Directions for supplementation of un-supplemented G-Series™ media can be found in the G-Series Manual on www.vitrolife.com. Once supplemented, the media should be used as the G-Series PLUS media described below.

**SPERM FREEZING**
Sperm freezing when using straws

**Freezing**

1. **Assess the semen sample**
   Ensure that both liquefied semen and SpermFreeze Solution are at ambient temp.

   Measure the total volume of semen and carry out semen analysis as required.

2. **Add SpermFreeze Solution**
   Dilute with equal volume of semen and SpermFreeze Solution. Add SpermFreeze Solution slowly and dropwise to the semen and then carefully tilt after each drop added. Close the lid tightly and turn the tube upside down 20 times, being careful not to create bubbles.

3. **Leave in room temperature**
   > 10 min

4. **Load straws**
   Mark straws with patient ID and load the semen mixture. Ensure that some air space is left in the lower part of the straw. Seal the straw according to the straw manufacturer’s instructions.

5. **Freezing**
   Place the straws horizontally on a 1-3 cm styrofoam board in a liquid nitrogen bath. Leave for 30 min.

6. **Store in N₂(l)**
   Transfer the straws quickly into liquid nitrogen and store at -196 °C.

**Thawing**

The day before thawing:

1. **Remove straws from N₂(l)**
   Remove straws from -196 °C and place them in a water bath at 35 ± 2 °C for 30 sec.

   Wipe the straws dry with a clean paper towel and open according to instructions from the manufacturer of the straws.

2. **Dilute with G-IVF PLUS**
   Expel semen mixture into clean test tubes and dilute with equal amount of equilibrated G-IVF PLUS. G-IVF PLUS should be added dropwise to the semen mixture and the solution carefully mixed after each addition.

3. **Gradient separation**
   Continue with gradient separation according to the G-series manual.

   - SpermGrad™
   - G-IVF PLUS
   - SpermGrad RTU

4. **Store in N₂(l)**
   Transfer the straws quickly into liquid nitrogen and store at -196 °C.

Note: Freezing program for machine:

- Start temperature: +20°C
- -5°C/min to -8°C
- Hold 1 min
- -10°C/min to -25°C
- -25°C/min to -150°C
- -196°C

*Optional, this step can be performed using a slow-freeze machine programmed for sperm freezing.*
Sperm freezing when using cryovials

**Freezing**

1. **Assess the semen sample**
   - Ensure that both liquefied semen and SpermFreeze Solution are at ambient temp.
   - Measure the total volume of semen and carry out semen analysis as required.

2. **Add SpermFreeze Solution**
   - Dilute with equal volume of semen and SpermFreeze Solution. Add SpermFreeze Solution slowly and dropwise to the semen and then carefully tilt after each drop added. Close the lid tightly and turn the tube upside down 20 times, being careful not to create bubbles.

3. **Leave in room temperature**
   - > 10 min

4. **Load vials**
   - Mark cryovials with patient ID and load the semen mixture into cryovials. Do not fill cryovials completely to allow for expansion.

5. **Freezing**
   - Place the cryovials upright on a 1-3 cm styrofoam board in a liquid nitrogen bath. Leave for 30 min.

6. **Store in N₂(l)**
   - Transfer the cryovials quickly into the liquid nitrogen and store at -196 °C.

**Thawing**

The day before thawing:

1. **Remove cryovials from N₂(l)**
   - Remove cryovials from -196 °C and place them in a water bath at 35 ± 2 °C for 10 min.
   - Wipe the cryovials dry with a clean paper towel.

2. **Dilute with G-IVF PLUS**
   - Transfer semen mixture to clean test tubes and dilute with equal amount of equilibrated G-IVF PLUS. G-IVF PLUS should be added dropwise to the semen mixture and the solution carefully mixed after each addition.

3. **Gradient separation**
   - Continue with gradient separation according to the G-series manual.

4. **Store in N₂(l)**
   - Transfer the cryovials quickly into the liquid nitrogen and store at -196 °C.

*Freezing program for machine:

- Start temperature: +20°C
  1. -8°C/min to -8°C
  2. Hold 1 min
  3. -10°C/min to -25°C
  4. -25°C/min to -150°C