**Preparation:**
1. Bring all reagents to room temperature.
2. Prepare fresh substrate by adding the following to a test tube:
   - 1 ml water
   - 250 µl Buffer
   - 40 µl DAB
   - exactly 1 drop Peroxide

**Procedure:**
1. Allow semen sample to liquefy.
2. Count spermatozoa.
3. Pipette 20 µl semen into a test tube.
4. Pipette 20 µl Peroxidase, a positive control, into a second test tube.
5. Pipette 20 µl water, a negative control, into a third test tube.
6. Add exactly 1 drop Fixative to each test tube.
7. Pipette 60 µl fresh substrate into each test tube and mix briefly.
8. Observe the test tubes with Peroxidase and water and note any color change. The test tube with Peroxidase should turn dark brown. This indicates that the fresh substrate is working properly. Proceed with the next step if the fresh substrate is working properly.
9. Prepare specimen for viewing using Method I or Method II.
   - **Method I:** Pipette 10 µl to 20 µl onto a glass slide and place a coverslip on top of the liquid. Examine at a total magnification of 400X using a microscope. Count brown cells and sperm within the same viewing area.
   - **Method II:** Pipette about 5 µl onto a Makler Chamber or a disposable counting chamber. Examine at a total magnification of 100X using a microscope. Count brown cells within the entire grid area.

**Calculation Of Granulocytes In Semen, Method I:**

Granulocytes/ml = Sperm Count \( \times \) Number of brown cells
\( / \) Number of sperm

**Example:** The following data were obtained for a tested semen specimen placed on a glass slide with a coverslip and viewed at 400X:
- Sperm Count = 73 X 10^6 cells/ml
- Number of brown cells = 4
- Number of sperm = 66

Applying the formula: 73 x 10^6 x 4/66 = ~4 x 10^6 granulocytes/ml

**Calculation Of Granulocytes In Semen, Method II:**

Granulocytes/ml = Number of brown cells X 5 X counting chamber factor where 5 is the dilution factor because semen was diluted 20 µl in a total volume of 100 µl.

**Example:** The following data were obtained for a tested semen specimen viewed at 100X in a Cell-VU:
- Number of brown cells in 100 squares = 16
- Cell-VU chamber factor = 10^2/2 = 5 cells/ml

Applying the formula: 16 x 5 x 10^3/2 = 4 x 10^3 granulocytes/ml

**Selected References:**
