Toll Free: 866-702-0260
Phone: 203-433-4329
Fax: 203-208-0664
www.phoenixsongsbio.com

Human FL-HCC Cancer Stem Cell Spheroids Growth Methods

Cryopreserved or fresh human FL-HCC Cancer Stem Cell Spheroids require special handling to ensure optimal viability and growth of fresh spheroids or following the freeze/thaw process. Please follow these methods closely to ensure the best performance of the spheroids

I. Required Equipment

1. All procedures should be conducted inside a biological safety cabinet such as a NuAire Class II Type A2.
2. FL-HCC Cancer Stem Cell Spheroids can be maintained with an antibiotic if required in order to prevent bacterial contamination. Our cells have been tested with gentamycin (30μg/ml).
3. Incubator should be controlled for humidity, temperature and CO_2 concentration such as a NuAire 4950 with the following settings
    a. 6% CO_2
    b. Normal O_2
    c. 95% humidity
    d. 37°C

    NOTE: It is best to fyrite incubators regularly to ensure appropriate gas concentration is maintained.

II. Required Supplies

1. Cryopreserved human FL-HCC Cancer Stem Cell Spheroids (Catalog #: 35002-101)
2. Kubota's FL-HCC Spheroid Growth Media (catalog #: 35101-250)
3. FL-HCC Spheroid Dissociation Media (catalog 35301-030)
4. Spheroid Wash Buffer (catalog 35301-500)
5. FL-HCC Spheroid Freezing Media (catalog # 35201-050)
6. Petri dishes or flasks
    a. Falcon/Corning petri dishes
        i. 35 x 10mm petri dish 351008
        ii. 60 x 15mm petri dish 351007
        iii. 100 x 15mm petri dish 351029
        iv. 15 x 150mm petri dish 351058
    b. Nunc petri Dishes
        i. 35 x 10mm petri dish 171099
        ii. 60 x 15mm petri dish174888
        iii. 100 x 20 mm petri dish 150679
        iv. 150 x 25mm petri dish 168381
    c. Falcon/Corning Petri Flasks BD 343133
    d. CELLSTAR© Suspension Culture Flasks
        i. T25 suspension filter cap flask 690-195
        ii. T75 suspension filter cap flask 658-195
        iii. T175 suspension filter cap flask 661-195
7. 70% ethanol
8. Chlorine Bleach

III. Safety Working with Human Cancer Cells

1. Assume that all cells isolated from human tissue are potentially hazardous even though they test negative for human pathogens
2. Use care in handling human cancer cells by preparing a 10% bleach solution and dip pipettes, pipette tips and plasticware into the bleach solution before placing in biohazard bag.
3. Always wear personal protective equipment
    a. Wear appropriate lab coat, gloves and safety glasses
4. All operations should be conducted using aseptic technique within a biosafety cabinet

IV. Receiving Cryopreserved Human FL-HCC Spheroids

1. Cryopreserved human FL-HCC spheroids will be shipped on dry ice.
    a. Upon receipt of the package, immediately transfer the cryopreserved vials into your liquid nitrogen freezer and store until ready for use
    b. For optimal performance of the cells, do not allow vials to warm where frost forms.

V. Thawing Cryopreserved Human FL-HCC spheroids

1 . The day prior to thawing FL-HCC spheroids remove the Kubota's FL-HCC Spheroid Growth Media from the -80°C and place in the 4°C refrigerator to allow media to slowly thaw.
3. Transfer 10ml Kubota's FL-HCC Spheroid Growth Media into 15ml conical tube.
4 . To thaw the spheroids, remove the vial of cells from the LN_2 freezer and take immediately to the water-bath. If the freezer is not in close proximity to the water-bath, transport the vial of cells in dry ice.
5. Holding onto the cap, submerge the vial into the 37°C waterbath continuously moving the vial in the water to ensure the water surrounding the vial remains warm.
6. Every 5-10 seconds remove the vial from the water and gently turn upside down to see if the frozen spheroid suspension has thawed. When the spheroid suspension becomes a slurry take to the biosafety cabinet
7. Douse the vial with 70% ethanol, dry and place inside the biosafety cabinet.
8. Open the vial then using a 5ml pipette aspirate 3ML media into the pipette then gently remove the spheroid suspension from the vial.
9. Transfer the spheroid suspension into the conical tube containing the Kubota's FL-HCC Spheroid Growth Media. Make sure to submerge the tip of the pipette below the surface of the media and gently express the spheroid suspension into the media.
10. Wash the vial with 1ml Kubota's FL-HCC Spheroid Growth Media and add to the conical tube then place the tube in the incubator with the cap loosened to allow the spheroids to settle to the bottom of the conical. This may take up to 2 hours.
11. Using a 10ml pipette remove the supernatant that contains unsettled cells leaving 1 ml above the spheroids taking care not to disturb the spheroids at the bottom of the conical and transfer to another 15ml tube.
12. Pellet the unsettled loose cells at 1500 RPM for 5 minutes
13. Add 10ml Kubota's FL-HCC Spheroid Growth Media gently to the tube containing the settled spheroids and resuspend the spheroids by gentle pipetting
14. Transfer the human FL-HCC spheroids into a petri dish/flask and place in the incubator.

15. Resuspend the pelleted unsettled cells in 10ml Kubota's FL-HCC Spheroid Growth Media and plate into a separate petri dish/flask, label unsettled and place in the incubator. The unsettled cells may form new spheroids.

VI. Feeding Human FL-HCC Spheroids and Unsettled Cells

1. Human FL-HCC spheroids should be fed twice a week for optimal growth.
2. Warm Kubota's FL-HCC Spheroid Growth Media to at least room temperature. If warming in water-bath, don't leave in water-bath longer than 5 minutes, as glutamine, proteins and growth factors easily degrade when left in water bath for extended periods of time. Douse with 70% ethanol and place in biosafety cabinet.
3. Remove the dishes/flasks from the incubator and transfer to the biosafety cabinet.
4. Using a pipette, remove the spheroid suspensions and transfer into conical tube. (Treat the cells in the unsettled petri dish/flask as spheroids so you collect the new spheroids)
5. Spheroid suspension from multiple dishes/flasks can be pooled into 50ml conical tubes. Once spheroids are collected transfer into incubator to allow spheroids to settle for up to 2 hours. Make sure the caps are loose to allow for gas exchange so the media can be equilibrated
6. Add half the media volume to each plate and return to the incubator (There may be some cells/spheroids left in the dish/flask)
7. Once spheroids have settled remove the media above the spheroids leaving 0.5-1ml at the bottom of the tube. Transfer the unsettled suspension into another conical tube. You may pool the unsettled suspension from multiple dishes/flasks.
8. Pellet the unsettled suspension by centrifugation at 1500 RPM for 5 minutes
9. Resuspend the spheroids in fresh Kubota's FL-HCC Spheroid Growth Media and return to petri dishes/flasks
10. After unsettled suspension has pelleted, remove the media and resuspend unsettled cells in fresh Kubota's FL-HCC Spheroid Growth Media and transfer into petri dishes/flasks labeled unsettled cells and transfer into the incubator.
11. At each feeding take note of the size of the settled spheroids and increase the number of dishes/flasks as needed.
12. When spheroids are larger than 200um, they will need to be dissociated to prevent the center of the spheroid from becoming necrotic.

VII. Dissociating Spheroids

1. Transfer the spheroid suspension from the dish/flask into a 50ml conical tube.
2. Wash the dish/flask with Wash Buffer and add to the tube containing the spheroid suspension.
3. Pellet by centrifugation at 1500 RPM for 5 minutes.
4. Remove supernatant taking care not to disturb the pellet.
5. Resuspend the pellet of spheroids in Wash Buffer. This step is to remove the hyaluronans containing media from the spheroids
6. Pellet by centrifugation at 1500 RPM for 5 minutes.
7. Resuspend the spheroid pellet in Dissociation Media and transfer the tube with the cap loose into the incubator and incubate 3 hours.
8. During the 3 hour incubation, examine the spheroid suspension every 30 minutes to see if the spheroids have broken apart. You may pipette up and down vigorously to mechanically dissociate the spheroids each time.

9. Continue this process until you can see with the naked eye that the spheroids seem smaller.
10. Once the spheroids appear to have disassembled, take a 20µ aliquot and drop it into the center of a fresh petri dish and take to the microscope to examine. (single cell suspension is not desirable because it will result in loss of cells).
11. When dissociation results in small (2-5 cell aggregates) add an equal volume of wash buffer to dilute the dissociation buffer.
12. Vigorously pipet up and down so you have a homogeneous cell suspension to ensure an accurate cell count.
    a. Take 20ul cell suspension using a wide bore pipette tip and add it to 20ul Trypan Blue. This provides a dilution factor of 2.
    b. Pipette up and down
    c. Take 10ul and add to the counting chamber of a hemacytometer
    d. Count the cells within the center grid
    e. Calculate the total cell count

       Cell count X 10,000 X 2 X Volume = Total cell count

       10,000 is a constant for the surface area of the counting chamber

       2 is the dilution factor

       Volume is the volume of the cell suspension being counted

    f. Determine the number of cells that can be plated for differentiation (refer to Table 2)
13. Pellet the cells by centrifugation at 1500 RPM for 5 minutes.
14. Determine the number of petri dishes/flasks you will for expansion using Table 1 as a guideline or determine the number of plates you will need for your assay (number of cells/multi-well plate x number of plates needed in the experiment using Table 2).
15. For Expansion, resuspend the pellet in Growth Media and return to petri dishes/flasks.
16. For assays refer to section VII.

VII. Plating the Cells for Assays

1. When the centrifuge stops remove the tube for the experiment, take it to the biosafety cabinet and aspirate the supernatant taking care not to disturb the cell pellet.
2. Resuspend the cell pellet in enough complete assay medium to plate the multi-well plates (use table 2 for plating density and volume)
3. FL-HCC cells will not remain attached and will reform spheroids so it is best to use multi-well insert systems for your assays so you can sample the media or replace the media during feeds.
    a. Falcon 24-well insert system Catalog # 351181 or 351183
    b. Falcon 96-well insert system Catalog # 351130
4. Deliver the cell suspension directly into the insert in the well.
5. Carefully transfer the plates to the humidified incubator set at 6% CO_2 and 37°C.
6. Feeding the Assay plates remove the media from the well though the port at the edge of the insert and replace with fresh Spheroid Growth Media. Feed at least twice per week.

VII. Cryopreserving Human FL-HCC Spheroids and Unsettled Cells

1. The day before cryopreserving place Spheroid Freezing Media in refrigerator to thaw.
2. When ready to cryopreserve the spheroids, remove the dishes/flasks from the incubator and transfer to the safety cabinet.
3. Using a pipette, remove the spheroid suspensions and transfer into conical tube. (Treat the cells in the unsettled dish/flask as spheroids so you collect the new spheroids)
4. Spheroid suspension from multiple dishes/flasks can be pooled into 50ml conical tubes.
5. Rinse each plate with media and add to the tube of spheroids.
6. Ensure there is a homogenous suspension then take an aliquot for counting.
7. Follow counting steps above in section V. 14-16.
8. Transfer tubes of spheroids into incubator to allow spheroids to settle for up to 2 hours.
9. Once spheroids have settled remove the media above the spheroids leaving 0.5-1ml at the bottom of the tube.
10. Transfer the unsettled suspension into another conical tube. You may pool the unsettled suspension from multiple dishes/flasks.
11. Pellet the unsettled suspension at 1500 RPM for 5 minutes.
12. Remove the supernatant taking care not to disturb the pellet.
13. Resuspend the pellet in enough Spheroid Freezing Media to freeze 1-2 million cells per vial.
14. Transfer the unsettled cells in freezing media into the tube of spheroids and gently resuspend into homogenous suspension
15. Transfer 1ml cryo spheroid suspension per tube to freeze.
16. Slow freeze in Styrofoam container in -80° ultralow freezer or in stepdown freezer
17. Store in liquid nitrogen freezer for long term storage.

Table 1. Plating densities for human FL-HCC cancer stem cells for spheroids formation

Petri Dish/Flask Size	Growth Area (cm^2)	Plating Volume (ml)	Maximum Volume (ml)	FL-HCC Spheroids/well
T25 Petri Flask	25	10	50	$3.0x\ 10^5$
T75 Petri Flask	75	30	250	1.0×10^6
T175 Petri Flask	175	60	550	2.0×10^6
35mm Petri Dish	9	3	5	1.0×10^5
60mm Petri Dish	21	6	10	2.0×10^5
100mm Petri Dish	58	10	12	7.0×10^5
150 mm Petri Dish	145	30	40	1.6×10^6
6-Well	9.6	2	3	1.0×10^5
24-Well	2.0	1	1.5	2.0×10^4
96-Well	.32	0.1	.2	4.0×10^3

Table 2. Plating density of human cancer stem cells on collagen type III coated plates for assays.

Dish/MEA Size	Growth Area (cm^2)	Working Volume (ml)	Maximum Volume (ml)	NSCs /well	NSCs/Plate
100mm	58.1	10	5	6.74×10^6	6.74×10^6
60mm	21.3	6	3	2.47×10^6	2.47×10^6
35mm	9.6	2	1	1.11×10^6	1.11×10^6
6-Well	9.6	2	1	1.11×10^6	6.66×10^6
12-Well	3.8	1.5	0.75	4.41×10^5	5.29×10^6
4-Well	2.0	0.5	0.25	2.32×10^5	9.28×10^5
24-Well	2.0	1.0	0.5	2.32×10^5	5.57×10^6
48-Well	1.1	0.5	0.25	1.28×10^5	6.10×10^6
96-Well	.32	0.1	0.05	3.70×10^4	3.55×10^6
384-well	.086	0.05	0.025	1.00×10^4	3.84×10^6

