1 Protocol A: Cytospin Procedure

1.1 Introduction

Cytocentrifugation is the process in which a benchtop centrifuge with a specially designed rotor and sample chambers deposits cells onto a clearly defined area of a glass slide. Residual fluid is absorbed by the sample chamber’s filter card, which flattens the cells onto the slide, making single sperm cells easier to detect. The slide can then be air-dried, stained, and examined. This procedure is used in cases where a wet preparation fails to yield detectable spermatozoa [1–2].

1.2 Specimen Collection

The physician instructs the patient on proper semen collection technique. The patient collects the specimen into a sterile container and brings it to the laboratory (see “Semen Sample Collection and Labeling Procedure”).

1.3 Equipment and Materials

A. Sterile saline,
B. Cytospin centrifuge
C. Frosted glass slides
D. Sterile Pasteur transfer pipettes
E. Shandon Single Cytofunnel disposable sample chambers
F. Metal slide holders (Cytospin 4, equipped)
G. Sperm counting chamber

1.4 Quality Control

A blank control slide is prepared simultaneously with the patient slides and used for the detection of sperm contamination. The blank slide is prepared by adding two drops of sterile saline into the attached Cytofunnel that is latched onto the metal slide holder. The slides are then stained using the Nuclear Fast/Picroindigocarmine stain (NF/PICS) staining procedure (see “NF/PICS” procedure).

1.5 Procedure

Note: Before proceeding, the patient sample should be spun at 300 g for 7–10 min. Aliquot off nearly all of the supernatant above the pellet and discard it. Next, vortex the specimen until it is well mixed. Plate a 5 μL drop of sample on a sperm counting chamber and view microscopically to rule out the presence of sperm before proceeding further. In cases where sperm is seen, the cytospin procedure is unnecessary. In cases where sperm is not seen, spinning the sample at high speed via cytocentrifugation increases the likelihood that sperm will be found on the NF/PICS stain.

A. Turn on the cytospin centrifuge. Lift the top cover and remove the protective lid on the inside of the centrifuge.
B. Obtain six of the metal slide holders specific to the cytospin. Position a pencil-labeled frosted slide holder specific to the cytospin. Position a pencil-labeled frosted slide (label should include patient’s name, medical record number, date) into the slide holder (Fig. 11.1) and place a Cytofunnel card directly on top of the slide. Lock the slide and Cytofunnel firmly in place with the slide holder’s locking mechanism. Stand the assembled apparatus upright.

Note: Five of the slides should be labeled “C” for the cytospin patient sample and one labeled “B” for the blank control slide.

C. Using a sterile transfer pipette, add one drop of the well-mixed patient sample into the top opening of five of the six Cytofunnels with slides labeled cytospin (C) (Fig. 11.2). Add one equal drop of sterile saline to each of...
the same five funnels (if the specimen is highly viscous, place one drop in the cytospin chamber and add two drops of sterile saline). Do not add the semen sample to the sixth Cytofunnel with the slide labeled blank (B).

Note: One slide holder will be used for the blank control slide as mentioned above. This slide will be prepared by adding two drops of sterile saline into the Cytofunnel (Fig. 11.3). It will be prepared the same way as the above patient slides except it will include no patient sample.

Caution: When adding the patient sample, make sure the fluid lies in the horizontal tunnel and not in the vertical cone-shaped part of the funnel. Use no more than 0.5 mL of fluid, as this is the maximum amount the tunnel can hold. All excess fluid in the cone-shaped vertical portion of the funnel will be forced up the cone during centrifugation and not onto the slide as expected.

D. Place closure caps onto the top of the Cytofunnel and place each of the assembled slides and Cytofunnel apparatuses into the appropriate slots inside the centrifuge (Fig. 11.4).

Note: Do not place your finger over the small hole in the center of the closure cap when placing it onto the funnel. This will force the fluid in the horizontal tunnel immediately onto the slide before centrifugation and minimizes cell recovery.

E. Replace the protective lid inside the centrifuge and lock it into place. Close the top cover. The indicator light will come on if the cover is properly locked (otherwise the centrifuge will not start).

F. Set the centrifuge to 350 g for 9 min and press “Start” (Fig. 11.5).
When the centrifuge stops spinning, the head will come to rest. The centrifuge will emit an audible beep. Open the top cover; remove the protective lid and the slide apparatuses.

Lay the slide holder with the attached Cytofunnel horizontally on a table. With the slides still locked inside, allow them to dry for approximately 5–10 min. Next, unlock the mechanism holding the Cytofunnel to the slide, remove the Cytofunnel, and discard into a biohazard bin. Allow the slides to air-dry for another 15 min before staining.

**Note:** If fluid remains in the sample chamber after spinning, do not spin again.

### 2 Protocol B: Nuclear Fast Red and Picroindigocarmine Stain

#### 2.1 Introduction

This staining technique is used to identify rare spermatozoa in the event that no sperm is seen during semen analysis. Sperm heads are stained red and tails green, allowing spermatozoa to be more easily identified when viewed microscopically [1–3].

#### 2.2 Equipment and Materials

A. Indigo carmine dye
B. Nuclear Fast Red biological stain
C. Picric acid, reagent grade
D. Absolute ethanol (reagent grade) (Fig. 11.6)
E. Aluminum sulfate
F. Nuclear Fast Red solution (NF) – Dissolve 5 g of aluminum sulfate in 200 mL of hot distilled water. Immediately add 0.1 g of Nuclear Fast Red and stir with a glass rod. Allow to cool then filter using filter paper. Store at room temperature. This solution is stable for 6 to 8 months.
G. Picroindigocarmine solution (PICS) – Add 150 mL of saturated picric acid solution to a glass beaker. Dissolve 0.5 g of indigo carmine dye in the solution. Filter and store in a brown bottle (indigo carmine dye is light sensitive). Store at room temperature. This solution is stable for one year.
H. Mounting Media
I. Coverslips
J. Staining rack
K. Sterile Pasteur pipettes
2.3 Quality Control

A. A blank cytospin control slide is prepared simultaneously with the patient specimen. The blank slide is prepared by adding two drops of sterile saline into the cytospin funnel. It is used to detect sperm contamination.

B. A normal donor slide is also used as a control slide to assess stain quality. Donor slides should be fixed in ethanol well in advance and used during the addition of the NFPIC stain.

C. Working components are checked microscopically for contaminants monthly.

D. Stains are checked weekly (or as required) for quality of the nuclear DNA and cytoplasmic RNA color.

2.4 Procedure

A. Place the patient/blank slides (Fig. 11.2) faceup and level on the staining rack (Fig. 11.7).

B. Using a sterile Pasteur pipette, place a sufficient amount of absolute ethanol (fixative) to fully cover each of the slides. Let slides sit in ethanol for 15 min (Figs. 11.3 and 11.8).

C. Place slides upright allowing the ethanol (fixative) to draw off and let the slides air-dry for 15 min (Figs. 11.4 and 11.9).

D. Lay the slides flat, again, on the staining rack. Place sufficient NF solution to cover each slide and allow the slides to sit for 15 min in solution (Fig. 11.5).

Note: The donor QC slide should be used during this step (Fig. 11.10).

E. Carefully remove the NF solution from the slide by tilting the slide and allowing the solution to run off.
F. Lay slides flat, again, on the staining rack. Place several drops of PICS on the NF-stained slide. After 30 s of staining with the PICS (Fig. 11.6), quickly proceed to the next step (Fig. 11.11).

G. Holding the slides at the top, carefully rinse with absolute ethanol.
H. Stand each slide upright and allow to air-dry for 15 min.
I. Mount each slide using a coverslip and mounting media.

References

Cytospin for Azoospermic Sample

Procedure

In cases where sperm is seen, the Cytospin procedure is unnecessary. In cases where sperm is not seen, spinning the sample at high speed via cytocentrifugation increases the likelihood that sperm will be found on the NF/PICS stain.

A. Turn on the Cytospin centrifuge. Lift the top cover and remove the protective lid on the inside of the centrifuge.

B. Using a pencil, label a frosted slide with patient name, medical record number and date. Obtain six metal slide holders specific to the Cytospin. Position the slide into the slide holder (Figure 1). Place a Cytofunnel® card directly on top of the slide. Lock the slide and Cytofunnel® firmly in place with the slide holder’s locking mechanism.

Note: 5 slides should be labeled “C” for the Cytospin patient sample and 1 labeled “B” for the blank control slide.

C. Using a sterile transfer pipette add one drop of the well-mixed patient sample into the top opening of 5 of the 6 Cytofunnels® with slides labeled cytopsin (C) (Figure 2). Add one equal drop of sterile saline to each of the 5 funnels. If the specimen is highly viscous, place one drop in the cytopsin chamber and add 2 drops of sterile saline. Do not add the semen sample to the 6th Cytofunnel® with the slide labeled blank (B).

Note: 1 slide holder should be used for the blank control slide. This slide will be prepared by adding two drops of sterile saline into the Cytofunnel® (Figure 3). It is prepared the same way as the above patient slides except it will include no patient sample.

D. Place closure caps onto the top of the Cytofunnel® and place each of the assembled slides and Cytofunnel® apparatuses into the appropriate slots inside the centrifuge (Figure 4).

E. Replace the protective lid inside the centrifuge and lock it into place. Close the top cover. The indicator light will come on if the cover is properly locked (otherwise the centrifuge will not start).

F. Set the centrifuge to 1800 rpm for 9 minutes and press “Start.” (Figure 5)

G. When the centrifuge stops spinning, it will emit an audible beep. Open the top cover, remove the protective lid and the slide apparatus.

H. Lay the slide holder with the attached Cytofunnel® horizontally on a table. With the slides still locked inside, allow them to dry for approximately 5-10 minutes. Next, unlock the mechanism holding the Cytofunnel® to the slide, remove the Cytofunnel® and discard. Allow the slides to air-dry for another 15 minutes before staining.

Figure 1. Slide mounted on metal Cytospin slide holder.

Figure 2. Drop of semen sample added to Cytofunnel.

Figure 3. Two drops of sterile saline added to Cytofunnel of “blank” control slide.

Figure 4. Proper insertion of Cytospin slide apparatus into centrifuge.

Figure 5. Cytospin centrifuge with required settings.
Nuclear Fast Red and Picroindigocarmine Stain

Procedure

A. Place the patient/blank slides (Figure 1) face-up and level on the staining rack.

B. Using a sterile Pasteur pipette, place a sufficient amount of absolute ethanol (fixative) to fully cover each of the slides. Let slides sit in ethanol for 15 minutes (Figure 2).

C. Place slides upright allowing the ethanol (fixative) to drain off and let the slides air-dry for 15 minutes (Figure 3).

D. Lay the slides flat, on the staining rack. Place sufficient Nuclear Fast (NF) solution to cover each slide and allow the slides to sit for 15 minutes in solution (Figure 4).

   Note: A donor smear acting as positive control should be used during this step.

E. Carefully remove the NF solution from the slide by tilting the slide and allowing the solution to run off.

F. Lay slides flat, again, on the staining rack. Place several drops of picroindigocarmine stain (PICS) on the NF-stained slide. After 30 seconds of staining with the PICS quickly proceed to the next step (Figure 5).

G. Holding the slides at the top, carefully rinse with absolute ethanol.

H. Stand each slide upright and allow to air dry for 15 minutes.

I. Mount each slide using a coverslip and mounting media.

J. Slides are examined under brightfield oil magnification.

K. Sperm heads are stained red and tails green.