

Animal Cell Culture Protocol

Aseptic Technique and Good Cell Culture Practice *To ensure all cell culture procedures are performed to a standard that will prevent contamination from bacteria, fungi and mycoplasma and cross contamination with other cell lines.*

Procedure

- 1 Sanitize the cabinet using 70% ethanol before commencing work.
- 2 Sanitize gloves by washing them in 70% ethanol and allowing to air dry for 30 seconds before commencing work.
- 3 Put all materials and equipment into the cabinet prior to starting work after sanitizing the exterior surfaces with 70% ethanol.
- 4 While working, do not contaminate hands or gloves by touching anything outside the cabinet (especially face and hair). If gloves become contaminated re-sanitize with 70% ethanol as above before proceeding.
- 5 Discard gloves after handling contaminated cultures and at the end of all cell culture procedures.
- 6 Equipment in the cabinet or that which will be taken into the cabinet during cell culture procedures (media bottles, pipette tip boxes, pipette aids) should be wiped with tissue soaked with 70% ethanol prior to use.
- 7 Movement within and immediately outside the cabinet must not be rapid. Slow movement will allow the air within the cabinet to circulate properly.
- 8 Speech, sneezing and coughing must be directed away from the cabinet so as not to disrupt the airflow.
- 9 After completing work disinfect all equipment and material before removing from the cabinet. Spray the work surfaces inside the cabinet with 70% ethanol and wipe dry with tissue. Dispose of tissue by autoclaving.
- 10 Sanitize the cabinet with 10 – 30 min UV light. Warning – plastics will crack and become brittle over time with repeated exposure to UV light. Only some cabinets have timed UV lights. Ensure they are not left on for extended periods.

Resuscitation of Frozen Cell Lines *It is vital to thaw cells correctly in order to maintain the viability of the culture and enable the culture to recover more quickly. Some cryoprotectants, such as DMSO, are toxic above 4°C therefore it is essential that cultures are thawed quickly and diluted in culture medium to minimize the toxic effects.*

Procedure

1. Remove ampule from liquid nitrogen and place in a waterbath at an appropriate temperature for your cell line e.g. 37°C for mammalian cells. Submerge only the lower half of the ampule. Allow to thaw until a small amount of ice remains in the vial - usually 1-2 minutes. Transfer to class II safety cabinet.
2. Wipe the outside of the ampule with a tissue moistened (not excessively) with 70% alcohol hold tissue over ampule to loosen lid.
3. Slowly, dropwise, pipette cells into pre-warmed growth medium to dilute out the DMSO.
4. Incubate cells overnight. Change media the next morning. Removal of DMSO is critical.
5. Examine cells microscopically (phase contrast) after 24 hours and sub-culture as necessary.

Subculture of Adherent (monolayer) Cell Lines *Adherent cell lines will grow in vitro until they have covered the surface area available or the medium is depleted of nutrients. Prior to this point the cell lines should be sub-cultured in order to prevent the culture dying. To subculture the cells they need to be brought into suspension. The degree of adhesion varies from cell line to cell line but in the majority of cases proteases, e.g. trypsin, are used to release the cells from the flask. However, this may not be appropriate for some lines where exposure to proteases is harmful or where the enzymes used remove membrane markers/receptors of interest. In these cases cells should be brought into suspension into a small volume of medium mechanically with the aid of cell scrapers.*

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- 1 Eyeball the cells - View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
 - Check the pH of the culture medium by looking at the color of the indicator, phenol red. As a culture becomes more acid the indicator shifts from red to yellow-red to yellow. As the culture becomes more alkaline the color shifts from red to fuchsia (red with a purple tinge). As a generalization, cells can tolerate slight acidity better than they can tolerate shifts in pH above pH 7.6.
 - Cell attachment. Are most of the cells well attached and spread out? Are the floating cells dividing cells or dying cells which may have an irregular appearance?
 - Percent confluency. The growth of a culture can be estimated by following it toward the development of a full cell sheet (confluent culture). By comparing the amount of space covered by cells with the unoccupied spaces you can estimate percent confluency.
 - Cell shape is an important guide. Round cells in an un—crowded culture is not a good sign unless these happen to be dividing cells. Look for doublets or dividing cells. Get to know the effect of crowding on cell shape.
 - Look for giant cells. The number of giant cells will in—crease as a culture ages or declines in "well-being." The frequency of giant cells should be relatively low and con—stant under uniform culture conditions.
 - One of the most valuable guides in assessing the success of a "culture split" is the rate at which the cells in the newly established cultures attach and spread out. Attachment within an hour or two suggests that the cells have not been traumatized and that the in vitro environment is not grossly abnormal. Longer attachment times are suggestive of problems. Nevertheless, good cultures may result even if attachment does not occur for four hours.
 - Keep in mind that some cells will show oriented growth patterns under some circumstances while many transformed cells, because of a lack of contact inhibition may "pile up" especially when the culture becomes crowded. Get to recognize the range of cells shapes and growth patterns exhibited by each cell line.
- 2 Remove spent medium.
- 3 Wash the cell monolayer with 1-2 ml of PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (CMF-PBS).
- 4 Pipette trypsin onto the washed cell monolayer using 1 ml per 25cm^2 of surface area. (eg. 1 ml – T25, 3 ml T75). Rotate flask to cover the monolayer with trypsin.
- 5 Incubate in hood for 2-10 minutes. Some cells may need to be sit in the incubator. Too long of a period of trypsinisation and the cells will die. Not enough and you will not transfer enough cells.
- 6 Once the cells start to sheet and the media becomes cloudy, move on to the next step. You may examine the cells using an inverted microscope to ensure that many (~40%) the cells are detached and floating. It may help to "slap or tap" the flasks gently to release any remaining attached cells.
- 7 Resuspend the cells in a small volume of fresh serum-containing medium to inactivate the trypsin.
- 8 Triturate the cells (this is a process to disaggregate clumps or sheets of cells) by running the suspended cells in the medium three to four times with the tip of the pipet on the bottom corner of the flask. BE CAREFUL not to aspirate your media into the pipet aid. If this happens, the filter MUST be replaced.
- 8 Transfer the required number of cells to a new labeled flask containing pre-warmed medium

Key Points

- 1 Trypsin is inactivated in the presence of serum. Therefore, it is essential to remove all traces of serum from the culture medium by washing the monolayer of cells with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$
- 2 Cells should only be exposed to trypsin/EDTA long enough to detach cells. Prolonged exposure could damage surface receptors.
- 3 Trypsin should be neutralized with serum prior to seeding cells into new flasks otherwise cells will not attach.

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Subculture Short Protocol and Splitting Guide:

Daily Culture Check

- Check Media Color
- Check Media Clarity
- Microscopic Check
 - Confluence
 - Morphology
 - Contamination

Cell Culture Splits – a 1:2 indicates that one-half of the cells are transferred into a new flask. A 1:10 split indicates that one tenth of the original cells are subcultured into a new flask. There is no magic number or formula for how to do this. BUT there are two key points. Ensure you add enough complete media to stop the action of trypsin and work to avoid wasting media and plastics, but ensure aseptic techniques are maintained.

Trypsinizing T25 flasks:

- Remove media from flask
- Wash with 1-2 mL CMF-PBS
- Remove CMF-PBS
- Add 1 mL 1X Trypsin to each flask
- Let stand 5-10 minutes* Read detailed protocol to ensure over trypsinizing does not occur!

1:2 Split

- Add 9 ml complete media to flask with trypsin.
- Pipette cells up and down to mix.
- Add 5 ml of cell suspension from original flask to each of the new flasks and pipette up and down to mix cells.

1:3 Split

- Add 2 ml complete media to flask with trypsin.
- Pipette cells up and down to mix.
- Add 1 ml of cell suspension from original flask to each of the new flasks
- QS flask to the appropriate volume and pipette up and down to mix cells

1:10 Split

- Add 9 ml complete media to flask with trypsin.
- Add 1 ml of cell suspension from original flask to each of the new flasks.
- QS flask to the appropriate volume and pipette up and down to mix cells

T25 to Two 6-Well Plates

- Add 11 ml of complete media to flask with trypsin.
- Pipette up and down numerous times to mix cells
- Add an additional 12 ml growth media to the flask.
- Pipette up and down numerous times to mix cells
- Add 2 ml of cell suspension to each well of two 6-well plates.
- Works best if you mix cell suspension and add suspension to first 6-well plate, then mix cell suspension again and add cells to second 6-well plate.

T25 to 12 - 35 mm Dishes

- Add 11 ml of growth media to flask with trypsin.
- Pipette up and down numerous times to mix cells
- Add an additional 12 ml growth media to the flask.
- Pipette up and down numerous times to mix cells
- Add 2 ml of cell suspension to each 35 mm dish.