**Sperm Preparation**

Density gradient centrifugation method

**Day -1**
The day before oocyte pick-up

- **G-IVF™ PLUS**
  - Pre-equilibrate G-IVF PLUS at 37°C 6% CO2 overnight

- **G-RINSE™**
  - Pre-rinse all utensils, including tubes and dishes, with G-RINSE.

**Day 0**

1. **Assess the semen sample**

2. **Prepare gradient solutions**
   - **SpermGrad™**
   - **G-IVF™ PLUS**
   - or
   - **SpermGrad™ RTU**

   If you use SpermGrad RTU go to paragraph 3.

3. **Prepare gradients**
   - Pipette 1.5 mL of the 90% solution into the rinsed tube first and then slowly pipette 1.5 mL of the 45% solution on top of it. Finally, 1.0 mL of the semen is layered on the top.
   - Make up 2-4 gradient tubes. Before use, allow the stock solutions to warm to ambient temperature.

4. **Centrifuge the gradients at**
   - 300-600g 10-20 min

5. **Wash I**
   - Remove the two top layers. Transfer the pellets to new rinsed tubes and re-suspend with 5 mL equilibrated G-IVF PLUS and centrifuge again at 300-600g 10 min

6. **Wash II**
   - Aspirate and discard the supernatant. Transfer the pellets to new rinsed tubes and re-suspend with 5 mL equilibrated G-IVF PLUS and centrifuge again at 300-600g 10 min

7. **Assess sperm preparation**
   - Aspirate and discard the supernatants. Combine all pellets in a new rinsed tube and re-suspend in 0.5-1.0 mL of equilibrated G-IVF PLUS depending on sample quality.
   - Determine motility and concentration of spermatozoa in the washed sample.

8. **Dilution**
   - Dilute with equilibrated G-IVF PLUS to a final concentration of 75,000-200,000 motile sperms/mL.

9. **Preparation of insemination dishes**
   - Prepare rinsed insemination centre well dishes with 0.5-1.0 mL of sperm solution and pre-equilibrate.

   - 37°C 6% CO2 >2 h

   - If oil overlay is used, droplets of at least 100 µL volume are recommended. Equilibrate the dishes at 37°C 6% CO2 >6 h

10. **Insemination**
    - Transfer the oocytes to the insemination centre well dishes and leave at 37°C 6% CO2 overnight

    - Alternatively: Add equilibrated sperm suspension to equilibrated centre well dishes with the oocytes already present.