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Short Communication

# Eosin Nigrosin staining technique in assessment of sperm vitality in medical laboratories – A snippet from our experience on implementing the staining, interpretation and quality control procedures

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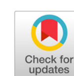


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ABSTRACT

Eosin Nigrosin staining for assessment of sperm vitality is an essential component of basic semen analysis as it helps differentiate between dead and immotile sperms, and has clinical implications in terms of patient treatment and follow up. This staining technique involves minimal use of reagents and simple procedural steps. Standardization of the same is pertinent to warrant accurate and reproducible results in medical laboratories, even those not specialized in infertility care. We wish to share our hands on experience through various stages in implementing this staining technique from challenges faced to putting quality control processes in place as reference for peers.

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## 1. Introduction

Semen analysis is an investigation ordered commonly for assessment of fertility/ infertility, effectiveness of vasectomy, suitability of semen for artificial insemination, follow up for fertility after cancer treatment and for medico-legal purposes. Laboratory seminology is still in its stage of infancy as many medical laboratories still use manual methods of analysis.[1] Of the various parameters in basic semen analysis, sperm vitality is essential to determine if immotile sperms are alive or dead and is indicated especially when motility in the ejaculate is less than 40% as per the $6^{th}$ (2021) edition of WHO.[2] Presence of a large proportion of live but immotile sperms may be indicative of structural defects in the flagellum, a high percentage of immotile and dead cells may indicate epididymal pathology or an immunological reaction due to an infection.

One of the standard recommended staining techniques for vitality testing is the Eosin Nigrosin staining. Any technique before being put into use must be standardized with a quality control process in place. This has several advantages in the routine laboratory in terms of producing reliable results. We wish to describe how the Eosin nigrosin staining techniques was implemented and put into use for routine semen analysis in our laboratory.

## 2. Materials and Methods

To begin with, reference literature regarding the staining technique was collected from various sources like standard guidelines of World Health Organization, text books and indexed journal articles. Steps in the process of staining were formulated. Stains procured were Eosin Y 2% solution (colour index 45380) and Nigrosin 10% (colour index 50420). Eosin was purchased as a solution for ease of use considering the eventual scaling of semen samples being received for testing.

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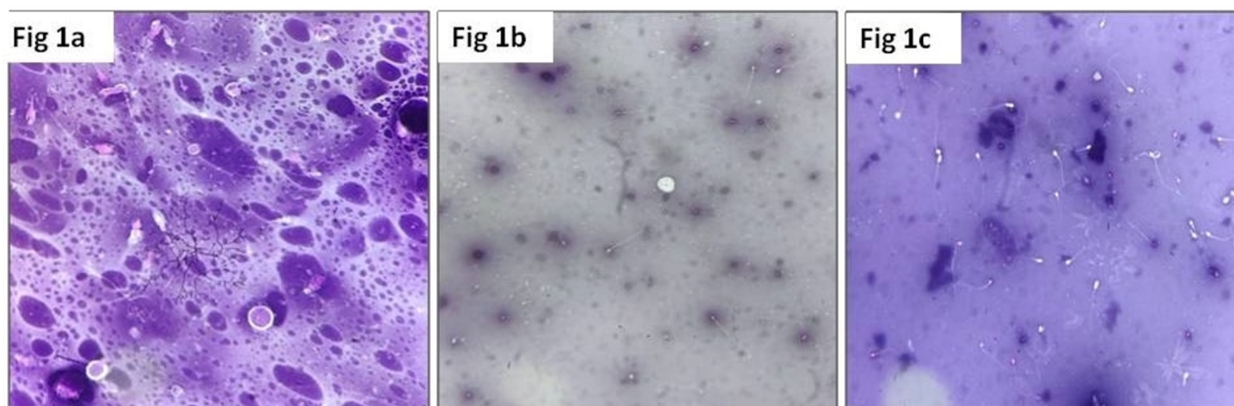


**Fig. 1: a)**: Clumps caused due to improper mixing. Eosin Nigrosin x 400; **b)**: Smear with insufficient contrast, Eosin Nigrosin x 400; **c)**: An ideal well stained slide with good contrast. Eosin Nigrosin x 100

Optimization of staining technique: Sperm vitality was assessed immediately after liquefaction of the semen sample, preferably at 30 minutes, but in any case, within one hour of ejaculation, to limit deleterious effects of dehydration.[3]

**Step 1:** WHO recommends 0.67% eosin. However, to counteract the hypo osmolality we used 1% eosin which was prepared by adding 1ml of 2% eosin to 1ml distilled water.[2]

**Step 2**: 1ml of this mixture was added to 1ml 10% nigrosin in at test tube which was the working stock solution.

**Step 3**: In a clean test tube, 50 microlitres semen was taken with a calibrated Finnpipette with yellow plastic tips in a clean test tube.

**Step 4:** To this was added 50 microlitres of the working stock solution.

**Step 5:** The solution was mixed and incubated for 3o seconds at 37 degrees.

**Step 6**: Two smears were prepared by transferring a 12 $\mu$l droplet with the pipette to a labeled microscope slide like for peripheral blood smears, air dried, mounted and observed under oil immersion and brightfield optics.

**Interpretation:** A total of 200 sperms were counted per slide. Eosin stained only the dead sperms, turning them a dark pink, whereas live sperm appeared white. Nigrosin increased the contrast between the background and sperm heads, making the sperm easier to visualize. Percentage of live and dead sperms was reported.[4] At every step variation was tried till optimization was reached like varying the incubation period, reducing/ increasing the stain concentrations etc.(Figure 1) Once standardized a Standard operating procedure (SOP) and quality check points were drafted and documented for workbench reference.

## 3. Discussion

Sperm vitality, as estimated by assessing the membrane integrity of cells requires a standardized technique to be clinically relevant. Eosin Nigrosin staining technique recommended by the WHO is easy to perform and is widely used in medical laboratories. There have been many comparative studies accounting the different variations in techniques- One step eosin alone, one step Eosin Nigrosin, and two step Eosin Nigrosin with different eosin concentrations.[5] The technique followed by us was the one step Eosin Nigrosin.

The procedure of introducing a new staining technique comes with trial and errors before standardization. A few problems faced with solutions encountered is as below.(Table 1)

Quality control procedures: Check on reporting can be done by replicate testing (same sample split into two and processed in independently by two technicians) and assuring an inter individual variation in reporting of less than 10% once in every three months.

Other options that can be considered: 1. Vitality test using eosin alone. This method is simple and rapid, but wet preparations cannot be stored for quality control purposes, and negative phase contrast optics are required to obtain reliable results. These optics are very difficult to source, and the more common positive phase contrast makes faint pink heads difficult to discern. 2. Vitality test using hypo-osmotic swelling- This is useful when staining of spermatozoa must be avoided, e.g. when choosing spermatozoa for intracytoplasmic sperm injection (ICSI). The hypo-osmotic swelling test presumes that only cells with intact membranes (live cells) can swell in hypotonic solutions. Spermatozoa with intact membranes swell within 5 minutes in hypo-osmotic medium, and all flagellar shapes are stabilized by 30 minutes. This technique is recommended in labs performing invitro or invivo assisted reproductive procedures.

**Table 1:** Problems faced during standardization of staining procedure

| Issue | Remarks |
|---|---|
| Quality of staining is not adequate (stain not taken up, dull background) | Prepare the stock solution fresh for each batch of staining. Stock solution as per recommendations can be prepared and stored in dark coloured bottles. But we found that preparing it fresh gave good quality of stain and allowed prolonged storage without fading of slides. For a good contrast background the nigrosin concentration can be increased. |
| Thick proteinaceous clumps are seen in the background? | Thorough mixing of the stock solution and semen sample dissolves the clumps giving a clear background. |
| Live unstained sperms not standing out against the background inspite of increasing Nigrosin concentration? | Incubating the mix of semen sample and stock solution for 30 seconds optimally gives a good contrast. |
| How to validate that the staining technique ? | Number of dead sperms (Eosin nigrosin slide) + number of motile sperms (from the wet mount motility) must be less than 100% |
| If the stain is limited to only a part of the neck region, and the rest of the head area is unstained? | This is considered a “leaky neck membrane”, not a sign of cell death and total membrane disintegration. These cells should be assessed as live |
| How to avoid subjectivity in random distribution? | Count 200 sperms instead of 100 in each slide to achieve an achieve an acceptably low sampling error. |

## 4. Conclusion

Assessment of sperm vitality is an important component of basic semen analysis that can be performed with minimal resources without any sophisticated equipment in medical laboratories. However, it is important to standardize the technique and ensure quality checks at each step from staining to interpretation for the results to have clinical decision-making implications.

## 5. Conflict of Interest

None.

## Acknowledgments

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