12.1 Basic Techniques - The "Do's and Don'ts" of Cell Culture

Given below are a few of the essential "do's and don'ts" of cell culture. Some of these are mandatory e.g. use of personal protective equipment (PPE). Many of them are common sense and apply to all laboratory areas. However some of them are specific to tissue culture.

The Do's

1. Use personal protective equipment, (laboratory coat/gown, gloves and eye protection) at all times. In addition, thermally insulated gloves, full-face visor and splash-proof apron should be worn when handling liquid nitrogen.
2. Always use disposable caps to cover hair.
3. Wear dedicated PPE for tissue culture facility and keep separate from PPE worn in the general laboratory environment. The use of different colored gowns or laboratory coats makes this easier to enforce.
4. Keep all work surfaces free of clutter.
5. Correctly label reagents including flasks, medium and ampules with contents and date of preparation.
6. Only handle one cell line at a time. This common-sense point will reduce the possibility of cross contamination by mislabeling etc. It will also reduce the spread of bacteria and mycoplasma by the generation of aerosols across numerous opened media bottles and flasks in the cabinet.
7. Clean the work surfaces with a suitable disinfectant (e.g. 70% ethanol) between operations and allow a minimum of 15 minutes between handling different cell lines.
8. Wherever possible maintain separate bottles of media for each cell line in cultivation.
9. Examine cultures and media daily for evidence of gross bacterial or fungal contamination. This includes medium that has been purchased commercially.
10. Quality Control all media and reagents prior to use.
11. Keep cardboard packaging to a minimum in all cell culture areas.
12. Ensure that incubators, cabinet, centrifuges and microscopes are cleaned and serviced at regular intervals.
13. Test cells for mycoplasma on a regular basis.
1. Do not continuously use antibiotics in culture medium as this will inevitably lead to the appearance of antibiotic resistant strains and may render a cell line useless for commercial purposes.
2. Don’t allow waste to accumulate particularly within the microbiological safety cabinet or in the incubators.
3. Don’t have too many people in the lab at any one time.
4. Don’t handle cells from unauthenticated sources in the main cell culture suite. They should be handled in quarantine until quality control checks are complete.
5. Avoid keeping cell lines continually in culture without returning to frozen stock.
6. Avoid cell culture becoming fully confluent. Always sub-culture at 70-80% confluency or as advised on ECACC’s cell culture data sheet.
7. Do not allow media to go out of date. Shelf life is only 6 weeks at +4ºC once glutamine and serum is added.
8. Avoid water baths from becoming dirty by using Sigma Clean (Prod. No. S5525).
9. Don’t allow essential equipment to become out of calibration. Ensure microbiological safety cabinets are tested regularly.

**12.2 Protocol 1 - Aseptic Technique and Good Cell Culture Practice**

**Aim**
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.

**Materials**
- Chloros / Presept solution (2.5g/l)
- 1% formaldehyde based disinfectant e.g. Virkon, Tegador
- 70% ethanol in water (Prod. No. R8382)

**Equipment**
- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Microbiological safety cabinet at appropriate containment level

**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. Whilst working do not contaminate 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. Cell culture discard in chloros (10,000) ppm must be kept in the cabinet for a minimum of two hours (preferably overnight) prior to discarding down the sink with copious amounts of water.
11. Periodically clean the cabinet surfaces with a disinfectant such as Presept, Tegador or Virkon or fumigate the cabinet according to the manufacturers instructions. However you must ensure that it is safe to fumigate your own laboratory environment due to the generation of gaseous formaldehyde, consult your on-site Health and Safety Advisor.

**12.3 Protocol 2 - Resuscitation of Frozen Cell Lines**

**Aim**
Many cultures obtained from a culture collection, such as ECACC, will arrive frozen and in order to use them the cells must be thawed and put into culture. 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 (Prod. No. D2650), are toxic above 4ºC therefore it is essential that cultures are thawed quickly and diluted in culture medium to minimize the toxic effects.

**Materials**
- Media– pre-warmed to the appropriate temperature (refer to the ECACC Cell Line Data Sheet for the correct medium and size of flask to resuscitation into.)
- 70% ethanol in water (Prod. No. R8382)
- DMSO (Prod. No. D2650)
Equipment
- Personal protective equipment (sterile gloves, Laboratory coat, safety visor)
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at appropriate containment level
- CO₂ incubator
- Pre labeled flasks
- Marker Pen
- Pipettes
- Ampule Rack
- Tissue

Procedure
1. Read Technical data sheet to establish specific requirements for your cell line.
2. Prepare the flasks as appropriate (information on technical data sheet). Label with cell line name, passage number and date.
3. Collect ampule of cells from liquid nitrogen storage wearing appropriate protective equipment and transfer to laboratory in a sealed container.
4. Still wearing protective clothing, remove ampule from container 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.
5. Wipe the outside of the ampule with a tissue moistened (not excessively) with 70% alcohol hold tissue over ampule to loosen lid.
6. Slowly, dropwise, pipette cells into pre-warmed growth medium to dilute out the DMSO (Prod. No. D2650) (flasks prepared in Step 2).
7. Incubate at the appropriate temperature for species and appropriate concentration of CO₂ in atmosphere.
8. Examine cells microscopically (phase contrast) after 24 hours and sub-culture as necessary.

Key Points
1. Most text books recommend washing the thawed cells in media to remove the cryoprotectant. This is only necessary if the cryoprotectant is known to have an adverse effect on the cells. In such cases the cells should be washed in media before being added to their final culture flasks. See Protocol 7 for further details.
2. Do not use an incubator to thaw cell cultures since the rate of thawing achieved is too slow resulting in a loss of viability.
3. If a CO₂ incubator is not available gas the flasks for 1-2 minutes with 5% CO₂ in 95% air filtered through a 0.25m filter.
4. For some cultures it is necessary to subculture before confluence is reached in order to maintain their characteristics e.g. the contact inhibition of NIH 3T3 (Prod. No. 93061524) cells is lost if they are allowed to reach confluence repeatedly.

12.4 Protocol 3 - Subculture of Adherent Cell Lines

Click here for a schematic diagram of "Subculture of Adherent Cell Lines"

Aim
Adherent cell lines will grow in vitro until they have covered the surface area available or the medium is depleted of nutrients. At 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.

Materials
- Media– pre-warmed to 37°C (refer to the ECACC Cell Line Data Sheet for the correct medium)
- 70% ethanol in water (Prod. No. R8382)
- PBS without Ca²⁺/Mg²⁺ (Prod. No. D8537)
- 0.25% trypsin/EDTA in HBSS, without Ca²⁺/Mg²⁺ (Prod. No. T4049)
- Trypsin (Prod. No. T4424)
- Soybean trypsin Inhibitor(Prod. No. T6414)

Equipment
- Personal protective equipment (sterile gloves, Laboratory coat, safety visor)
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at appropriate containment level
- CO₂ incubator
- Pre-labeled flasks
- Marker Pen
- Pipettes
- Ampule Rack
- Tissue

Procedure
1. View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
2. Remove spent medium.
Wash the cell monolayer with PBS without 
Ca$^{2+}$/Mg$^{2+}$ (Prod. No. D8537) using a volume equivalent to half the volume of culture medium. Repeat this wash step if the cells are known to adhere strongly.

4. Pipette trypsin/EDTA (Prod. No. T4049) onto the washed cell monolayer using 1ml per 25cm$^2$ of surface area. Rotate flask to cover the monolayer with trypsin. Decant the excess trypsin.

5. Return flask to the incubator and leave for 2-10 minutes.

6. Examine the cells using an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.

7. Resuspend the cells in a small volume of fresh serum-containing medium to inactivate the trypsin. Remove 100-200uL and perform a cell count (Protocol 6 - Cell Quantification).

8. Transfer the required number of cells to a new labeled flask containing pre-warmed medium (refer to ECACC Cell Line Data Sheet for the required seeding density).

9. Incubate as appropriate for the cell line.

10. Repeat this process as demanded by the growth characteristics of the cell line.

**Key Points**

1. Some cultures whilst growing as attached lines adhere only lightly to the flask, thus it is important to ensure that the culture medium is retained and the flasks are handled with care to prevent the cells detaching prematurely.

2. Although most cells will detach in the presence of trypsin alone the EDTA is added to enhance the activity of the enzyme.

3. 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 
Ca$^{2+}$/Mg$^{2+}$ (Prod. No. D8537).

4. Cells should only be exposed to trypsin/EDTA (Prod. No. T4049) long enough to detach cells. Prolonged exposure could damage surface receptors.

5. Trypsin should be neutralized with serum prior to seeding cells into new flasks otherwise cells will not attach.

6. Trypsin may also be neutralized by the addition of soybean trypsin inhibitor (Prod. No. T6414), where an equal volume of inhibitor at a concentration of 1mg/ml is added to the trypsinised cells. The cells are then centrifuged, resuspended in fresh culture medium and counted as above. This is especially necessary for serum-free cell culture.

7. If a CO$_2$ incubator is not available gas the flasks for 1-2min with 5% CO$_2$ in 95% air filtered through a 0.25m filter.

**12.5 Protocol 4 - Subculture of Semi-Adherent Cell Lines**

Click here for a schematic diagram of "Subculture of Semi-Adherent Cell Lines"

**Aim**

Some cultures grow as a mixed population (e.g. B95-8 - marmoset) where a proportion of cells do not attach to the tissue culture flask and remain in suspension. Therefore to maintain this heterogeneity both the attached cells and the cells in suspension must be subcultured.

**Materials**

- Media– pre-warmed to 37°C (refer to the ECACC Cell Line Data Sheet for the correct medium)
- 70% ethanol in water (Prod. No. R8382)
- PBS without 
Ca$^{2+}$/Mg$^{2+}$ (Prod. No. D8537)
- 0.25% trypsin/EDTA in HBSS, without 
Ca$^{2+}$/Mg$^{2+}$ (Prod. No. T4049)
- Trypsin (Prod. No. T4424)
- Soybean trypsin Inhibitor(Prod. No. T6414)

**Equipment**

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet at the appropriate containment level
- Centrifuge
- Inverted phase contrast microscope
- CO$_2$ incubator
- Haemocytometer (Bright-line, Prod. No. Z359629, Improved Neubauer Grid, Camlab CCH.AC1)
- Pre-labeled flasks
- Tissues

**Procedure**

1. View cultures using an inverted phase contrast microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants. Give the flask a gentle knock first, this may dislodge the cells from the flask and remove the need for a trypsinisation step with the subsequent loss of some cells due to the washings.

2. Decant spent medium into a sterile centrifuge tube and retain.

3. Wash any remaining attached cells with PBS without 
Ca$^{2+}$/Mg$^{2+}$ (Prod. No. D8537) using 1-2ml for each 25cm$^2$ of surface area. Retain the washings.

4. Pipette trypsin/EDTA (Prod. No. T4049) onto the washed cell monolayer using 1ml per 25cm$^2$ of surface area. Rotate flask to cover the monolayer with trypsin. Decant the excess trypsin.

5. Return flask to incubator and leave for 2-10 minutes.

6. Examine the cells using an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.

7. Transfer the cells into the centrifuge tube containing the retained spent medium and cells.

8. Centrifuge the remaining cell suspension at 150g for 5 minutes. Also centrifuge the washings from Number 3 above if they contain
significant numbers of cells.

9. Decant the supernatants and resuspend the cell pellets in a small volume (10-20mls) of fresh culture medium. Pool the cell suspensions. Count the cells.

10. Pipette the required number of cells to a new labeled flask and dilute to the required volume using fresh medium (refer to ECACC Cell Line Data Sheet for the required seeding density).

11. Repeat this process every 2-3 days as necessary.

Key Points

1. Although most cells will detach in the presence of trypsin alone the inclusion of EDTA is used to enhance the activity of the enzyme.
2. 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 Ca\(^{2+}\)/Mg\(^{2+}\) (Prod. No. D8537). Repeated warming to 37°C also inactivates trypsin.
3. Cells should only be exposed to trypsin/EDTA (Prod. No. T4049) long enough to detach cells. Prolonged exposure could damage surface receptors. In general a shorter time of exposure to trypsin is required for semi adherent cell lines.
4. Trypsin should be neutralized with serum prior to seeding cells into new flasks otherwise cells will not attach.
5. Trypsin may also be neutralized by the addition of Soybean trypsin Inhibitor (Prod. No. T6414), where an equal volume of inhibitor at a concentration of 1mg/ml is added to the trypsinised cells. The cells are then centrifuged, resuspended in fresh culture medium and counted as above.
6. If a CO\(_2\) incubator is not available gas the flasks for 1-2 minutes with 5% CO\(_2\) in 95% air filtered through a 0.25m filter.

12.6 Protocol 5 - Subculture of Suspension Cell Lines

Click here for a schematic diagram of "Subculture of Suspension Cell Lines"

Aim

In general terms cultures derived from blood (e.g. lymphocytes) grow in suspension. Cells may grow as single cells or in clumps (e.g. EBV transformed lymphoblastoid cell lines). For these types of lines subculture by dilution is relatively easy. But for lines that grow in clumps it may be necessary to bring the cells into a single cell suspension by centrifugation and resuspension by pipetting in a smaller volume before counting.

Materials

- Media– pre-warmed to 37°C (refer to the ECACC Cell Line Data Sheet for the correct medium)
- 70% Ethanol in water (Prod. No. R8382)

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- CO\(_2\) incubator
- Inverted phase contrast microscope
- Haemocytometer (Bright-line, Prod. No. Z359629, Improved Neubauer, Camlab CCH.AC1)
- Pre-labeled flasks

Procedure

1. View cultures using an inverted phase contrast microscope. Cells growing in exponential growth phase should be bright, round and refractile. Hybridomas may be very sticky and require a gentle knock to the flask to detach the cells. EBV transformed cells can grow in very large clumps that are very difficult to count and the center of the large clumps may be non-viable.
2. Do not centrifuge to subculture unless the pH of the medium is acidic (phenol red = yellow) which indicates the cells have overgrown and may not recover. If this is so, centrifuge at 150g for 5 minutes, re-seed at a slightly higher cell density and add 10- 20% of conditioned medium (supernatant) to the fresh media.
3. Take a small sample of the cells from the cell suspension (100-200uL - Protocol 6 - Cell Quantification). Calculate cells/ml and re-seed the desired number of cells into freshly prepared flasks without centrifugation just by diluting the cells. The data sheet will give the recommended seeding densities.
4. Repeat this every 2-3 days.

Key Points

1. If the cell line is a hybridoma or other cell line that produces a substance (e.g. recombinant protein or growth factor) of interest retain the spent media for analysis.

12.7 Protocol 6 - Cell Quantification

Click here for a schematic diagram of "Cell Quantification"

Aim

For the majority of manipulations using cell cultures, such as transfections, cell fusion techniques, cryopreservation and subculture
routines it is necessary to quantify the number of cells prior to use. Using a consistent number of cells will maintain optimum growth and also help to standardize procedures using cell cultures. This in turn gives results with better reproducibility.

Materials
- Media—pre-warmed to appropriate temperature (refer to the ECACC Cell Line Data Sheet for the correct medium and temperature)
- 70% ethanol in water (Prod. No. R8382)
- 0.4% Trypan Blue Solution (Prod. No. T8154)
- Trypsin/EDTA (Prod. No. T4049)

Equipment
- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- CO₂ incubator
- Haemocytometer (Bright-line, Prod. No. Z359629, Improved Neubauer, Camlab CCH.AC1)
- Inverted phase contrast microscope
- Pre-labeled flasks

Procedure
1. Bring adherent and semi adherent cells into suspension using trypsin/EDTA (Prod. No. T4049) as above (Protocol 3 and 4) and resuspend in a volume of fresh medium at least equivalent to the volume of trypsin. For cells that grow in clumps centrifuge and resuspend in a small volume and gently pipette to break up clumps.
2. Under sterile conditions remove 100-200uL of cell suspension.
3. Add an equal volume of Trypan Blue (Prod. No. T8154) (dilution factor =2) and mix by gentle pipetting.
4. Clean the haemocytometer.
5. Moisten the coverslip with water or exhaled breath. Slide the cover-slip over the chamber back and forth using slight pressure until Newton’s refraction rings appear (Newton’s refraction rings are seen as rainbow-like rings under the cover-slip).
6. Fill both sides of the chamber (approx. 5-10uL) with cell suspension and view under a light microscope using x20 magnification.
7. Count the number of viable (seen as bright cells) and non-viable cells (stained blue) - (see below). Ideally >100 cells should be counted in order to increase the accuracy of the cell count (see notes below). Note the number of squares counted to obtain your count of >100.
8. Calculate the concentration of viable and non-viable cells and the percentage of viable cells using the equations below.

Where:
- A is the mean number of viable cells counted, i.e. Total viable cells counted divided by Number of squares
- B is the mean number of non-viable cells counted, i.e. Total non-viable cells counted divided by Number of squares
- C is the dilution factor and
- D is the correction factor (this is provided by the haemocytometer manufacturer).

Concentration of viable cells (cells/ml) = A x C x D
Concentration of non-viable cells (cells/ml) = B x C x D
Total number of viable cells = concentration of viable cells x volume
Total number of cells = number of viable + number of dead cells
Percentage Viability = (No of viable cells x 100) divided by Total No of cells

Key Points
1. Trypan Blue (Prod. No. T8154) is toxic and is a potential carcinogen. Protective clothing, gloves and face/eye protection should be worn. Do not breathe the vapor.
2. The central area of the counting chamber is 1mm². This area is subdivided into 25 smaller squares (1/25mm²). Each of these is surrounded by triple lines and is then further divided into 16 (1/400mm²). The depth of the chamber is 0.1mm.
3. The correction factor of 10⁴ converts 0.1mm³ to 1ml (0.1mm³ = 1mm² x 0.1mm)
4. There are several sources of inaccuracy:
   - The presence of air bubbles and debris in the chamber.
   - Overfilling the chamber such that sample runs into the channels or the other chamber
   - Incomplete filling of the chamber.
   - Cells not evenly distributed throughout the chamber.
   - Too few cells to count. This can be overcome by centrifuging the cells, resuspending in a smaller volume and recounting.
   - Too many cells to count. This can be overcome by using a higher dilution factor in trypan blue e.g. 1:10

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12.8 Protocol 7 - Cryopreservation of Cell Lines

Click here for a schematic diagram of "Cryopreservation of Cell Lines"

Aim
The protocol below describes the use of passive methods involving an electric -80°C freezer for the cryopreservation of cell cultures. ECACC routinely use a programmable rate controlled freezer (Planer Series Two) from Planer Products. This is the most reliable and reproducible way to freeze cells but as the cost of such equipment is beyond the majority of research laboratories the methods below are
Freeze medium (commonly 70% basal medium, 20% FCS, 10% DMSO (Prod. No. D2650) or glycerol, check ECACC data sheets for details).
- 70% ethanol in water (Prod. No. R8382)
- PBS without Ca²⁺ Mg²⁺ (Prod. No. D8537)
- 0.25% trypsin/EDTA in HBSS, without Ca²⁺/Mg²⁺ (Prod. No. T4049)
- DMSO (Prod. No. D2650)
- Trypsin/EDTA (Prod. No. T4049)
- HL60 (Prod. No. 98070106-1v1)

Equipment
- Personal protective equipment (sterile gloves, Laboratory coat)
- Full-face protective mask/visor
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- Haemocytometer (Sigma Bright-line Prod. No. Z359629, Improved Neubauer – Camlab CCH.AC1)
- Pre labeled ampules/cryotubes
- Cell Freezing Device (e.g. Nalgene Mr. Frosty Prod. No. C1562)

Procedure
1. View cultures using an inverted microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants.
2. Bring adherent and semi adherent cells into suspension using trypsin/EDTA (Prod. No. T4049) as above (Protocol 3 and 4 – Subculture of adherent/attached and semi-adherent cell lines) and re-suspend in a volume of fresh medium at least equivalent to the volume of trypsin. Suspension cell lines can be used directly.
3. Remove a small aliquot of cells (100-200uL) and perform a cell count (Protocol 6 – Cell Quantification). Ideally the cell viability should be in excess of 90% in order to achieve a good recovery after freezing.
4. Centrifuge the remaining culture at 150g for 5 minutes.
5. Re-suspend cells at a concentration of 2-4x10⁶ cells per ml in freeze medium.
6. Pipette 1ml aliquots of cells into cryoprotective ampules that have been labeled with the cell line name, passage number, cell concentration and date.
7. Place ampules inside a passive freezer e.g. Nalgene Mr. Frosty (Prod. No. C1562). Fill freezer with isopropyl alcohol and place at –80°C overnight.
8. Frozen ampules should be transferred to the vapor phase of a liquid nitrogen storage vessel and the locations recorded.

Key Points
1. The most commonly used cryoprotectant is dimethyl sulphoxide (DMSO Prod. No. D2650), however, this is not appropriate for all cell lines e.g. HL60 (Prod. No. 98070106-1v1) where DMSO is used to induce differentiation. In such cases an alternative such as glycerol should be used (refer to ECACC data sheet for details of the correct cryoprotectant).
2. ECACC freeze medium recommended above has been shown to be a good universal medium for most cell types. Another commonly used freeze medium formulation is 70% basal medium, 20% FCS, 10% DMSO but this may not be suitable for all cell types. Check it works for your cells before using on a regular basis (Prod. No. C6164).
3. It is essential that cultures are healthy and in the log phase of growth. This can be achieved by using pre-confluent cultures (cultures that are below their maximum cell density) and by changing the culture medium 24 hours before freezing.
4. The rate of cooling may vary but as a general guide a rate of between –1°C and –3°C per minute will prove suitable for the majority of cell cultures.
5. An alternative to the Mr. Frosty system is the Taylor Wharton passive freezer where ampules are held in liquid nitrogen vapor in the neck of Dewar. The system allows the ampules to be gradually lowered thereby reducing the temperature. Rate controlled freezers are also available and are particularly useful if large numbers of ampules are frozen on a regular basis.
6. As a last resort if no other devices are available ampules may be placed inside a well insulated box (such as a polystyrene box with sides that are at least 1cm thick) and placed at –80°C overnight. It is important to ensure that the box remains upright throughout the freezing process. Once frozen, ampules should be transferred to the vapor phase of a liquid nitrogen storage vessel and the locations recorded.
7. If using a freezing method involving a -80°C freezer it is important to have an allocated section for cell line freezing so that samples are not inadvertently removed. If this happens at a crucial part of the freezing process then viability and recovery rates will be adversely affected.

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12.9 Protocol 8 - Testing for Bacteria and Fungi

Aim
In cases of gross contamination the naked eye may identify the presence of bacteria and fungi. However, it is necessary to detect low-level infections by incubation of cell cultures and/or their products in microbiological broth. Equally these sterility tests can be used to confirm the absence of bacteria and fungi from the preparation which is important when preparing cell banks or cell culture products.

Materials
Soybean Casein Digest (Tryptone Soya Broth, TSB) (15ml aliquots) (Prod. No. S1674) TSB Powder (Prod. No. T8907)
Bacillus subtilis NCTC*
Candida albicans NCTC*
Clostridium sporogenes NCTC*

Equipment
- Personal protective equipment (latex medical gloves, laboratory coat, safety glasses)
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- Incubator set at 32°C
- Incubator set at 22°C

Click here for Figure 12. Flow Scheme for Bacteria and Fungi Testing

Procedure
1. Culture cell line in the absence of antibiotics for 2 passages prior to testing.
2. Bring attached cells into suspension with the use of a cell scraper. Suspension cell lines may be tested directly.
3. Inoculate 2 x Thioglycollate Medium (TGM) (Prod.No. F4797) and 2 x Tryptone Soya broth (TSB) (Prod.No. T8907) with 1.5ml test sample.
4. Inoculate 2 (TGM) and 2 (TSB) with 0.1ml C.albicans (containing 100 colony forming units, cfu).
5. Inoculate 2 (TGM) and 2 (TSB) with 0.1ml B. subtilis (containing 100cfu).
6. Inoculate 1 TGM with 0.1ml C. sporogenes (containing 100cfu).
7. Leave 2 (TGM) and 2 (TSB) un-inoculated as negative controls.
8. Incubate broths as follows:
   - For TSB, incubate one broth of each pair at 32°C the other at 22°C for 14 days
   - For TGM, incubate one broth of each pair at 32°C the other at 22°C for 14 days
   - For the TGM inoculated with C.sporogenes incubate at 32°C for 14 days
9. Examine Test and Control broths for turbidity after 14 days.

Criteria for a Valid Result
All positive control broths show evidence of bacteria and fungi within 14 days of incubation and the negative control broths show no evidence of bacteria and fungi.

Criteria for a Positive Result
Test broths containing bacteria or fungi show turbidity.

Criteria for a Negative Result
Test broths should be clear and show no evidence of turbidity.

Notes
1. The positive controls should be handled in a laboratory remote from the main tissue culture laboratory.
2. Control organisms (Bacillus subtilis, Clostridium sporogenes and Candida albicans) are also available from the National Collection Type Cultures (NCTC), UK*.
3. This test procedure should be carried out in a microbiology laboratory away from the cell culture laboratory.

12.10 Protocol 9 - Detection of Mycoplasma by Culture

Aim
Detection of mycoplasma by culture is the reference method of detection and has a theoretical level of detection of 1 colony-forming unit (cfu). However there are some strains of mycoplasma that are non-cultivable (certain strains of Mycoplasma hyorhinis). The method is suitable for the detection of mycoplasma in both cell cultures and cell culture reagents and results are obtained within 4 weeks. Mycoplasma colonies observed on agar plates have a ‘fried egg’ appearance (see Figure 14).

Materials
- 70% ethanol in water (Prod. No. R8382)
- Mycoplasma Pig Agar plates (in 5cm petri dishes)
- Mycoplasma Pig Agar broths (in 1.8ml aliquots)
- M. orale NCTC* 10112
- M. pneumoniae NCTC* 10119

Equipment
- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- CO₂ Incubator set at 32°C
- Gas Jar (Gallenkamp)
Click here for Figure 13. Flow Scheme for Detection of Mycoplasma by Culture

Procedure

1. Inoculate 2 agar plates with 0.1ml of test sample.
2. Inoculate 1 agar plate with 100cfu \textit{M. pneumoniae}.
3. Inoculate 1 agar plate with 100cfu \textit{M. orale}.
4. Leave 1 agar plate un-inoculated as a negative control.
5. Inoculate 1 broth with 0.2 ml of test sample.
6. Inoculate 1 broth with 100cfu \textit{M. pneumoniae}.
7. Inoculate 1 broth with 100cfu \textit{M. orale}.
8. Leave 1 agar plate un-inoculated as a negative control.
9. Incubate agar plates anaerobically for 14 days at 37ºC using a gas jar with anaerobic gas pak and catalyst.
10. Incubate broths aerobically for 14 days at 37ºC.
11. Between 3 and 7 days and 10 and 14 days incubation, subculture 0.1 ml of test broth onto an agar plate and incubate plate anaerobically as above.
12. Observe agar plates after 14 days incubation at x300 magnification using an inverted microscope for the presence of mycoplasma colonies (see Figure 14).

Criteria for a Valid Result

All positive control agar plates and broths show evidence of mycoplasma by typical colony formation on agar plates and usually a color change in broths.
All negative control agar plates and broths show no evidence of mycoplasma.

Criteria for a Positive Result

Test agar plates infected with mycoplasma show typical colony formation.

Criteria for a Negative Result

The test agar plates show no evidence of mycoplasma.

Notes

1. Mycoplasma colonies have a typical colony formation commonly described as "fried egg" (See Figure 8) due to the opaque granular central zone of growth penetrating the agar surrounded by a flat translucent peripheral zone on the surface. However in many cases only the control zone will be visible.
2. Positive controls may be included at a concentration to give 100 colony-forming units. These controls should obviously be handled in a laboratory remote from the main tissue culture laboratory.
3. Control organisms (\textit{M. pneumoniae}, and \textit{M. orale}) are available from National Collection of Type Cultures (UK).
4. \textit{Mycoplasma pneumoniae} is a potential pathogen and must be handled in a class II microbiological safety cabinet operating to ACDP Category 2 Conditions.
5. This test procedure should be carried out in a microbiology laboratory away from the cell culture laboratory.

Click here for Figure 14 - Typical "fried egg colonies" \textit{Mycoplasma pneumoniae}

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12.11 Protocol 10 - Testing for Mycoplasma by Indirect DNA Stain (Hoechst 33258 stain)

Aim

DNA staining methods such as Hoechst staining techniques are quick with results available within 24 hours, which compares favorably with 4 weeks for detection by culture. However the staining of cultures directly with a DNA stain, results in a much-reduced sensitivity (~10^6 cfu/ml). This may be improved by co-culturing the test cell line in the presence of an indicator cell line such as Vero (Prod.No. 84113001-1v1). This enrichment step results in a sensitivity of 10^4 cfu/ml of culture. This step also improves sensitivity by increasing the surface area upon which mycoplasma can adhere. Like detection by culture, DNA staining methods are suitable for the detection of mycoplasma from cell cultures or cell culture reagents.

Materials

- Media– pre-warmed to 37ºC (refer to the ECACC Cell Line Data Sheet for the correct medium)
- 70% ethanol in water (Prod. No. R8382)
- Methanol (Prod. No. 175)
- Acetic Acid Glacial (Prod. No. A6283)
- Hoechst 33258 stain solution (Prod. No. H6024)
- Vero cells (Prod. No. 84113001-1v1)
- Mountant (Autoclave 22.2ml 0.2M citric acid with 27.8ml 0.2M disodium phosphate. Add 50ml glycerol. Filter sterilize and store at 4ºC) (Prod. No. M1289)
- \textit{Mycoplasma hyorhinis} NCTC* 10112

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37ºC
- Microbiological safety cabinet of appropriate containment level
Click here for Figure 15. Testing for Mycoplasma by Indirect DNA Stain

Procedure Equipment

1. For each sample and control sterilize 2 cover slips in a hot oven at 180°C for 2 hours or by immersing in 70% ethanol (Prod. No. R8382) and flaming in a blue Bunsen flame until the ethanol has evaporated. Also sterilize 2 cover slips to use as a negative control.
2. Place the cover slips in 35mm culture dishes (Prod. No. C6296) (1 per dish).
3. Store until needed.
4. To prepare the Vero (Prod. No. 84113001-1v1) indicator cells add 2x104 cells in 2ml of antibiotic-free growth medium to each tissue culture dish.
5. Incubate at 37°C in 5% CO₂ for 2 – 24 hrs to allow the cells to adhere to the cover slips.
6. Bring attached test cell lines into suspension using a cell scraper. Suspension cell lines may be tested directly.
7. Remove 1ml of culture supernatant from duplicate dishes and add 1ml of test sample to each. Inoculate 2 dishes with 100cfu M. hyorhinis and 2 with 100cfu M. orale. org.uk.
8. Leave duplicate tissue culture dishes un-inoculated as negative controls.
9. Incubate dishes at 37°C in 5% CO₂ for 1-3 days.
10. After 1 day observe one dish from each pair for bacterial or fungal infection. If contaminated discard immediately. Leave the remaining dish of each pair for a further 2 days.
11. Fix cells to cover-slip by adding a minimum of 2ml of freshly prepared fixative (1:3 glacial acetic acid: absolute methanol) to the tissue culture dish and leave for 3 to 5 minutes.
12. Decant used fixative to toxic waste bottle. Add another 2ml aliquot of fixative to cover-slip and leave for a further 3 to 5 min. Decant used fixative to toxic waste.
13. Air dry cover-slip by resting it against the tissue culture dish for 30-120 min.
15. Decant used and unused stain to toxic waste.
16. Add 1 drop of mountant to a pre-labeled microscope slide and place cover-slip (cell side down) onto slide.
17. Keep slide covered with aluminum foil (Prod. No. Z18514-0), allowing it to set for at least 15 min at 37°C or for 30 min at room temperature.
18. Observe slide under uv Epi-Fluorescence at x1000.

Criteria for a Valid Result

Negative controls show no evidence of mycoplasma infection
Positive controls show evidence of mycoplasma infection
Vero cells clearly seen as fluorescing nuclei.

Criteria for a Positive Result

Samples infected with mycoplasma are seen as fluorescing nuclei plus extra-nuclear fluorescence of mycoplasma DNA (small cocci or filaments).

Criteria for a Negative Result

Uninfected samples are seen as fluorescing nuclei against a dark background. There should be no evidence of mycoplasma.

Notes

1. DNA stains such as Hoechst stain (Prod. No. H6024) bind specifically to DNA. In all cultures cell nuclei will fluoresce.
2. Uncontaminated cultures will show only fluorescent nuclei whereas mycoplasma positive cultures contain small cocci or filaments which may or may not be adsorbed onto the cells (see figure 16).
3. Hoechst stain is toxic and should be handled and discarded with care.
4. Culture dishes should be placed in a sealed box or cultured in large petri dishes to reduce evaporation.
5. Positives should obviously be handled in a laboratory remote from the main tissue culture laboratory.
6. Control organisms (M. hyorhinis) are available from the National Collection of Type Cultures (UK).
7. In some instances results may be difficult to interpret for the following reasons:
   - Bacterial/yeast/fungal contamination
   - Too much debris in the background
   - Broken nuclei as cells are all dead
   - Too few or no live cells
8. Although this procedure recommends the setting up of positive controls, this may not necessarily be feasible nor desirable in a cell culture facility with limited resources. If positive controls are to be set up they should be done so in a separate laboratory from the main tissue culture facility. If this is not possible then positive slides can be purchased from ECACC. If positive controls are not being used then it is strongly recommended that you get an independent testing laboratory to periodically test your cell lines.

Click here for Figure 16. Hoechst Positive Culture
Click here for Figure 17. Hoechst Negative Culture
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