VALIDATION PROTOCOL

A. Sperm Concentration

Basic haemocytometer (v SAMi) protocol should therefore resemble the following:

1. *As far as possible follow the directions of the WHO (2010)*
2. *Using a positive displacement pipette make your dilution (a good tip – an average dilution is 1 in 10 using 50µl semen to 450µl of diluent). We advise trying to obtain a suitable dilution which enables you to assess the whole gride of 25 haemocytometer squares.*
   a. *Use an 'air-displacement' pipette to dispense the appropriate amount of diluent/fixative into two vials. Mix well*
   b. *Aspirate the appropriate volume of semen immediately after mixing, allowing no time for the spermatozoa to settle out of suspension*
   c. ***Wipe the semen off the outside of the pipette tip, taking care not to touch the opening – V. important***
   d. *Dispense the semen into the fixative and rinse the pipette tip by re-aspirating and expressing*
   e. *Repeat for the replicate*
3. *Attach a coverslip to an improved Neubauer haemocytometer ensuring that iridescent 'Newton's rings' can be seen. OR use a disposable haemocytometer such as those from Cellvision (www.cellvision.nl).*
   a. *Use an 'air-displacement' pipette to dispense the appropriate amount of diluent/fixative into two vials. Mix well*
   b. *Aspirate the appropriate volume of semen immediately after mixing, allowing no time for the spermatozoa to settle out of suspension*
   c. *Mix the first dilution thoroughly by vortexing for 10 seconds at maximum speed and immediately remove approximately 10 µl of fixed suspension*
   d. *Depress the plunger of the pipette slowly, allowing the chamber to fill by capillary action.*
   e. *The coverslip should not be moved during filling, and the chamber should not be overfilled (when the coverslip may be seen to move) or under filled*
   f. *Mix the second dilution, as above and load the second chamber of the haemocytometer following the steps*
   g. *Store the haemocytometer horizontally for at least 4-5 minutes at room temperature in a humid chamber*
   h. *Examine the haemocytometer with phase-contrast optics at ×200 or ×400 magnification*
   i. *Count at least 200 spermatozoa in each replicate, in order to achieve an acceptably low sampling error*
   j. *Continue counting until at least 200 spermatozoa have been observed and a complete row (of 5 large squares) has been examined. We advise counting the whole grid of 25 squares since when counting in 5 or 10 squares can lead to errors associated with to over-counting sperm lying in tram lines*

SAMi (CASA) protocol

   a. *Mix sample well (swirling the pot for 10 seconds may be adequate but use of a vortex increases reliability*
   b. *Fill cell vision slide (remove any excess on the 'fill port' of this slide as it prevents drifting)*
   c. *Allow sample to settle for at least 1 minute*
   d. *Measure 400 sperm in at least 2 fields*
   e. *If initial analysis is more than 75x10^6/ml, dilute samples 1:1 or 1:2 in a suitable wash buffer and re-analyse remembering to account for the dilution factor*

Remember that parity may be different depending on which end of the range of results you examine, however ideally results should be close for the entire range if these basic rules are followed

Recent results from n=80 samples

Sperm count range x10^6/ml	**SAMi**	**Haemocytometer**
<10	4.1	3.99
10-80 million	39	37.5
>80 million	113	106