Cell culture guidelines

The following is a general guideline for culturing of cell lines. All cell culture must be undertaken in microbiological safety cabinet using aseptic technique to ensure sterility.

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% Foetal 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 always 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	5 ml
100 U penicillin / 0.1 mg/ml streptomycin	5 ml

3. Creating the correct culturing environment

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

1. 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 colour.

2. Discard cells if:
   - They are detaching in large numbers (attached lines) and/or look shrivelled and grainy/dark in colour.
   - They are in quiesence (do not appear to be growing at all).

5. Sub-culturing

Sub-culturing should always be done with aseptic technique in sterile conditions

1. Split ratios can be used to ensure cells should be ready for an experiment on a particular day, or just to keep the cell culture running for future use or as a backup. Suspension cell lines often have a recommended sub-culture seeding density. Always check the guidelines for the cell line in use. Some slow growing cells may not grow if a high split ratio is used. Some fast growing cells may require a high split ratio to make sure they do not overgrow. Note that most cells must not be split more than 1:10 as the seeding density will be too low for the cells to survive.

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.

Attached cell line split ratios are done on volume of flask surface area:

1 X 25^2cm flask → **Split 1:3** → 3 X 25^2 cm flasks
Or
1 X 75^2 cm

Suspension cell line split ratios are done on volume of culture cell suspension:

100 ml cell suspension → **Split 1:4** → 25 ml cell suspension + 75 ml fresh media in 5 separate new flasks
Or
50ml cell suspension + 150 ml Fresh media in 2 larger flasks

2. If cells are less then 70-80% confluent but you wish to subculture them on (e.g. Friday before the weekend) then they should be split at a lower split ratio in order to seed the cells at a high enough density to survive e.g. use 1:2 or 1:5 split.

6. Splitting

1. When the cells are approximately 80% confluent (80% of surface of flask covered by cell monolayer) they should still be in the log phase of growth and will require sub-culturing. (Do not let cells become over confluent as they will start to die off and may not be recoverable).
2. To sub-culture, first warm the fresh culture medium at 37°C water bath or incubator for at least 30 minutes. Then carry out one of the appropriate following procedures:

3. Make sure flasks are labelled with the cell line, passage number, split ratio, date, operator initials and the vial number of the cells. Place flask(s) straight into 37°C CO_2 incubator. Write down the details of the sub-culturing in the culture record log sheet. There should be a separate log sheet for each vial of cells resuscitated and in use.

7. Sub-culturing loosely attached cell lines requiring cell scraping for sub-culture

1. When ready, carefully pour off media from flask of the required cells into waste pot (containing approximately 100ml 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 pre-warmed fresh culture media into the flask.

3. Using 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.
   e.g. 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.
   e.g. in 25 cm^2 flask approx 5-10 ml
   75 cm^2 flask approx 10-30 ml
   175 cm^2 flask approx 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 trypsinize and these will need more washes to get rid of any residual FBS to help trypsinization).

3. Using pipette, add enough trypsin EDTA to cover the cells at the bottom of the flask.
   e.g. in 25 cm^2 flask approx 1 ml
   75 cm^2 flask approx 5 ml
   175 cm^2 flask approx 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.
   e.g. in 25 cm^2 flask approx 5-10 ml
   75 cm^2 flask approx 10-30 ml
   175 cm^2 flask approx 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.
   e.g. 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.
   e.g. For 1:2 split from 100 ml add 50mls 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 (e.g. 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 media change, warm up fresh culture media (section 5.1) at 37°C in water bath or incubator for at least 30 mins. 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 CO_2 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.