Eosin-Nigrosin Staining Procedure

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Ashok Agarwal, Sajal Gupta, and Rakesh Sharma

1 Introduction

Eosin-Nigrosin is a staining technique that assesses the vitality of a sperm sample when the initial motility is less than 25 % [1–3]. Nigrosin increases the contrast between the background and sperm heads, making the sperm easier to visualize. Eosin stains only the dead sperm, turning them a dark pink, whereas live sperm appears white (Fig. 8.1). This staining technique must be performed immediately after motility is assessed using sperm from the same semen sample. Stained slides can be stored for reevaluation and quality control purposes [1–5].

2 Specimen Collection

The physician instructs the patient on proper collection technique (for details, see *Semen Sample Collection and Labeling Procedure*). The patient collects a specimen into a sterile container and brings it to the laboratory at the appointed time.

A. Agarwal, PhD, HCLD (ABB), ELD (ACE) (✉)
S. Gupta, MD (Ob/Gyn), MS (Embryology), TS (ABB)
R. Sharma, PhD
Andrology Center and Reproductive Tissue Bank,
American Center for Reproductive Medicine,
Cleveland Clinic, Cleveland, OH, USA
e-mail: agarwaa@ccf.org; guptas2@ccf.org; sharmar@ccf.org

3 Equipment and Materials

A. Stain components: Eosin Y disodium; Nigrosin (water soluble)
B. Disposable Pasteur pipettes
C. Porcelain Boerner slide
D. Wooden applicators
E. Glass slides
F. Coverslips
G. Mounting media

4 Reagent Preparation

Eosin Y 1 % Solution (Fig. 8.2):

1. Weigh 0.5 g of Eosin Y and add to 50 mL of deionized water.
2. Dissolve this solution using gentle heat.
3. Cool the liquid to room temperature and filter using filter paper.
   Note: This reagent is stable for 3 months at room temperature.

Nigrosin 10 % Solution (Fig. 8.3):

1. Weigh 5 g of Nigrosin and add it to 50 mL of deionized water.
2. Dissolve this solution using gentle heat.
3. Cool the liquid to room temperature and filter using filter paper.
   Note: This reagent is stable for 3 months at room temperature.

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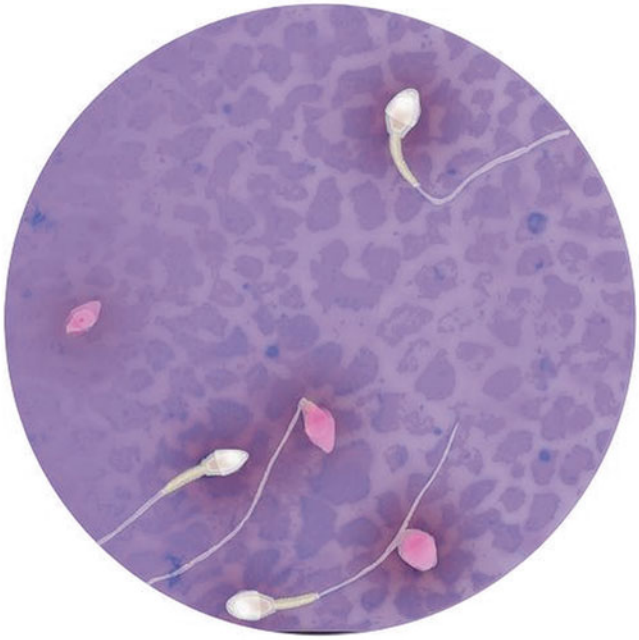

Fig. 8.1 E/N stained seminal smear showing live and dead sperm [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

Fig. 8.2 Eosin Y 1 % solution. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

Fig. 8.3 Nigrosin 10 % solution. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

5 Quality Control

1. A monthly patient control should be run to check the quality of reagents. The motility should be assessed prior to the QC run.
2. When new reagents are prepared, QC must be performed before they can be used for a new patient specimen.
3. The specimen should be stained with the old lot of reagents as well as the new lot of reagents. Both sets of slides should be scored for the vitality percent results.

Criteria: The viable sperm percentage (determined by scoring the E/N slides) should be greater than or equal to the motility of the specimen used. The comparison of the two lots of reagents should be within 10 % of each other.

Response: If results are not within the acceptable range, repeat using another specimen.

6 Procedure

1. Label two frosted slides with an accession number, the patient's name, Medical record#, and date.
2. Place 1 drop of well-mixed semen on a Boerner slide.
3. Add 2 drops of 1 % aqueous Eosin Y.
4. Mix well with a wooden stirrer for 15 s.

Fig. 8.4 Boerner slide well. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

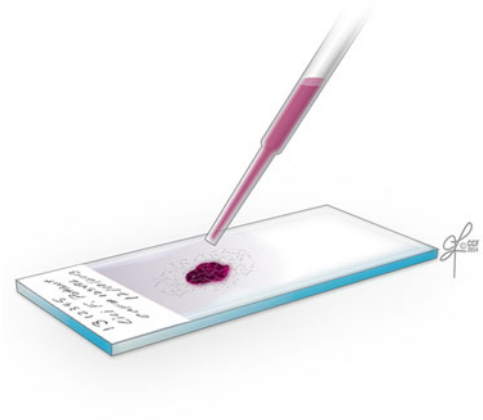

Fig. 8.5 Placement of E/N suspension on glass slide. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

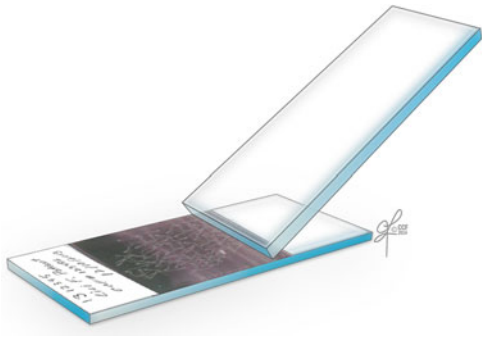

Fig. 8.6 Preparation of E/N smear. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

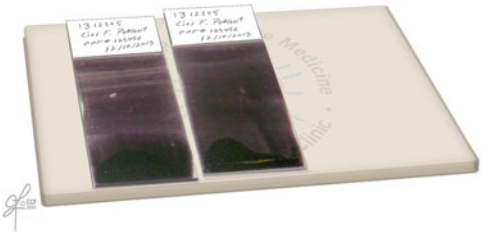

Fig. 8.7 E/N slides ready for scoring. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

5. Add 2 drops of 10 % aqueous Nigrosin.
6. Mix well with a wooden stirrer in Boerner slide well (Fig. 8.4).
7. Immediately make two thin smears from this mixture by pipetting 10–20 μL onto each labeled slide (Figs. 8.5 and 8.1). Air-dry semen smears.
8. Coverslip with Cytoseal mounting media (Fig. 8.2).

7 Scoring

1. The Nigrosin provides a dark background that makes it easier to observe faintly stained spermatozoa.
2. With bright-field optics, live spermatozoa have white or faint pink heads, and dead spermatozoa have heads that are stained red or dark pink.
3. If the stain is limited to only part of the neck region, and the rest of the head area is unstained, this is considered a "leaky neck membrane" and is not a sign of cell death.
4. Record the percentage of viable sperm on the patient worksheet.
5. File slide in the appropriate file box.

8 Normal Range

>58 % viability in normal sperm specimens (fifth centile, 95 % CI 55–63).

Note: For specimens with <25 % motility, the viability should be greater than or equal to the specimen motility.

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Eosin-Nigrosin Staining Procedure

Procedure

Vitality test is performed only when sperm motility is equal or less than 25%

Preparation

Eosin Y-1% Solution (Figure 1)

1. Weigh 0.5 grams of Eosin-Y and add to 50mL of deionized water.
2. Dissolve this solution using gentle heat.
3. Cool the liquid to room temperature and filter using filter paper.

Note: This reagent is stable for 3 months at room temperature.

Figure 1. Eosin-Y 1% solution.

Nigrosin 10% Solution (Figure 2)

1. Weigh 5 grams of Nigrosin and add it to 50mL of deionized water.
2. Dissolve this solution using gentle heat.
3. Cool the liquid to room temperature and filter using filter paper.

Note: This reagent is stable for 3 months at room temperature.

Figure 2. Nigrosin 10% solution.

1. Label two frosted slides with an accession number, patient's name, medical record number and date.
2. Place 1 drop of well-mixed semen into Boerner slide well.
3. Add 2 drops of 1% aqueous Eosin-Y.
4. Mix well with a wooden stirrer for 15 seconds.
5. Add 2 drops of 10% aqueous Nigrosin.
6. Mix well with a wooden stirrer in slide well (**Figure 3**).

Figure 3. Boerner slide well.

7. Immediately make 2 thin smears from this mixture by pipetting 10-20μL onto each labeled slide (**Figure 4 & 5**). Air dry.

Figure 4. Semen mixed with Eosin-Nigrosin.

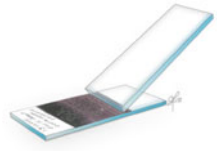

Figure 5. Eosin-Nigrosin smear.

8. Coverslip with Cytoseal mounting media (**Figure 6).**

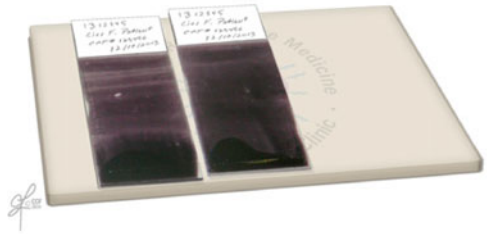

Figure 6. Final preparation of Eosin-Nigrosin slides.

Scoring

1. The Nigrosin provides a dark background that makes it easier to identify lightly stained spermatozoa.
2. Using oil at 1000X magnifcation, observe spermatozoa under brightfield microscopy. White or light pink heads indicate viable spermatozoa while red or dark pink heads indicate non-viable spermatozoa.
3. If the stain is limited to only part of the neck region, and the rest of the head area is unstained, this is considered a "leaky neck membrane" and is not indicative of cell death.
4. Record the percentage of viable sperm on the patient worksheet.
5. File slide in the appropriate file box.

Normal Range

>58% viability in normal sperm specimens

References

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