total motility: > 40%

Progressive motility: > 32%

Viability: > 58%

Aggregation: absent

Agglutination: absent

Total ejaculated spermatozoa count: > 39 million/ejaculate (calculated by LIS)

Spermatozoa count: >15 million/ mm3

Spermatozoa morphology: > 4% normal spermatozoa

**METHOD LIMITATIONS**

* Bacteria may be present due to prolonged specimen storage or an inflammatory process of the male reproductive tract.
* Results are dependent on patient self-reporting of proper specimen collection techniques. Deviations from techniques not reported by patients could result in spurious test results.

**REFERENCES:**

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