**Spheroid Culture**

**Materials**

**Reagents**

Complete media

Sterile PBS

Trypsin

Ice

Poly-NIPAM gel

Trypan Blue

**Plastics/ glassware**

24 well plate

15 mL tube (2)

1.5 mL Eppendorf tube

P-1000 positive displacement pipette

Large beaker

Cell counting slide

**Procedure**

1. Grow cells in 2D culture until confluent
2. Aspirate media from flask and rinse cells twice with sterile PBS
3. Add Trypsin to flask, gently agitating cells to ensure contact is made with Trypsin
4. Place flask in incubator for 5 minutes, or until cells are sufficiently removed from plate; however, do not exceed 20 minutes, as Trypsin is cytotoxic
5. After Trypsinization, add media to Trypsin in a 2:1 ratio
6. Transfer cells suspended in the Trypsin/ media solution to a 15 mL tube and spin in centrifuge for 5 minutes at 200 g
7. During this time, label and place a new 24 well plate into the incubator
8. Add ice to a beaker and place Poly-NIPAM gel flask and a 15 mL tube on ice
9. Aspirate Trypsin/ media solution from 15 mL tube and resuspend cells in 3 mLs media
10. Using a 1.5 mL tube, add 20 uLs of cell suspension
11. Subsequently add 20 uLs of Trypan blue and resuspend to mix with cells thoroughly
12. Add 20 uLs of this solution to a cell counting slide and calculate live cell population
13. Calculate number of cells needed to achieve 5,000 cells per well in a 600 uL volume of Poly-NIPAM gel
14. Remove 24 well plate from incubator
15. Aliquot 600 uL of Poly-NIPAM to the chilled 15 mL tube using a positive displacement pipette
16. Add appropriate amount of cells to gel and stir with tip (do not resuspend with this tip as the gel is very viscous)
17. Add 140 uLs of cell/ Poly-NIPAM suspension to one well in the 24 well plate using positive displacement pipette
18. Hold pipette as vertical as possible, aiming to place the gel in the center of the well; try not to add any bubbles to the gel by pipetting at a steady pace both when taking the suspension from the 15 mL tube and when placing the gel in the well
19. Once all the gels have been pipetted into their wells, place plate in incubator for 10 minutes
20. When ready, add 800 uLs of media to each well and return to incubator to allow spheroid formation