Sub-Cultivating Cells in 2D

Protocol

To prepare a cell culture for growth in the ClinoStar®, the cells are initially cultured in a traditional monolayer system (2D). This protocol describes the sub-culture of the HEPG2-C3A (C3A (ATCC® CRL-10741™)) immortal cell line in 2D. The protocol steps and reagents should be individually optimized for other types of cells and application of choice.

Reagents and Materials

- Cell culture media (DMEM, low glucose, with pyruvate, no glutamine, no phenol red) (e.g. Gibco 11880-DMEM)
- Dimethyl sulfoxide (DMSO), sterile filtered
- 70 % Ethanol solution
- Foetal bovine serum (FBS)
- Hanks´ Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}
- MEM Non-Essential Amino Acids Solution
- Penicillin-Streptomycin [10 000 U /mL]
- Stable glutamine supplement (e.g., GlutaMax™)
- 0.4% Trypan Blue solution
- Trypsin-EDTA (0.5 %)
- Isopropanol

Additional information

It is immensely important to maintain a sterile environment. Flowcharts are available below.

Protocol

Preparation of growth media

1. Thaw the components for the media using a 37°C water bath or according to manufactures protocol.
2. Wipe the reagents and containers with 70 % Ethanol solution and move them to the laminar flow bench.
3. Mix all the medium components in a suitable sterile bottle starting with addition of the basal medium according to ***Table 1***
4. Mix well by pipetting up and down many times and then aliquot the supplemented medium into sterile 50 mL conical centrifuge tubes.
5. Label the tubes with the right medium name, date, and initials.
6. Place the medium in a fridge at 4°C (use the media within 14 days).

Table 1: Cell culture media composition for HEPG2-C3A cell line

DMEM medium mL	***100***	***200***	***400***	***500***
87.5 % DMEM basal medium mL	87.5	175	350	437.5
1 % Non-Essential Amino Acids mL	1	2	4	5
10 % Foetal Calf Serum mL	10	20	40	50
0.5 % Penicillin-Streptomycin mL	0.5	1	2	2.5
1 % Stable Glutamine mL	1	2	4	5

Thawing cells

1. Preheat the cell culture medium to 37°C by placing 50 mL aliquots for at least 15 min in a water bath.
2. Transfer the cryovial with cells from the cryogenic storage into an appropriate container filled with dry ice.
3. Loosen the lid on the cryovial momentary to relieve overpressure.
4. Place the closed tube in the water bath and thaw the frozen pellet AS QUICKLY AS POSSIBLE. Gently agitate the tube to speed up the process but avoid shaking that will disturb the cells.
5. Wipe the tube carefully with a tissue soaked in 70 % Ethanol solution.
6. Prepare a sample for cell viability testing by mixing the cell suspension gently and transferring 20 µL into a 1.5 mL sterile micro test tube. Place it in the incubator until testing.
7. Transfer the remaining cell suspension/freezing medium to the already prepared 15 mL conical centrifuge tube.
8. Add 9 mL of the preheated cell culture medium to the 15 mL conical centrifuge tube.
9. Centrifuge the cells at 140 x g for 5 min at room temperature.
10. Add the cell culture media to a cell culture flask (e.g.: 4 mL / 9 mL per T25 / T75 flask).
11. Remove the supernatant from 15 mL conical centrifuge tube.
12. Resuspend the cell pellet in 1 mL of the cell culture medium.
13. Transfer the cell suspension to a culture flask pre-filled with the cell culture medium and place it in the incubator.
14. Change the medium the following day to remove any remaining cryoprotective additive and not viable cells from culture.

Evaluation of cell culture viability via haemocytometer

1. Collect the sample for cell viability testing from the incubator.
2. Add 30 µL of DMEM and 50 µL of trypan blue to the micro test tube containing 20 µL of the cell suspension.
3. Incubate for 3 min and then pipette 10 µL of the cell suspension to a haemocytometer cell counting chamber.
4. Count total number of cells and then count only the dead cells (stained in blue).
5. Calculate the viability: (live cells / total cells) * 100 % = % of viable cells.

Sub-culture of 2D cell culture

1. Preheat the needed reagents (media etc.) to 37°C.
2. The trypsin solution should be defrosted at room temperature to avoid the gradual autodigestion that occurs at 37°C.
3. Ensure the laminar flow cabinet is switched on and cleaned according to manufactures guidelines e.g. with 70 % Ethanol solution.
4. Collect the cells from the incubator and perform a visual inspection of cells grown in cell culture flasks using the optical microscope. Select a culture flask that is 70-90% confluent.
5. Tilt the flask and remove the medium.
6. Carefully add a suitable amount of HBSS without Ca^{2+} and Mg^{2+} (see **Table 2**) in the corner of the flask and carefully wash all sides of the flask (floor, walls, and ceiling) by gently swirling and rotation of the flask.
7. Remove HBSS from the flask and repeat the washing step.
8. Remove HBSS and carefully add the suitable amount of trypsin (see **Table 2**) to the flask by dripping directly on the cell monolayer.
9. Close the flask and incubate in the sterile hood for 3 to 4 min.

10. Detach cells from the bottom surface of the flask by gently tapping the flask against the hand.
11. When cells are fully detached add the suitable amount of FBS (see **Table 2**) to the flask to stop the enzymatic activity of trypsin (We recommend adding FBS instead of growth media as this facilitates single cell suspension).
12. Transfer the cell suspension to a sterile 15 mL conical centrifuge tube.
13. If needed wash the flask with the suitable amount of medium (see **Table 2**) and transfer it to the tube.
14. Centrifuge the cells at 140 x g for 5 min at room temperature.
15. Remove the supernatant and resuspend the cell pellet in a suitable amount of medium (see **Table 2**).
16. Prepare new cell culture flasks by filling them with an appropriate amount of fresh medium (see **Table 2**).
17. Mix the cell suspension well and transfer the appropriate number of cells into the new flasks. NOTE: For sub-culturing C3A cells every 4th day, the recommended seeding density is equal to 0.3 x 10^5 cells / cm^2.
18. Gently swirl the flasks back and forward, and left-right a couple of times to ensure equal redistribution of cells within the cell growth area (avoid circular movements).
19. Place the new culture flasks in the incubator.

Table 2: Cell culture flask size and reagent volume

Flask type	*HBSS mL*	*0,05 % trypsin mL*	*FCS mL*	*Medium wash mL*	*Final volume mL*
T25	2 x 5	3	1	5	9
T75	2 x 15	5	3	5	13
T150	2 x 30	10	5	10	25
T175	2 x 35	15	10	10	35

Preparation of freezing media for cryopreservation of cells

1. Prepare an appropriate amount of freezing medium by adding DMSO to the cell culture medium at 5 % (v/v) final concentration (see **Table 3**).
2. Aliquot the supplemented medium into sterile falcon 15 mL and/or 50 mL conical centrifuge tubes.
3. Label the tube(s) with the freezing medium name, date, and initials.
4. Place the freezing medium in a fridge at 4°C. Do not store for more than 14 days.

Table 3: Freezing medium composition

Freezing medium (mL)	*10*	*20*	*50*	*100*
95 % Cell Culture Medium mL	9.5	19	47.5	95
5 % Dimethyl Sulfoxide mL	0.5	1	2.5	5

Preparation of cell culture stocks for storage in liquid nitrogen

1. Prepare appropriate amount of freezing medium and bring it to 4°C before use.
2. Sub-cultivate the cells in 2D according to laboratory specifications and cell line.
3. Resuspend cell pellet in COLD (4°C) freezing medium.
4. Evaluate the cell count and viability using either a haemocytometer or an automated cell counter.
5. Add additional freezing medium to the cell suspension so that its final cell concentration is either $3x10^6$ or $6x10^6$ cells / mL.
6. Aliquot cell suspension into cryotubes by adding 1 mL of the suspension to each tube.
7. Write the ampoule number, cell-line name, cell concentration and date on each tube.
8. Place the cryotubes in the freezing container filled with isopropanol.
9. Place the container in the - 80°C freezer.
10. The following day move the cells to a liquid nitrogen tank.

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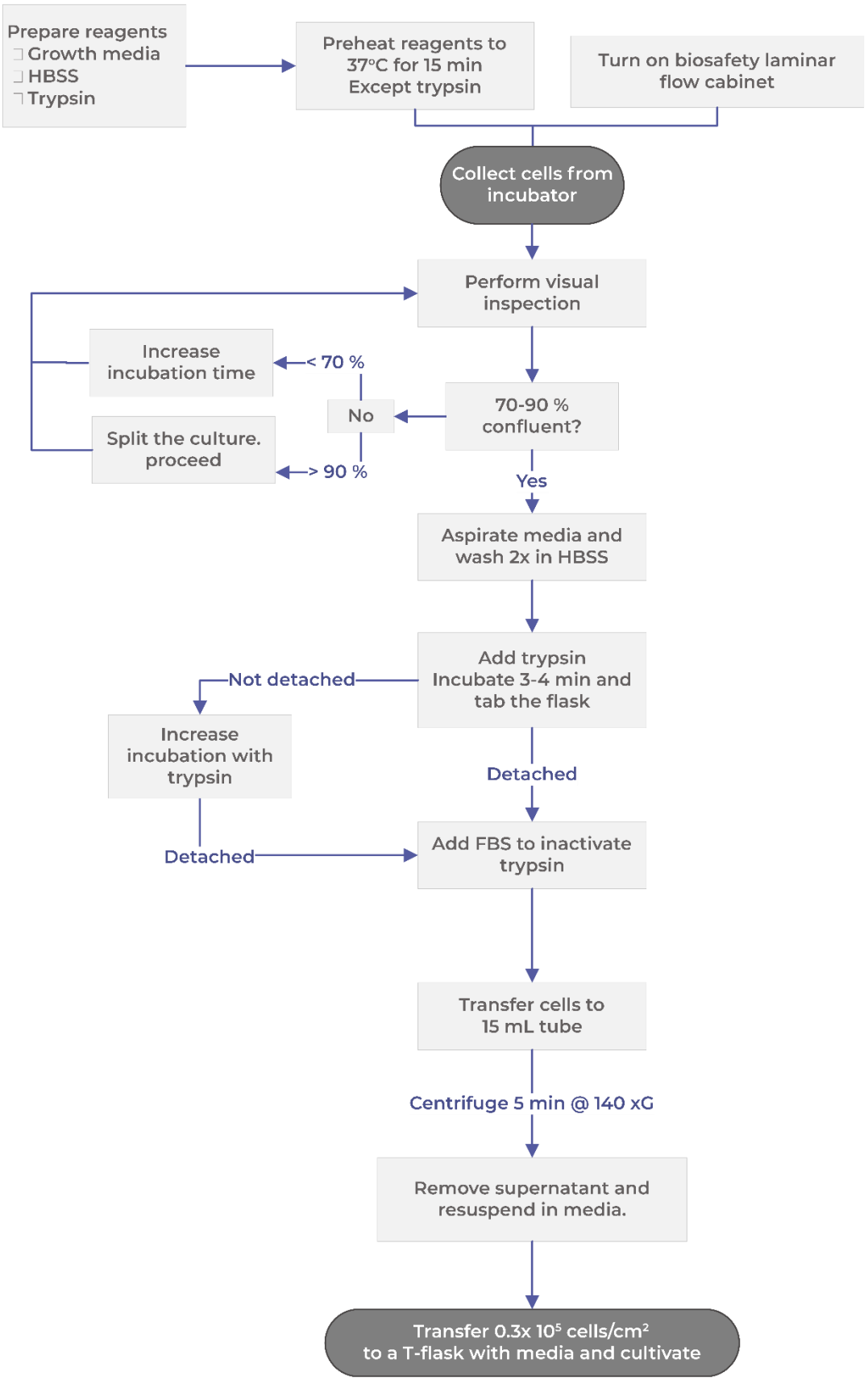
Prepare reagents
Growth media
HBSS
Trypsin
Preheat reagents to 37°C for 15 min Except trypsin
Turn on biosafety laminar flow cabinet
Collect cells from incubator
Perform visual inspection
70-90 % confluent?
No
< 70 %
Increase incubation time
> 90 %
Split the culture. proceed
Yes
Aspirate media and wash 2x in HBSS
Add trypsin Incubate 3-4 min and tab the flask
Not detached
Increase incubation with trypsin
Detached
Detached
Add FBS to inactivate trypsin
Transfer cells to 15 mL tube
Centrifuge 5 min @ 140 xG
Remove supernatant and resuspend in media.
Transfer 0.3x 10⁵ cells/cm² to a T-flask with media and cultivate