User's Manual

Note, the following user's manual was developed using MCF-7 cell line grown in cell growth media which consisted of DMEM/F12 + Glutamax ™ basal media supplemented with 5%v/v fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). Adjustments may be required depending on the cell-line you are using.

SAFETY NOTICE

The provided 3DCellMaker material is non-toxic and not derived from animal or human origin. It is critical to remember, however, that the cell-lines and reagents you may be working with could potentially require Bio Safety Level (BSL) protocols and equipment due to their pathological or chemical dangers. FOLLOW all BSL requirements established by your institution for the work process you are doing. 3DCellMaker is not designed to improve the safety of your process and it is the customer responsibility to maintain safety of their cell-growth activities at all times especially when handling potentially hazardous cell lines (e.g. primate-derived cultures) or known pathological/toxic materials. Dispose of contaminated items/liquids in accordance with your institutions policy and relevant BSL requirements.

DISCLAIMER

The following equipment and reagents have been utilized at Akina for the development of this user's manual. Inclusion of these items here does not constitute an endorsement of the equipment or particular manufacturers but simply indicates the materials used in development of this manual. Note that 3DCellMaker may be utilized with other equipment and reagents and is not specific to any particular make or manufacturer.

Equipment

1. Serological pipettes (Falcon 5mL ref no. 357551, 10mL ref no. 357543, 25mL ref no. 357535)
2. Autopipettor *(Drummond Pipet-Aid)*
3. Mechanical Pipettes (Eppendorf Research Plus 1000uL, Eppendorf Research 20uL)
4. Culture Flasks (Corning, 75 cm^2)
5. Laminar Flow Hood (Esco class II type A2 biological safety cabinet)
6. 5% CO2 incubator (VWR Symphony 3405 air jacketed incubator)
7. Microscope (Amscope IN300t-FL phase contrast microscope)
8. Bright-Line™ Hemacytometer
9. Hot (37 °C) Water Bath (Thermo Scientific Model 2870)
10. Centrifuge (Eppendorf Model 5702R)
11. Well Plates (flat bottom polystyrene multi-well tissue culture plates with low evaporation lid)

Reagents

1. DMEM/F12 + Glutamax TM basal media (Invitrogen, catalog no. 10565-018)
2. Fetal bovine serum (Invitrogen, catalog no. 16000-044)
3. Penicillin/Streptomycin (Invitrogen, catalog no. 15140-122)
4. Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen, catalog no. 14040-182)
5. Trypsin-EDTA (Invitrogen, catalog no. 25300-062)
6. MCF-7 Cells (ATCC catalog no. HTB-22)
7. Ethanol (or other appropriate sanitizer)

Aseptic Preparation/Techniques

Perform all work inside a laminar flow hood. Prior to initiating work, spray and wipe down all surfaces, gloves, and equipment with 70% ethanol or other sanitizer. Note the contents of the provided 3DCellMaker bag have been sterilized by ethylene oxide however the external portion of the bag is not sterile due to handling. Prior to opening it is suggested to spray and wipe down the external bag with 70% ethanol or other sanitizer. Additionally, if desired, the bag and its contents may be exposed to UV radiation inside the laminar flow hood as part of sterilization procedure. The polymer is minimally UV sensitive and as such this process will not affect its performance when exposed to UV for periods of no more than 1 hour. If outer bag is damaged or surface of vial is contaminated by the end user it can also be sprayed with 70% ethanol. Follow typical aseptic techniques including use of gloves, sterile serological pipettes, not touching wetted surfaces, and using wetted components only once.

Media Preparation

Prepare cell-culture media per typical protocol for the cell line you are working with. For MCF-7 growth at Akina this was done utilizing DMEM/F12 + Glutamax ™ (500ml) to which 25ml of Fetal Bovine Serum and 5 ml of Penicillin/Streptomycin is added.

1. Carefully open bag and interior vial of 3DCell Maker

2. Pipette into interior vial either 5ml or 10ml of cell-growth media depending on volume of kit purchased.

3. Seal container and close bag around vial.

4. Transfer to refrigerator or cold (not frozen) storage. Note storage of solution at -20C or in liquid nitrogen (vapor or liquid) is not suggested. Optimal storage is at 4-6 °C.

5. Leave solution in refrigerator overnight. Periodically remove and swirl vial by hand to aid dissolution. Avoid vigorous mixing as this can cause foaming. Once fully dissolved use solution within 4 weeks for best results.

Note: A3DH and A3DC can be stored for up to 2 years prior to dissolving in media. A3DC can be stored at ambient conditions but A3DH should be stored at -20°C

Cell Preparation

Prepare cells per typical protocol for the cell line you are working with. For MCF-7 preparation the following protocol was utilized.

1. Culture cells in 75 cm^2 flasks until ~70-90% confluence is reached

2. Remove media and wash twice with 5 mL of DPBS

3. Rinse flask with 1 mL of 0.025% Trypsin in EDTA and remove, then add 2 mL of 0.025% Trypsin in EDTA and incubate 3-5 min at 37 ^{o}C/5% CO_2 to detach cells from the surface of the flask.

4. Add 8 mL of fresh media to neutralize trypsin and re-suspend cells. Rinse surface of container with this media using pipette to help dislodge cells.

5. Transfer cell suspension to 15ml conical bottom tube and centrifuge at 200g for 3 minutes to obtain cell pellet.

6. Re-suspend cell pellet in fresh media. Note cells may need diluted to reach appropriate loading concentration. See details below for loading concentrations.

Cell Loading

There are two primary techniques for cell loading which differ by product. There is "hot loading" technique in which the hydrogel is already pre-warmed and solidified prior to cell addition and there is "cold loading" technique in which the thermogel is a cold liquid prior to cell addition. Both techniques are detailed below.

Hot Loading

1. Pipette hydrogel solution into a sterile flat bottom multi-well culture plate. The volume added should be sufficient to coat the bottom of the plate. For example, for a 12 well plate pipette in 1.0 ml

2. Place plate in 37°C incubator for 90 minutes to solidify the thermogel to a solid.

3. On top of gel gently add cell suspension. The following was developed for growth of MCF-7 cells on top of A3DH gel.

Growth on 1.0 ml of solidified A3DH gel	Volume of Cell Suspension Added (ul)				
Concentration of Cell Suspension (cells/ml)	10	50	100	200	500
100,000	Too few cells	Too few cells	Too few cells	Too few cells	Too much dilution
250,000	Too few cells	Too few cells	Too few cells	Good growth	Too much dilution
500,000	Too few cells	Too few cells	Good growth	Optimal	Too much dilution
1,000,000	Too few cells	Good growth	Optimal	Optimal	Too much dilution
2,000,000	Too few cells	Optimal	Optimal	Good growth	Too much dilution
5,000,000	Good growth	Optimal	Good growth	Good growth	Too much dilution

4. After seeding, cover and return to 5% CO_2 incubator.

Cold Loading

1. Pipette hydrogel solution into a sterile flat bottom multi-well culture plate. The volume added should be sufficient to coat the bottom of the plate. For example, for a 12 well plate pipette in 1.0 ml

2. On top of gel gently add cell suspension. The following was developed for growth of MCF-7 cells on top of A3DC gel.

Growth on 1.0 ml of solidified A3DC gel	Volume of Cell Suspension Added (ul)				
Concentration of Cell Suspension (cells/ml)	10	50	100	200	500
100,000	Too few cells	Too few cells	Too few cells	Too few cells	Too few cells
250,000	Too few cells	Too few cells	Too few cells	Too few cells	Good growth
500,000	Too few cells	Too few cells	Too few cells	Good growth	Optimal
1,000,000	Too few cells	Too few cells	Good growth	Optimal	Good growth
2,000,000	Too few cells	Good growth	Optimal	Good growth	Too many cells
5,000,000	Too few cells	Optimal	Good growth	Too many cells	Too many cells

3. After seeding, the solution may be transferred or pipetted into other sterile containers

4. Transfer solutions in to 37°C, 5%CO2 incubator to solidify gel and grow cells

Note: Resuspension of cell-pellet directly into thermogel solution is not recommended at this time.

Maintenance

1. Cells will grow into spheroids over the course of approximately 1-2 days for hot seeding and 4-5 days for cold seeding.

2. While growing, maintain media at regular intervals by replacing 0.5 ml of media with fresh media (for 12-well plate, scale appropriately for other containers). Make sure to pipette carefully from the top so as to not disturb the gel layer. Initial media change will only be an addition of fresh media after the first day of incubation.

3. Replace media at regular intervals per cell maintenance cycle or as indicated by drop in pH. Generally every 1 to 2 days.

Spheroid harvesting

Spheroids may be utilized in the gel or liquefied and harvested. Note that the gels will break up and re-dissolve naturally if not harvested after 7-14 days depending on the frequency which media is changed.

1. After growth of spheroids, bring plate into sterile hood and allow to cool to room temperature.

2. Remove as much old media as possible without removing spheroids. When spheroids reach adequate size they will be visible to the naked eye. Add small amount of fresh, media to each well. Shake/pipette to distribute spheroids in media.

3. Pipette out spheroid solution and apply to desired test as a representative, physiologically relevant 3D tumor spheroid for drug screening and or other applications.