Cell Culture

Cell culture

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been already established.

Specimen

Type: Cell line/cell strain

Materials:

1. Cell line
2. Media (RPMI culture media, DMEM culture media)
3. Fetal Bovine Serum (FBS)
4. 2 mM glutamine and antibiotics
5. General example using DMEM media:

Equipment and supplies:

1. Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)
2. Incubator (humid CO2 incubator recommended)
3. Water bath
4. Centrifuge
5. Refrigerator and freezer (–20°C, -80°C)
6. Cell counter (e.g., Countess® Automated Cell Counter or hemocytometer)
7. Inverted microscope
8. Liquid nitrogen (N2) freezer or cryostorage container
9. Sterilizer (i.e., autoclave
10. Aspiration pump (peristaltic or vacuum)
11. pH meter
12. Confocal microscope
13. Flow cytometer
14. Cell culture vessels (e.g., flasks, Petri dishes, roller bottles, multi-well plates)
15. Pipettes and pipettors
16. Syringes and needles
17. Waste containers

Safety (Warning and Biohazard consideration):

In addition to the safety risks common to most everyday work places such as electrical and fire hazards, a cell culture laboratory has a number of specific hazards associated with handling and manipulating human or animal cells and tissues, as well as toxic, corrosive, or mutagenic solvents and reagents. The most common of these hazards are accidental punctures with syringe needles or other contaminated sharps, spills and splashes onto skin and mucous membranes, ingestion through mouth pipetting, and inhalation exposures to infectious aerosols .

The fundamental objective of any biosafety program is to reduce or eliminate exposure of laboratory workers and the outside environment to potentially harmful biological agents. The most important element of safety in a cell culture laboratory is the strict adherence to standard microbiological practices and techniques.

Procedure:

1. Preparing an aseptic environment:

1. Hood regulations

(a) Close hood sash to proper position to maintain laminar air flow

(b) Avoid cluttering

2. Autoclaving

(a) Pipette tips (or can be purchases pre-autoclaved, DNase/RNase free)

(b) Glass 9” Pasteur pipettes

(c) 70% ethanol. Be sure to spray all surface areas

All media, supplement and reagents must be sterile to prevent microbial growth in the cell culture. Some reagents and supplements will require filter sterilization if they are not provided sterile.

2. Preparation of cell growth medium:

Before starting work check the information given with the cell line to identify what media type, additives and recommendations should be used.
Most cell lines can be grown using DMEM culture media or RPMI culture media with 10% Fetal Bovine Serum (FBS), 2 mM glutamine and antibiotics can be added if required (see table below) .

Check which culture media and culture supplements the cell line you are using requires before starting

cultures. Culture media and supplements should be sterile. Purchase sterile reagents when possible, only use under aseptic conditions in a culture hood to ensure they remain sterile.
General example using DMEM media:

DMEM Remove 50 ml from 500 ml bottle then add the other constituents	450 ml
10% FBS 50 ml 2 mM glutamine	50 ml
100 U penicillin/ 0.1 mg/ml streptomycin	5ml
2 mM glutamine	5ml

3. Creating the correct culturing environment:

Most cell lines can be grown using DMEM culture media or RPMI culture media with 10% Fetal Bovine Serum (FBS), 2 mM glutamine and antibiotics can be added if required.

Check which culture media and culture supplements the cell line you are using requires before starting cultures. Culture media and supplements should always be sterile. Purchase sterile reagents when possible, only use under aseptic conditions in a culture hood to ensure they remain sterile.
Most cell lines will grow on culture flasks without the need for special matrixes etc. However, some cells, particularly primary cells, will require growth on special matrixes such as collagen to promote cell attachment, differentiation or cell growth. We recommend reviewing the relevant literature for further information on the cells you are culturing .

The following is an example for endothelial and epithelial cells :
For human cells, coat flasks with 1% gelatin. Alternatively, for other cell types such as BAEC, flasks can be coated with 1% fibronectin.

1.Prepare 10mL of coating solution composed of 1% gelatin or 1% fibronectin by diluting with distilled water followed by filtration. This is efficient to coat about 5 flasks.
2. Pipette coating solution into flask. Rock back and forth to evenly distribute the bottom of the flask. Let sit in incubator for 15-30 minutes.
3.Completely remove coating solution by aspirating before seeding.

4. Checking cells:

Cells should be checked microscopically daily to ensure they are healthy and growing as expected. Attached cells should be mainly attached to the bottom of the flask, round and plump or elongated in shape and refracting light around their membrane. Suspension cells should look round and plump and refracting light around their membrane. Some suspension cells may clump. Media should be pinky orange in color .
Discard cells if:
• They are detaching in large numbers and/or look shriveled and grainy/dark in color. • They are in quiescence (do not appear to be growing at all) .

5. Sub-culturing:

Also referred to as cell splitting and cell passaging.

Split ratios or seeding densities can be used to ensure cells are ready for an experiment on a particular day or maintain cell cultures for future use or as a backup. Suspension cell lines are seeded based on volume so

seeding densities will be calculated as cells/mL, whereas adherent cell lines are seeded based on flask surface area so will be calculated as cells/cm2. Cell lines often require specific seeding densities so always check the

guidelines for the cell line in use. Slow growing cells may not grow if a high split ratio is used. Fast growing cells may require a high split ratio to make sure they do not overgrow .

Adherent cell lines can be split using cell line specific split ratios or seeding densities (cells/cm^2) :

1:2 split should be 70-80% confluent and ready for an experiment in 1 to 2 days - 1:5 split should be 70-80% confluent and ready for an experiment in 2 to 4 days - 1:10 split should be 70-80% confluent and ready for sub-culturing or plating in 4 to 6 days .

Split ratios are based on flask surface area, eg:

As a general guide, from a confluent flask of cells :

1:2 split should be 70-80% confluent and ready for an experiment in 1 to 2 days.

1:5 split should be 70-80% confluent and ready for an experiment in 2 to 4 days.

1:10 split should be 70-80% confluent and ready for sub-culturing or plating in 4 to 6 days .

Split ratios are based on flask surface area :

1 x 25 cm^2 flask Split 1:3 would yield 3 x 25 cm^2 flasks or 1 x 75 cm^2

Suspension cell lines should be maintained using cell line specific seeding densities (cells/mL) :

-2e5 should be ready for an experiment in 3-4 days

-1e6 should be ready for an experiment in 1-2 days

If cells are to be left unattended for longer periods (i.e. holiday weekends) it is recommended to use a lower than normal seeding density/split ratio.

6. Adherent subculture protocol (using dissociation reagent) :

When the cells are approximately 80% confluent (80% of the flask surface is covered by cell monolayer), cells should still be in their log phase of growth and will require sub-culturing. It is not recommended to allow cells to become over confluent as this may negatively affect gene expression and cell viability.

1. Remove cell culture media and dissociation reagent from the fridge and place in a 37oC incubator and allow to come to temperate 37oC.

- Do not leave media in the incubator for longer than is necessary as the media components will degrade over time.

2. Switch on and perform a basic clean for your biological safety cabinet.

- Spray all media bottles, pipettes and centrifuge tubes with ethanol before placing in the biological safety cabinet.

3. Under the biological safety cabinet, remove the conditioned media and gently wash the cell monolayer with room temperature DPBS.

- Carefully add DPBS to side of flask so not to forcefully dislodge adherent cells.

4. Remove the DPBS using a sterile serological pipette and add pre-warmed dissociation reagent (Trypsin-EDTA) to the flask and place in an incubator for ~2 mins (dissociation times can vary between cell lines). Check flask frequently to ensure all cells have dissociated from flask surface. - Not all cells will require trypsinization, and to some cells it can be toxic. Trypsin can also induce temporary internalization of some membrane proteins, which should be taken into consideration when planning experiments. Other methods such as gentle cell scraping or using a very mild dissociation reagent (Versene) can often be used as a substitute in these circumstances.

5. When all cells are detached, neutralize the dissociation reagent with serum containing growth medium appropriate to the cell line in culture.

6. Transfer cell suspension to a centrifuge tube. Using sterile media, wash flask and transfer to centrifuge tube, ensuring all cells have been harvested from flask.

7. Centrifuge the cell suspension for 5min @ 1000rpm, room temperature.

8. Discard the supernatant and gently flick the cell pellet (to break up pellet), then resuspend cells in sterile media to a suitable volume for counting.

9. Based on count and viability data, seed cell suspension for an appropriate flask and density, e.g. T175, 30mL at 2e4 cells/cm^2.

10. Immediately incubate the newly seeded cultures in a 37oC/5% CO2 air humidified incubator.

7. Sub-culturing loosely attached cell lines requiring cell scrapping for subculture:

1. When ready, carefully pour off media from flask of the required cells into waste pot (containing approximately 100 ml of 10% sodium hypochlorite) taking care not to increase contamination risk with any drips.

2. Replace this immediately by carefully pouring an equal volume of prewarmed fresh culture media into the flask.

3. Using a cell scraper, gently scrape the cells off the bottom of the flask into the media. Check all the cells have come off by inspecting the base of the flask before moving on.

4. Take out required amount of cell suspension for required split ratio using a serological pipette.

eg for 1:2 split from 100 ml take 50 ml into a new flask

1:5 split from 100 ml take 20 ml into a new flask

1:10 split from 100 ml take 10 ml into a new flask

5. Top the new flasks up to required volume (taking into account split ratio) with pre-warmed fresh culture media eg in 25 cm2 flask approximately 5-10 ml

75 cm^2 flask approximately 10-30 ml

175 cm^2 flask approximately 40-150 ml

8. Sub-culturing attached cell lines requiring trypsin:

Note

-not all cells will require trypsinization, and to some cells it can be toxic. It can also induce temporary internalization of some membrane proteins, which should be taken into consideration when planning experiments. Other methods such as gentle cell scraping or using very mild detergent can often be used as a substitute in these circumstances .

1. When ready, carefully pour off media from flask of the required cells into waste pot (containing approximately 100 ml 10% sodium hypochlorite) taking care not to increase contamination risk with any drips.

2. Using aseptic technique, pour/pipette enough sterile PBS into the flask to give cells a wash and get rid of any FBS in the residual culture media. Tip flask gently a few times to rinse the cells and carefully pour/pipette the PBS back out into waste pot. This may be repeated another one or two times if necessary (some cell lines take a long time to tyrosinase and these will need more washes to get rid of any residual FBS to help trypsinization)

3. Using a pipette, add enough trypsin EDTA to cover the cells at the bottom of the flask. eg in 25 cm^2 flask approximately 1 ml

75 cm^2 flask approximately 5 ml 175 cm^2 flask approximately 10 ml

4. Roll flask gently to ensure trypsin contact with all cells. Place flask in 37°C incubator. Different cell lines require different trypsinization times. To avoid over-trypsinization which can severely damage the cells, it is essential to check them every few minutes.
5. As soon as cells have detached (the flask may require a few gentle taps) add some culture media to the flask (the FBS in this will inactivate the trypsin)
6. Using this cell suspension, pipette required volume of cells into new flasks at required split ratio. These flasks should then be topped up with culture media to required volume
eg in 25 cm^2 flask approximately 5-10 ml
75 cm^2 flask approximately 10-30 ml
175 cm^2 flask approximately 40-150 ml
Leave cells overnight to recover and settle. Change media to get rid of any residual trypsin.

9. Sub-culturing of suspension cell lines:

1. Check guidelines for the cell line for recommended split ratio or sub-culturing cell densities.
2. Take out required amount of cell suspension from the flask using pipette and place into new flask.
eg for 1:2 split from 100 ml of cell suspension take out 50 ml
For 1:5 split from 100 ml of cell suspension take out 20 ml
3.Add required amount of pre-warmed cell culture media to fresh flask.
eg for 1:2 split from 100 ml add 50 ml fresh media to 50 ml cell suspension
For 1:5 split from 100 ml add 80mls fresh media to 20 ml cell suspension.

10. Changing media:

1.If cells have been growing well for a few days but are not yet confluent (eg if they have been split 1:10) then they will require media changing to replenish nutrients and keep correct pH. If there are a lot of cells in suspension (attached cell lines) or the media is staring to go orange rather than pinky orange then media change them as soon as possible.

2.To change media, warm up fresh culture media (section 5.1) at 37°C in water bath or incubator for at least 30 min. Carefully pour of the media from the flask into a waste pot containing some disinfectant. Immediately replace the media with 100 ml of fresh pre-warmed culture media and return to CO2 37°C incubator.

11. Passage number:

The passage number is the number of sub-cultures the cells have gone through. Passage number should be recorded and not get too high. This is to prevent use of cells undergoing genetic drift and other variations.

References:

1.Basic Principles of Cell Culture, R. Ian Freshney Centre for Oncology and Applied Pharmacology, Cancer Research UK Beatson. Laboratories, Garscube Estate, Bearsden, Glasgow G61 1BD, Scotland, UK.

2.https//abcam.com/ mammalian-cell-tissue-culture-techniques-protocol.pdf

3. https//abcam.com/ Cell culture guidelines.

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