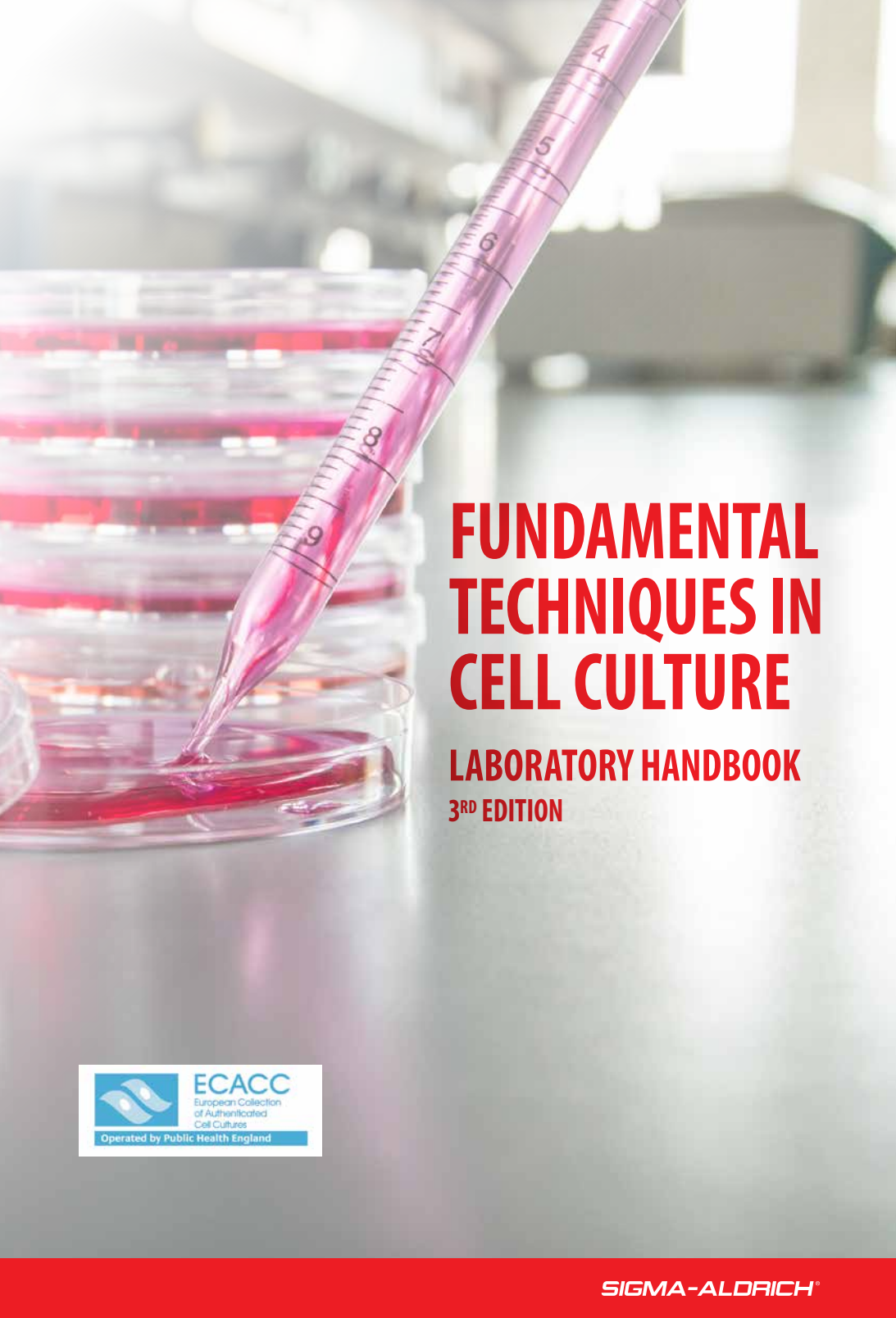




FUNDAMENTAL TECHNIQUES IN CELL CULTURE

LABORATORY HANDBOOK
3RD EDITION



ECACC
European Collection
of Authenticated
Cell Cultures

Operated by Public Health England

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The European Collection of Authenticated Cell Cultures (ECACC)

The European Collection of Authenticated Cell Cultures (ECACC) was established in 1984 as a cell culture collection to service the research community and provide an International Depository Authority recognised patent depository for Europe. Over the last three decades ECACC has expanded and diversified to become one of the premier collections of authenticated cell cultures in the world and this remains the core of ECACC's business. The collections currently hold over 40,000 cell lines representing 45 different species, 50 tissue types, 300 HLA types, 450 monoclonal antibodies and at least 800 genetic disorders.

The development and maintenance of such a diverse collection has inevitably produced a high level of specialist knowledge and this has enabled ECACC to position itself as a centre of expertise in all aspects of cell culture. ECACC has developed a comprehensive range of cell culture services and diversified into new product areas such as extracted from cell lines.

ECACC is the largest of four collections of clinically significant organisms, maintained by Public Health England (PHE). The other collections are the National Collection of Type Cultures (NCTC), the National Collection of Pathogenic Viruses (NCPV), and the National Collection of Pathogenic Fungi (NCPF).

*See page 37 for more information on the cell lines available.

Products and Services available from the PHE ECACC Culture Collections:

Products

- **Cell Lines and Hybridomas**
- General Cell Collection
- Hybridoma Collection
- Human Genetic Collection
- Chromosomal Abnormality Collection
- HLA-Typed Collection
- Human Random Control Collection
- European Bank for induced pluripotent Stem Cells (EBiSC)
- HipSci Collection
- Neuron Culture Kits
- **DNA**
- Human Random Control DNA Panels
- Ethnic Diversity DNA Panel
- Primate DNA Panel
- Diabetes UK Warren DNA Panels
- Custom Prepared Cell Line DNA
- **RNA**
- **cDNA**

Services

- Contract Cell Banking
- Cell Line Authentication
- DNA Extraction
- Bio-Banking
- Mycoplasma Testing
- Patent Deposits
- Safe Deposits
- Sterility Testing
- Training

Visit www.phe-culturecollections.org.uk for more information!

FUNDAMENTAL TECHNIQUES IN CELL CULTURE



1.0 Introduction

Over ten years ago, Sigma® Life Science and the European Collection of Authenticated Cell Cultures (ECACC) formed a working partnership to bring together the most diverse selection of cell culture products and services available commercially. We did this with researchers like you in mind, to ensure that you have the necessary quality products to further your research goals. We continue to expand upon this partnership, and now are able to offer an even greater array of cell lines, cell culture products, knowledge, and services to the global research community.

The field of cell culture has advanced greatly over the years. For more than 30 years, Sigma and ECACC have both been part of and contributed to that advancement. Early cell culture research focused on discovering methods for culturing a diverse array of cells from many species. Today cell culture methods are vital to broad areas of life science research. With the number of researchers adding cell culture to their repertoire of techniques expanding daily, we believe there are many who can benefit from Sigma's and ECACC's combined knowledge and experience in cell culture.

To that aim, we have assembled this updated laboratory handbook of cell culture techniques. For the researcher new to cell culture, this handbook provides a wealth of information from the sourcing of cell lines, safety and laboratory design to aspects of cryopreservation quality control and cell line authentication. The issue of cell line misidentification and cross contamination has been recognised as a significant problem in recent years. Additionally, a series of detailed protocols are provided, which are routinely used in the ECACC laboratories. Protocols for the use of induced pluripotent stem cells and for growing cells in 3D cell culture, two areas of growing importance to cell culture research, have been added to this latest edition of the handbook. The handbook is intended as a guide rather than an in-depth text book of cell culture and you are encouraged to consult relevant specialised literature to obtain more detailed information.

2.0 Design and Equipment for the Cell Culture Laboratory

2.1 Laboratory Design

Perhaps one of the most undervalued aspects of tissue culture is the need to design the facility to ensure that good quality material is produced in a safe and efficient manner. Most tissue culture is undertaken in laboratories that have been adapted for the purpose and in conditions that are not ideal. However, as long as a few basic guidelines are adopted this should not compromise the work.

There are several aspects to the design of good tissue culture facilities. Ideally work should be conducted in a single use facility which, if at all possible, should be separated into an area reserved for handling newly received material (quarantine area) and an area for material which is known to be free of contaminants (main tissue culture facility). If this is not possible work should be separated by time with all manipulations on clean material being completed prior to manipulations involving the 'quarantine material'. Different incubators should also be designated. In addition, the work surfaces should be thoroughly cleaned between activities.

All new material should be handled as 'quarantine material' until it has been shown to be free of contaminants such as bacteria, fungi and particularly mycoplasma. Conducting tissue culture in a shared facility requires considerable planning and it is essential that a good aseptic technique is used throughout to minimise the risk of contamination occurring.

For most cell lines the laboratory should be designated to at least Category 2 based on the Advisory Committee on Dangerous Pathogens (ACDP) guidelines (ACDP, 1995)[†]. However, the precise category required is dependent upon the cell line and nature of the work proposed. The guidelines make recommendations regarding the laboratory environment including lighting, heating, the type of work surfaces and flooring and provision of hand washing facilities. In addition, it is recommended that laboratories should be run at air pressures that are negative to corridors to contain any risks within the laboratory.

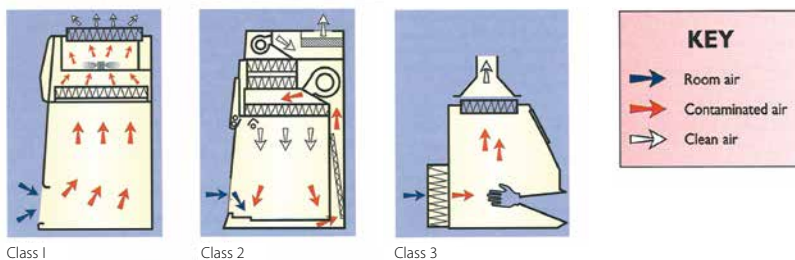
[†] Advisory Committee on Dangerous Pathogens (1995) Categorisation of Biological Agents According to Hazard and Categories of Containment, 4th edition, Health & Safety Executive (HSE) books, Sudbury, UK, (www.hse.gov.uk).

2.2 Microbiological Safety Cabinets

A microbiological safety cabinet is probably the most important piece of equipment for cell culture since, when operated correctly, it will provide a clean working environment for the product, whilst protecting the operator from aerosols. In these cabinets operator and/or product protection is provided through the use of HEPA (high efficiency particulate air) filters. The level of containment provided varies according to the class of cabinet used. Cabinets may be ducted to atmosphere or re-circulated through a second HEPA filter before passing to atmosphere see figure 1.



Figure 1. Diagram of microbiological safety cabinet airflow patterns



Environmental monitoring with Tryptose Soya Broth agar settle plates inside the cabinet for a minimum of four hours is a good indicator of how clean a cabinet is (refer to '9.5 Environmental Monitoring'). There should be no growth of bacteria, after incubation for 3 days, or fungi after incubation for 5 days, on such plates.

In most cases a class 2 cabinet is adequate for animal cell culture. However, each study must be assessed for its hazard risk and it is possible that additional factors, such as a known virus infection or an uncertain provenance may require a higher level of containment.

2.3 Centrifuges

Centrifuges are used routinely in tissue culture as part of the subculture routine for most cell lines and for the preparation of cells for cryopreservation. By their very nature centrifuges produce aerosols and thus it is necessary to minimise this risk. This can be achieved by purchasing models that have sealed buckets. Ideally, the centrifuge should have a clear lid so that the condition of the load can be observed without opening the lid. This will reduce the risk of the operator being exposed to hazardous material if a centrifuge tube has broken during centrifugation. Care should always be taken not to over-fill the tubes and to balance them carefully. These simple steps will reduce the risk of aerosols being generated. The centrifuge should be situated where it can be easily accessed for cleaning and maintenance. Centrifuges should be checked frequently for signs of corrosion.

A small bench-top centrifuge with controlled braking is sufficient for most purposes. Cells sediment satisfactorily at 80 – 150 x g. Higher gravitational forces may cause damage and promote agglutination of the cell pellet.

2.4 Incubators

Cell cultures require a strictly controlled environment in which to grow. Specialist incubators are used routinely to provide the correct growth conditions, such as temperature, degree of humidity and CO₂ levels in a controlled and stable manner. Generally, they can be set to run at temperatures in the range of 28°C (for insect cell lines) to 37°C (for mammalian cell lines) and set to provide CO₂ at the required level (e.g. 5-10%). Some incubators also have the facility to control the O₂ levels. Copper-coated incubators are also now available. These are reported to reduce the risk of microbial contamination within the incubator due to the microbial inhibitory activity of copper. The inclusion of a bactericidal agent in the incubator water trays will also reduce the risk of bacterial and fungal growth. However, there is no substitute for regular cleaning.

2.5 Work Surfaces and Flooring

In order to maintain a clean working environment the laboratory surfaces including bench-tops, walls and flooring should be smooth and easy to clean. They should also be waterproof and resistant to a variety of chemicals (such as acids, alkalis, solvents and disinfectants). In areas used for the storage of materials in liquid nitrogen, the floors should be resistant to cracking if any liquid nitrogen is spilt. Refer to Section 7.4 for safety considerations on the use of liquid nitrogen. In addition, the floors and walls should be continuous with a



coved skirting area to make cleaning easier and reduce the potential for dust to accumulate. Windows should be sealed. Work surfaces should be positioned at a comfortable working height.

2.6 Plasticware and Consumables

Almost every type of cell culture vessel, together with support consumables such as tubes and pipettes, are commercially available as single use, sterile packs. Suppliers include Sigma-Aldrich, Nunc, Greiner, Bibby Sterilin and Corning. The use of such plasticware is more cost effective than recycling glassware, enables a higher level of quality assurance and removes the need for validation of cleaning and sterilisation procedures. Plastic tissue culture flasks are usually treated to provide a hydrophilic surface to facilitate attachment of anchorage dependent cells.

2.7 Care and Maintenance of Laboratory Areas

In order to maintain a clean and safe working environment tidiness and cleanliness are key. All spills should be dealt with immediately. Routine cleaning should be undertaken involving the cleaning of all work surfaces both inside and outside of the microbiological safety cabinet, the floors and all other pieces of equipment e.g. centrifuges. Humidified incubators are a particular area for concern due to the potential for fungal and bacterial growth in the water trays. This will create a contamination risk that can only be avoided by regular cleaning of the incubator. All major pieces of equipment should be regularly maintained and serviced by qualified engineers, for example:

- Microbiological safety cabinets should be checked every six months to ensure that they are safe to use in terms of product and user protection. These tests confirm that the airflow is correct and that the HEPA filters are functioning properly.
- The temperature of an incubator should be regularly checked with a NAMAS (National Accreditation of Measurement and Sampling, UK), or equivalent calibrated thermometer and temperature adjusted as necessary.
- Incubator CO₂ and O₂ levels should also be regularly checked to ensure the levels are being maintained correctly.



T flasks available from Corning

3.0 Safety Aspects of Cell Culture

3.1 Risk Assessment

The main aim of risk assessment is to prevent injury, protect property and avoid harm to individuals and the environment. In many countries the performance of risk assessment is a legal requirement. For example, this is the case in the UK under the Health and Safety at Work Act, UK (1974). There are also European Community directives covering Health and Safety at work. You can visit the European Agency for Safety and Health at Work website (www.europe.osha.eu.int) for information on legislation and standards or you should contact your on-site Health and Safety representative. Consequently risk assessments must be undertaken prior to starting any activity. The assessment consists of two elements:

1. Identifying and evaluating the risks.
2. Defining ways of avoiding or minimising the risk.

For animal cell culture the level of risk is dependent upon the cell line to be used and is based on whether the cell line is likely to cause harm to humans. The different classifications are given below:

Low risk	– Non human/non primate continuous cell lines and some well characterised human continuous lines.
Medium risk	– Poorly characterised mammalian cell lines.
High risk	– Primary cells derived from human/primate tissue or blood. – Cell lines with endogenous pathogens (the precise categorisation is dependent upon the pathogen) – refer to ACDP guidelines, for details†. – Cell lines used following experimental infection where the categorisation is dependent upon the infecting agent – refer to ACDP guidelines, for details.

†Advisory Committee on Dangerous Pathogens (ACDP) (1995) Categorisation of Biological Agents According to Hazard and Categories of Containment, 4th edition, HSE books, Sudbury, UK. The fifth update to the 1995 document was produced in 2013. An update to the Approved List of Biological agents was issued in 2013, available at: <http://www.hse.gov.uk/pubns/misc208.pdf>.

Note: The U.S. Department of Health and Human Services (Centers for Disease Control and Prevention) publishes a similar list, in its Biosafety in Microbiological and Biomedical Laboratories (BMBL) document (2009), available at: <http://www.cdc.gov/biosafety/publications/>. The U.S. system uses Biological Safety Levels in place of the UK ACDP hazard groups.

A culture collection such as ECACC will recommend a minimum containment level required for a given cell line based upon its risk assessment. For most cell lines the appropriate level of containment is Level 2 requiring a class 2 microbiological safety cabinet. However, this may need to be increased to containment Level 3 depending upon the type of manipulations to be carried out and whether large culture volumes are envisaged. For cell lines

derived from patients with HIV or Human T-Lymphotropic Virus (HTLV) Level 3 containment is required.

Containment is the most obvious means of reducing risk. Other less obvious measures include restricting the movement of staff and equipment into and out of laboratories. Good laboratory practice and good bench techniques such as ensuring work areas are uncluttered, reagents are correctly labelled and stored, are also important for reducing risk and making the laboratory a safe environment in which to work. The risk of exposure to aerosols or splashes can be limited by avoiding rapid pipetting, scraping and pouring. In addition, it is recommended that people working in laboratories where primary human material is used are vaccinated against Hepatitis B. Staff training and the use of written standard operating procedures and risk assessments will also reduce the potential for harm. Cell culture training courses covering the basics of tissue culture safety are offered by ECACC.



3.2 Biohazards

Viruses pathogenic for humans are one of the most likely biohazards presented by cell cultures. Where infection with an agent pathogenic for humans is known or suspected, the cell culture should be handled at a containment level appropriate for the agent concerned. Other potential biohazards should also be considered. These relate to components of the cell culture medium, other adventitious agents (e.g. contaminating mycoplasmas), and cell products, some of which may be biologically active molecules with pharmacological, immunomodulating or sensitising properties. In addition, the generation and use of modified cells, for example, hybrids, transformed cells and cells containing recombinant DNA can be hazardous. These procedures could potentially result in the appearance of modified or reactivated viruses, novel fusion/hybrid proteins (especially in cross-species hybrids) and the expression of viral or cellular oncogenes.

Laboratory workers should never culture cells derived from their own body or tissues. In vitro transformation or genetic modification could result in malignant disease or expression of an unusual pharmacologically active protein if they were to be accidentally inoculated into the donor. Therefore, human cells should be obtained from individuals having no association with the experimental work.

Biohazardous waste should be disposed of according to the methods described under '3.5 Waste Disposal'.

3.3 Genetically Modified Organisms

The generation and use of genetically modified organisms (GMOs) should be strictly controlled and regulated. Most countries have regulatory organisations to ensure the risks posed by GMOs are minimised. For example, in the UK all institutions that carry out work using and/or generating GMOs are required by law to have a Genetic Modification Safety Committee (GMSC). Prior to any work commencing proposals for the intended work should go through the committee and, if necessary, be approved by the Health and Safety Executive (HSE). There is a European Directive governing the regulation of GM work. Visit the European Agency for Safety and Health at Work website (www.europe-osh.eu.int) for information on legislation and standards, or contact your on-site Health and Safety representative.

It is the responsibility of the individual cell culture user and institution to ensure compliance with the regulations set by the authorities of the country they are operating in.



3.4 Disinfection

Methods designed for the disinfection/decontamination of culture waste, work surfaces and equipment represent important means for minimising the risk of harm. Always wear appropriate personal protective equipment (PPE) such as gloves and eye protection when using concentrated forms of disinfectants. The selected gloves should protect against the substance being handled and meet the European standard EN374-3. Manufacturers' charts will help to identify the best gloves for the work.

The major disinfectants fall into four groups and their relative merits can be summarised as follows:

Hypochlorites (e.g. Sodium Hypochlorite)

- Good general purpose disinfectant
- Active against viruses
- Corrosive against metals and therefore should not be used on metal surfaces e.g. centrifuges
- Readily inactivated by organic matter and therefore should be made fresh daily
- Should be used at 1000ppm for general use surface disinfection, 2500ppm in discard waste pots for disinfecting pipettes, and 10,000ppm for tissue culture waste and spillages

Note: When fumigating a cabinet or room using formaldehyde all the hypochlorites must first be removed as the two chemicals react together to produce carcinogenic products.

Phenolics

Phenolic based disinfectants should never be used as they are not supported as part of the EU Biocidal Products Directive review programme.

Alcohol (e.g. Ethanol, Isopropanol)

- Effective concentrations: 70% for ethanol, 60-70% for isopropanol
- Their mode of action is dehydration and fixation
- Effective against bacteria. Ethanol is effective against most viruses but not non-enveloped viruses
- Isopropanol is not effective against viruses
- Aldehydes (e.g. Formaldehyde)
- Aldehydes are irritants and their use should be limited due to problems of sensitisation
- Should only be used in well ventilated areas

Formaldehyde is used to fumigate laboratories. The formaldehyde is heated in a device so it will vaporise and all exposed surfaces are coated with the disinfectant.

Generally the use of aldehydes for disinfection and fumigation purposes can be hazardous. Check local regulations and with your safety advisor.

3.5 Waste Disposal

Every employer has a 'duty of care' to dispose of all biological waste safely in accordance with national legislative requirements. Given below is a list of ways in which tissue culture waste can be decontaminated and disposed of safely. One of the most important aspects of the management of all laboratory-generated waste is to dispose of waste regularly and not to allow the amounts to build up. The best approach is 'little and often'. Different forms of waste require different treatment.

- Tissue culture waste (culture medium) – inactivate for at least 2 hours in a solution of hypochlorite (10,000ppm) prior to disposal to drain with an excess of water
- Contaminated pipettes should be placed in hypochlorite solution (2500ppm) overnight before disposal by autoclaving and incineration
- Solid waste such as flasks, centrifuge tubes, contaminated gloves, tissues, etc., should be placed inside heavy-duty sacks for contaminated waste and incinerated
- If at all possible waste should be incinerated rather than autoclaved
- Waste from specially licensed laboratories e.g. those handling genetically modified level 3 (GM3) organisms requires specific treatment and tracking

Did You Know?

Every employer has a 'duty of care' to dispose of all biological waste safely in accordance with national legislative requirements

4.0 Sourcing of Cell Lines

Many cell lines look identical when viewed under a microscope. Cell lines with very different origins and biological characteristics typically cannot be separated on grounds of morphology or culture characteristics. Infection or contamination of a cell line with an adventitious virus or mycoplasma may not be apparent even when cells are viewed under a microscope, but can significantly change the characteristics of the cells. Cell lines will also change with time in culture, and to add to all these natural hazards it is all too easy to incorrectly label or cross-contaminate different cell lines in a busy cell culture laboratory.

The opportunities for inadvertently introducing error into a cell line are limitless and ever present. It is in the nature of the science that once introduced, an error will be propagated, compounded, consolidated and disseminated.

Did You Know?

The European Collection of Authenticated Cell Cultures (ECACC) is one of the world's largest Biological Resource Centres supplying a diverse range of authenticated cell lines.

The integrity and biological characteristics of a cell line have to be actively maintained by a well organised management system based on systematic cell banking supported by testing regimens in a structured quality assured environment. Such a controlled environment will only prevail in a dedicated professionally organised cell culture laboratory or cell bank. A small research laboratory with a high throughput of short-term research students, a minimum of permanent laboratory staff and no formal quality management programme will find it difficult to maintain its cell lines unchanged over many years.

For all these reasons it is recommended that new cell lines should ideally be acquired from a specialist, reputable culture collection such as ECACC. Moreover, if a laboratory believes it already has a certain cell line in its liquid nitrogen store, the identity and purity of such a cell line should be questioned in the absence of a well recorded culture history and recent test data. If there is a doubt, it is straightforward and cost effective to replace such cell stocks with authenticated material from a Culture Collection.

When a Culture Collection acquires a new cell line it will characterise the cell line using techniques such as isoenzyme analysis, STR profiling, DNA barcoding and SNP analysis so that the identity of the cell line subsequently can be verified. The Collection will then establish a hierarchy of Master and Working cell banks, cryopreserved in liquid nitrogen, that are demonstrated free from microbial contamination including mycoplasma. Customers are supplied from the authenticated Working Cell Banks (WCB). Replacement WCBs are manufactured from the original Master Cell Bank (MCB) and the new WCB will again be fully tested.

ECACC supplies its cell lines with advice on how to maintain the line. The technical support team can subsequently assist with difficulties and provide additional technical information about the cell line, if required. Culture Collections exist to ensure that animal cell research is conducted using standardised, authenticated material that ensures the work can be reproduced. An authenticated cell line of known provenance is the very bedrock of any cell based project. See p.37 for more info on cell lines available from ECACC.

5.0 Cell Types & Culture Characteristics

5.1 Primary Cultures

Primary cultures are derived directly from excised, normal animal tissue and cultures either as an explant culture or following dissociation into a single cell suspension by enzyme digestion. Such cultures are initially heterogeneous but later become dominated by fibroblasts. The preparation of primary cultures is labour intensive and they can be maintained in vitro only for a limited period of time. During their relatively limited lifespan primary cells usually retain many of the differentiated characteristics of the cell in vivo. Important Note: Primary cultures by definition have not been passaged, as soon as they are passaged they become a cell line and are no longer primary. 'Primary' cells sourced from most suppliers are in fact low-passage cell lines.

5.2 Continuous Cultures

Continuous cultures are comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely. Cell lines of a finite life are usually diploid and maintain some degree of differentiation. The fact that such cell lines senesce after approximately thirty cycles of division means it is essential to establish a system of Master and Working banks in order to maintain such lines for long periods.

Continuous cell lines that can be propagated indefinitely generally have this ability because they have been transformed into tumour cells. Tumour cell lines are often derived from actual clinical tumours, but transformation may also be induced using viral oncogenes or by chemical treatments. Transformed cell lines present the advantage of almost limitless availability, but the disadvantage of having retained very little of the original in vivo characteristics.

5.3 Culture Morphology

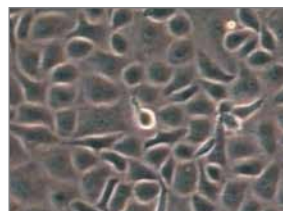
In terms of growth mode cell cultures take one of two forms, growing either in suspension (as single cells or small free floating clumps) or as a monolayer that is attached to the tissue culture flask. The form taken by a cell line reflects the tissue from which it was derived e.g. cell lines derived from blood (leukaemia, lymphoma) tend to grow in suspension whereas cells derived from solid tissue (lungs, kidney) tend to grow as monolayers. Attached cell lines can be classified as endothelial such as BAE-1, epithelial such as HeLa, neuronal such as SH-SY5Y, or fibroblasts such as MRC-5 and their morphology reflects the area within the tissue of origin.

The cell lines most commonly ordered from ECACC are listed in Table 1 (see next page).

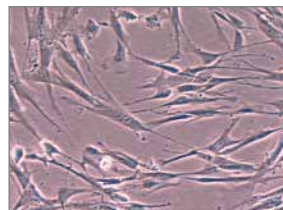
Figure 2. Examples of Cell Morphology



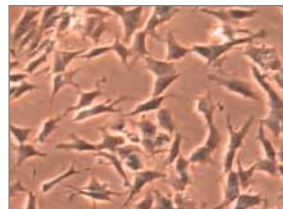
HeLa – epithelial



BAE-1 – endothelial



MRC-5 – fibroblast



SH-SY5Y – neuronal

Table 1. Commonly used cell lines of each culture type

Attached Cell Lines		
Name	Species and tissue of origin	Morphology
MRC-5	Human lung	Fibroblast
HeLa	Human cervix	Epithelial
Vero	African Green Monkey Kidney	Epithelial
NIH 3T3	Mouse embryo	Fibroblast
L929	Mouse connective tissue	Fibroblast
CHO	Chinese Hamster Ovary	Fibroblast
BHK-21	Syrian Hamster Kidney	Fibroblast
HEK 293	Human Kidney	Epithelial
Hep G2	Human Liver	Epithelial
BAE-1	Bovine aorta	Endothelial
SH-SY5Y	Human neuroblastoma	Neuroblast
Suspension Cell Lines		
Name	Species and tissue of origin	Morphology
NS0	Mouse myeloma	Lymphoblastoid
U937	Human Histiocytic Lymphoma	Lymphoblastoid
Namalwa	Human Lymphoma	Lymphoblastoid
HL60	Human Leukaemia	Lymphoblastoid
WEHI 231	Mouse B-cell Lymphoma	Lymphoblastoid
YAC 1	Mouse Lymphoma	Lymphoblastoid
U 266B1	Human Myeloma	Lymphoblastoid



There are some instances when cell cultures may grow as semi-adherent cells, e.g. B95-8, where there appears to be a mixed population of attached and suspension cells. For these cell lines it is essential that both cell types are subcultured to maintain the heterogeneous nature of the culture.

5.4 Phases of Cell Growth

It is important to know and record the growth characteristics of the cell line in use before starting any experiments. An alteration in cellular growth can indicate a significant problem within the cell line and if undetected can have detrimental effects on experimental results.

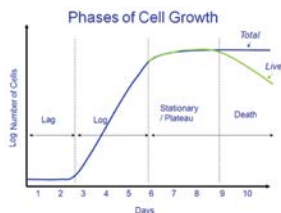
A typical growth curve for cultured cells displays a sigmoid pattern of proliferation. The growth phases associated with normal cells are defined as:

1. Lag Phase – at this stage the cells do not divide. During this period the cells adapt to the culture conditions and the length of this phase will depend upon the growth phase of the cell line at the time of subculture and also the seeding density.

2. Logarithmic (Log) Growth Phase – cells actively proliferate and an exponential increase in cell density arises. The cell population is considered to be the most viable at this phase, therefore it is recommended to assess cellular function at this stage. Each cell line will show different cell proliferation kinetics during the log phase and it is therefore the optimal phase for determining the population doubling time. Cells are also generally passaged at late log phase. Passaging cells too late, can lead to overcrowding, apoptosis and senescence.

3. Plateau (or Stationary) Phase – cellular proliferation slows down due to the cell population becoming confluent. It is at this stage the number of cells in the active cell cycle drops to 0-10% and the cells are most susceptible to injury.

4. Decline Phase – cell death predominates in this phase and there is a reduction in the number of viable cells. Cell death is not due to the reduction in nutrient supplements but the natural path of the cellular cycle.



Note: Different cell lines have different timescales for each phase, this graph is provided as a general example of a typical growth curve.

5.5 *In Vitro* Age of a Cell Culture

Two terms are predominantly used to define the age of a cell culture: (i) passage number - indicates the number of times the cell line has been sub-cultured and (ii) the population doubling (pd) number - indicates the number of cell generations the cell line has undergone i.e. the number of times the cell population has doubled. The *in vitro* age of a cell culture is particularly useful to know for cell lines with a finite lifespan or unstable characteristics that change over time in continuous culture.

Did You Know?

The cell population is at its most viable during the log growth phase.

6.0 The Cell Environment (including types of culture medium)

In general terms cultured cells require a sterile environment and a supply of nutrients for growth. In addition, the culture environment should be stable in terms of pH and temperature. Over the last 30 years various defined basal media types have been developed and are now available commercially. Originally, balanced salt solutions were used to maintain contractility of mammalian heart tissue and Tyrode's salt solution was designed for use in work with primary mammalian cells. These have since been modified and enriched with amino acids, vitamins, fatty acids and lipids. Consequently media suitable for supporting the growth of a wide range of cell types are now available. The precise media formulations have often been derived by optimising the concentrations of every constituent. Examples of the different media and their uses are given in Table 2 (see next page).



Table 2. Different types of culture medium and their uses

Media Type	Examples	Uses
Balanced salt solutions	PBS, Hanks' BSS, Earle's salts DPBS HBSS EBSS	Form the basis of many complex media
Basal media	MEM	Primary and diploid culture
	DMEM	Modification of MEM containing increased level of amino acids and vitamins. Supports a wide range of cell types including hybridomas
	GMEM	Glasgows modified MEM was defined for BHK-21 cells
Complex media	RPMI 1640	Originally derived for human leukaemic cells. It supports a wide range of mammalian cells including hybridomas
	Iscoves DMEM	Further enriched modification of DMEM which supports high density growth
	Leibovitz L-15	Designed for CO ₂ free environments
	TC 100 Graces insect medium Schneider's Insect medium	Designed for culturing insect cells
Serum free media	CHO HEK293	For use in serum free applications
	Ham F10 and derivatives Ham F12 DMEM/F12	Note: these media must be supplemented with other factors such as insulin, transferrin and epidermal growth factor. These media are usually HEPES buffered
Insect cells	Serum-Free Insect Medium 1 (Cat no. S3777)	Specifically designed for use with Sf9 insect cells

6.1 Basic Constituents of Media

- Inorganic salts
- Carbohydrates
- Amino Acids
- Vitamins
- Fatty acids and lipids
- Proteins and peptides
- Serum
- Trace Elements

Each type of constituent performs a specific function as outlined below:

6.2 Inorganic Salts

The inclusion of inorganic salts in media performs several functions. Primarily they help to retain the osmotic balance of the cells and help regulate membrane potential by provision of sodium, potassium and calcium ions. All of these are required in the cell matrix for cell attachment and as enzyme cofactors.

6.3 Buffering Systems

Most cells require pH conditions in the range 7.2-7.4 and close control of pH is essential for optimum culture conditions. There are major variations to this optimum. Fibroblasts prefer a higher pH (7.4-7.7) whereas, continuous transformed cell lines require more acid conditions pH (7.0-7.4).

Regulation of pH is particularly important immediately following cell seeding when a new culture is establishing and is usually achieved by one of two buffering systems; (i) a "natural" buffering system where gaseous CO_2 balances with the CO_3/HCO_3 content of the culture medium and (ii) chemical buffering using a zwitterion called HEPES.

Cultures using natural bicarbonate / CO_2 buffering systems need to be maintained in an atmosphere of 5-10% CO_2 in air usually supplied in a CO_2 incubator. Bicarbonate/ CO_2 is low cost, non-toxic and also provides other chemical benefits to the cells.

HEPES has superior buffering capacity in the pH range 7.2-7.4 but is relatively expensive and can be toxic to some cell types at higher concentrations (above ~100nMolar). HEPES buffered cultures do not require a controlled gaseous atmosphere.

Most commercial culture media include phenol red as a pH indicator so that the pH status of the medium is constantly indicated by the colour. Usually the culture medium should be changed/replenished if the colour turns yellow (acid) or purple (alkali).

6.4 Carbohydrates

The main source of energy is derived from carbohydrates generally in the form of sugars. The major sugars used are glucose and galactose, however, some media contain maltose or fructose. The concentration of sugar varies from basal media containing 1g/L to 4.5g/L in some more complex media. Media containing the higher concentration of sugars are able to support the growth of a wider range of cell types. Pyruvate is included in the formulation of some media, as an alternative energy source.

6.5 Amino Acids

Amino acids are the building blocks of proteins. 'Essential' amino acids must be added to culture media as cells are not able to synthesize these themselves. The concentration of amino acids in the culture medium will determine the maximum cell density that can be achieved - once depleted the cells will no longer be able to proliferate.

Did You Know?

In culture medium glutamine degrades rapidly to toxic ammonia at 37°C. Therefore aliquot your medium before use and only warm and use volumes you need that day. Discard opened bottles of prepared medium containing glutamine after 4-6 weeks.

In relation to cell culture, glutamine, an essential amino acid, is particularly significant. In liquid media or stock solutions glutamine degrades relatively rapidly. Optimal cell performance usually requires supplementation of the media with glutamine prior to use. Some media formulations include L-alanyl glutamine which is a more stable form of glutamine, and do not require supplementation.

Adding supplements of non-essential amino acids to media both stimulates growth and prolongs the viability of the cells in culture.

6.6 Vitamins

Serum is an important source of vitamins in cell culture. However, many media are also enriched with vitamins making them consistently more suitable for a wider range of cell lines. Vitamins are precursors for numerous co-factors. Many vitamins, especially B group vitamins, are necessary for cell growth and proliferation and for some lines the presence of B12 is essential. Some media also have increased levels of vitamins A and E. The vitamins commonly used in media include riboflavin, thiamine and biotin.

6.7 Proteins and Peptides

These are particularly important in serum free media. The most common proteins and peptides include albumin, transferrin, fibronectin and fetuin and are used to replace those normally present through the addition of serum to the medium.

6.8 Fatty Acids and Lipids

Like proteins and peptides these are important in serum free media since they are normally present in serum e.g. cholesterol and steroids essential for specialised cells.

6.9 Trace Elements

These include trace elements such as zinc, copper, selenium and tricarboxylic acid intermediates. Selenium is a detoxifier and helps remove oxygen free radicals.

6.10 Preparation of Media

Whilst all media may be made from the basic ingredients this is time consuming and may predispose to contamination. For convenience, many commonly used media are available as ready mixed powders or as 10x and 1x liquid media. All commonly used media are listed in the Sigma-Aldrich online catalogue. If powder or 10x media are purchased it is essential that the water used to reconstitute the powder or dilute the concentrated liquid is free from mineral, organic and microbial contaminants. It must also be pyrogen free and of tissue culture grade. In most cases water prepared by reverse osmosis and resin cartridge purification with a final resistance of 16-18MΩ is suitable. Once prepared the pH of the medium should be adjusted appropriately, and then the media filter sterilised before use.

6.11 Serum

Serum is a complex mix of albumins, growth factors and growth inhibitors and is probably one of the most important components of cell culture medium. The most commonly used serum is foetal bovine serum (FBS). Other types of serum are available including newborn calf serum and horse serum. The quality, type and concentration of serum can all affect the growth of cells and it is therefore important to screen batches of serum for their ability to support the growth of cells. In addition, there are other tests that may be used to aid the selection of a batch of serum including cloning efficiency, plating efficiency and the preservation of cell characteristics.

Serum is also able to increase the buffering capacity of cultures that can be important for slow growing cells or where the seeding density is low (e.g. cell cloning experiments). It also helps to protect against mechanical damage which may occur in stirred cultures or whilst using a cell scraper.

A further advantage of serum is the wide range of cell types with which it can be used despite the varying requirements of different cultures in terms of growth factors. In addition, serum is able to bind and neutralise toxins. However, serum is subject to batch-to-batch variation that makes standardisation of production protocols difficult.

There is also a risk of contamination associated with the use of serum. These risks can be minimised by obtaining serum from a reputable source since suppliers of large quantities of serum perform a battery of quality control tests and supply a certificate of analysis with the serum. In particular, serum is screened for the presence of bovine viral diarrhoea virus (BVDV) and mycoplasma. Heat inactivation of serum (incubation at 56°C for 30 minutes) can help to reduce the risk of contamination, since some viruses are inactivated by this process, however this process also denatures some proteins and destroys nutrients in the serum with modern production methods for serum, the routine use of heat inactivated serum is not an absolute requirement for cell culture. The use of serum also has a cost implication not only in terms of medium formulation but also in downstream processing. A 10% FBS supplement contributes 4.8mg of protein per millilitre of culture fluid which complicates downstream processing procedures such as protein purification.

6.12 Guidelines for Serum Use

Foetal bovine serum (FBS) has been used to prepare a number of biologicals and has an excellent record of safety. The recognition of Bovine Spongiform Encephalopathy (BSE) in 1986 and its subsequent spread into continental Europe along side the announcement of the probable link between BSE and a new variant of Creutzfeldt Jacob disease in Humans stimulated an increased concern about safe sourcing of all bovine materials. In 1993, the Food and Drug Administration (FDA) "recommended against the use of bovine derived materials from cattle which have resided in, or originated from countries where BSE has been diagnosed".

The current European Union (EU) guidelines on viral safety focus on sourcing, testing and paying particular attention to the potential risk of cross contamination during slaughtering or collection of the starting tissue. As far as BSE is concerned, the EU guidance on minimising the risk of BSE transmission

Did You Know?

The most commonly used serum is foetal bovine serum. Other types of serum are available including newborn calf serum and horse serum.



via medicinal products, EMEA/410/01 Rev. 3, recommends the main measures to be implemented in order to establish the safety of bovine material. Similarly the focus is on geographical origin, the age of the animals, the breeding and slaughtering conditions, the tissue to be used and the conditions of its processing.

The use of FBS in production processes of medicinal products is acceptable provided good documentation on sourcing, age of the animals and testing for the absence of adventitious agents is submitted. All responsible suppliers of FBS for bio-pharmaceutical applications will provide such documentation.

Regulatory requirements in Europe stress the importance of justifying the use of material of bovine, caprine or ovine origin in the production of pharmaceutical products. Thus, although FBS has been used for many years in the production process of many medicinal products such as viral vaccines and recombinant DNA products, at present there is a justified trend to remove all material of animal origin from manufacturing processes. Sigma-Aldrich has recognised this growing trend and works closely with customers to optimise animal free media formulations to meet each customer's cell culture requirements. Serum-free cell lines that have been adapted to media that do not contain serum are available from ECACC.

The United States Department of Agriculture (USDA) regulates all products that contain a primary component of animal origin. With specific reference to serum the USDA has declared that for materials which fall under their jurisdiction, only biological products manufactured using serum from approved countries of origin will be allowed in to USA.

6.13 Origin of Serum

ECACC only uses serum sourced from countries with a negligible risk of BSE. Historically Australian, New Zealand and USA sourced serum has offered the lowest risk of BSE contamination. It is essential to check the source country of serum used and their BSE risk status. Use of serum of the appropriate quality is particularly important if the intended use of the serum is in the production of medicinal or other products being sent to the USA.

Serum from a reputable supplier should have undergone various quality control tests which will be listed in the product information sheet. Most serum products are cell culture tested including growth promotion, cloning efficiency and plating efficiency tests.

Standard tests performed on serum commonly include tests to determine the presence and/or level of the following:

Sterility	Total Protein
Virus Contamination	Immunoglobulin
Mycoplasma Contamination	Hormone Testing
Endotoxin	pH (at room temperature)
Haemoglobin	Osmolality

7.0 Cryopreservation and Storage of Cells

7.1 Cryopreservation of Cell Lines

The aim of cryopreservation is to enable stocks of cells to be stored to prevent the need to have all cell lines in culture at all times. It is invaluable when dealing with cells of limited life span. The other main advantages of cryopreservation are:

- Reduced risk of microbial contamination
- Reduced risk of cross contamination with other cell lines
- Reduced risk of genetic drift and morphological changes
- Work conducted using cells at a consistent passage number (refer to section 8 'Good Cell Banking Practices')
- Reduced costs (consumables and staff time)

There has been a large amount of developmental work undertaken to ensure successful cryopreservation and resuscitation of a wide variety of cell lines of different cell types. The basic principle of successful cryopreservation and resuscitation is a slow freeze and quick thaw. Although the precise requirement may vary with different cell lines as a general guide cells should be cooled at a rate of -1°C to -3°C per minute and thawed quickly by incubation in a 37°C water bath for 3-5 minutes. If this and the additional points given below are followed then most cell lines should be cryopreserved successfully.

1. Cultures should be healthy with a viability of $>90\%$ and no signs of microbial contamination.
2. Cultures should be in log phase of growth (this can be achieved by using pre-confluent cultures i.e. cultures that are below their maximum cell density and by changing the culture medium 24 hours before freezing).
3. A high concentration of serum/protein ($>20\%$) should be used. In many cases serum is used at 90% .
4. Use a cryoprotectant such as dimethyl sulphoxide (DMSO) or glycerol to help protect the cells from rupture by the formation of ice crystals. The most commonly used cryoprotectant is DMSO at a final concentration of 10% , however, this is not appropriate for all cell lines e.g. 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). Sigma also offers ready made cell freezing media containing DMSO, glycerol and serum-free formulations containing DMSO.





7.2 Ultra-low Temperature Storage of Cell Lines

Following controlled rate freezing in the presence of cryoprotectants, cell lines can be cryopreserved in a suspended state for indefinite periods provided a temperature of less than -135°C is maintained. Such ultra-low temperatures can only be attained by specialised electric freezers or more usually by immersion in liquid or vapour phase nitrogen. The advantages and disadvantages can be summarised as follows:

Table 3. Comparison of ultra-low temperature storage methods for cell lines.

Method	Advantages	Disadvantages
Electric (-135°C) Freezer	• Ease of Maintenance • Steady temperature • Low running costs	• Requires liquid nitrogen back-up • Mechanically complex • Storage temperatures high relative to liquid nitrogen
Liquid Phase Nitrogen	• Steady ultra-low (-196°C) temperature • Simplicity and mechanical reliability	• Requires regular supply of liquid nitrogen • High running costs • Risk of cross-contamination via the liquid nitrogen
Vapour Phase Nitrogen	• No risk of cross-contamination from liquid nitrogen • Low temperatures achieved • Simplicity and reliability	• Requires regular supply of liquid nitrogen • High running costs • Temperature fluctuations between -135°C and -190°C

Storage in liquid phase nitrogen allows the lowest possible storage temperature to be maintained with absolute consistency, but requires the use of large volumes (depth) of liquid nitrogen which is a potential hazard. There have also been documented cases of cross contamination by virus pathogens via the liquid nitrogen medium. For these reasons ultra-low temperature storage is most commonly in vapour phase nitrogen.

For vapour phase nitrogen storage, the ampoules are positioned above a shallow reservoir of liquid nitrogen, the depth of which must be carefully maintained. A vertical temperature gradient will exist through the vapour phase, the extremes of which will depend on the liquid levels maintained, the design of the vessel, and the frequency with which it is opened. Temperature variations in the upper regions of a vapour phase storage vessel can be extreme if regular maintenance is not carried out. Modern designs of liquid nitrogen storage vessels are increasingly offering improved vapour phase storage technology.

Loss of entire cell stocks through inadequate storage maintenance is distressingly common. All liquid nitrogen storage vessels should minimally include alarms that warn of low liquid nitrogen levels and should also be constantly temperature monitored and alarmed. This is particularly true of vapour phase storage systems. The bulk liquid nitrogen storage vessel should not be allowed to become less than half full before it is re-supplied. This will ensure that at least one liquid nitrogen delivery can be missed without catastrophic consequences. It is highly recommended that valuable cell stocks should be backed up by storage at a second site. ECACC offers a Safe Deposit Service for this purpose.

7.3 Inventory Control

All ultra-low temperature storage vessels should include a racking / inventory system designed to organise the contents for ease of location and retrieval. This should be supported by accurate record keeping and inventory control incorporating the following:

- Each ampoule should be individually labelled, using “wrap around”, liquid nitrogen resistant labels with identity, lot number and date of freezing
- The location of each ampoule should be recorded ideally on an electronic database or spreadsheet, but also on a paper storage plan
- There should be a control system to ensure that no ampoule can be deposited or withdrawn without updating the records

7.4 Safety Considerations (Liquid Nitrogen)

General safety issues

It is important that staff are trained in the use of liquid nitrogen and associated equipment including the storage vessels which need to be vented safely and containers which may need to be filled. As with all laboratory procedures personal protective equipment should be worn at all times whilst handling nitrogen, including a full-face visor and thermally insulated gloves in addition to a laboratory coat and preferably a splash-proof plastic apron. Proper training and the use of protective equipment will minimise the risk of frostbite, burns and other adverse incidents.

Risk of asphyxiation

The single most important safety consideration is the potential risk of asphyxiation when escaped nitrogen vapourises and displaces atmospheric oxygen. This is critical since oxygen depletion can very rapidly cause loss of consciousness, without warning. Consequently liquid nitrogen refrigerators should be placed in well-ventilated areas in order to minimise this risk and be subject to planned preventative maintenance. Large volume stores should have low oxygen alarm systems.

Precautions for Dedicated Liquid Nitrogen Storage Areas

- Use oxygen alarms set to 18% oxygen (v/v)
- Staff training – staff should be trained to evacuate the area immediately on hearing the alarm and not return until the oxygen is back to normal levels (~ 20% v/v)
- Staff should work in pairs when handling liquid nitrogen
- Prohibit the use of nitrogen outside of normal working hours
- Mechanical ventilation systems should be installed if at all possible

Did You Know?

The glass transition temperature of water is -135°C , below this molecular movement ceases and all biological activity is suspended.





8.0 Good Cell Banking Practices

It is bad practice to maintain a cell line in continuous or extended culture for the following reasons:

- Risk of microbial contamination
- Loss of characteristics of interest (e.g. surface antigen or monoclonal antibody expression)
- Genetic drift particularly in cells known to have an unstable karyotype (e.g. CHO, BHK 21)
- Loss of cell line due to exceeding finite life-span e.g. human diploid cells such as MRC-5
- Risk of cross contamination with other cell lines
- Increased consumables and staff costs

All of these potential risk factors may be minimised by the implementation of a cell banking system as described below. This type of system is known as a tiered banking system or Master Cell Banking system (refer to Figure 3). On initial arrival into the laboratory a new cell culture should be regarded as a potential source of contamination e.g. harbouring bacteria, fungi and mycoplasma and should be handled under quarantine conditions until proven negative for such microbial contaminants. Following initial expansion 3-5 ampoules should be frozen as a Token Stock before a Master Cell Bank is prepared. One of the Token Stock ampoules should then be thawed and expanded to produce a Master Cell Bank of 10-20 ampoules depending upon the anticipated level of use.

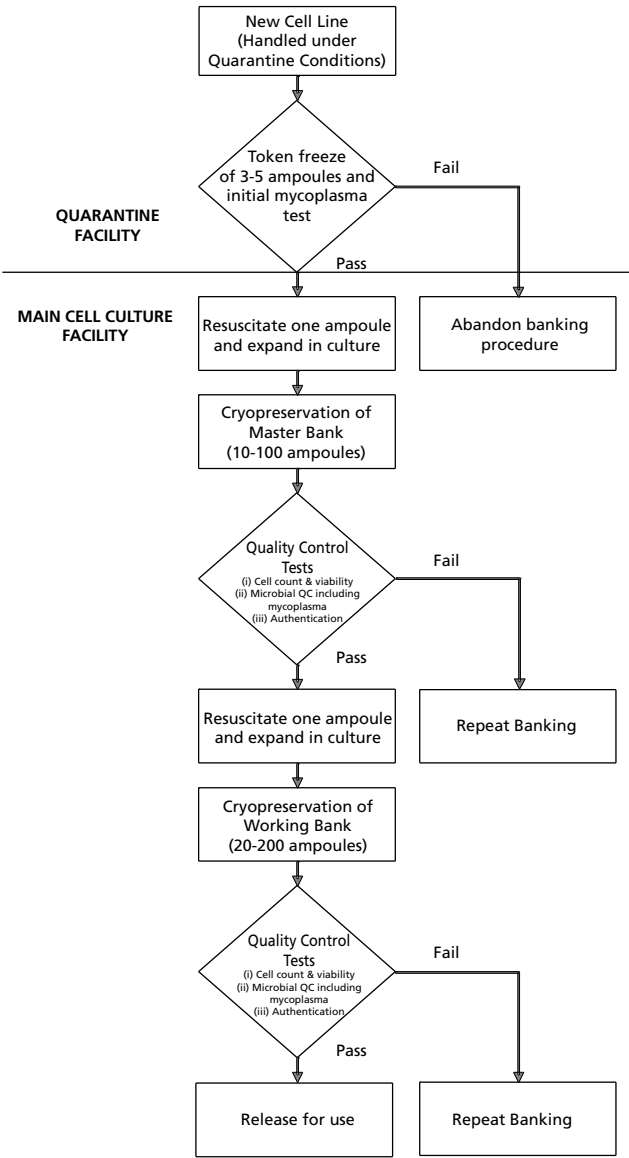
Ampoules of this Master Cell Bank (2-3) should be allocated for quality control comprising confirmation that the cell count and viability of the bank is acceptable and that the bank is free of bacteria, fungi and mycoplasma. Additional tests (such as viral screening and authenticity testing) may also be required. Once these tests have been completed satisfactorily an ampoule from the Master Cell Bank should be thawed and cultured to produce a Working Cell Bank. The size of this bank will again depend on the envisaged level of demand. Quality control tests (cell count and viability and the absence of microbial contaminants) are again required prior to using the cultures for routine experimentation or production.

It is also important at this stage to authenticate the Master and Working Cell Banks by STR profiling, DNA Bar-coding or SNP analysis.

Implementation of this banking system ensures:

- Material is of a consistent quality
- Experiments are performed using cultures in the same range of passage numbers
- Cells are only in culture when required
- The original cell line characteristics are retained

Figure 3. Schematic Representation of a Tiered Cell Banking System:



Did You Know?

It is bad practice to maintain a cell line in continuous or extended culture.



Notes

1. The number of ampoules prepared for Master and Working Cell Banks depends upon the forecast demand for their use.
2. The number of ampoules sampled for quality control is dependent upon the size of bank. Ideally 5-10% of the bank should be tested before use.
3. Ampoules from the Working Cell Bank should be used sequentially keeping cells in culture for not more than a predetermined number of cell doublings. This number will be least in the case of cell lines having a finite life-span (e.g. diploid lines).
4. The Working Cell Bank should be replenished from an ampoule of the Master Cell Bank. This should be done in sufficient time to allow the quality control to be completed.
5. A new Master Cell Bank should be prepared before the number of original Master stock drops below five ampoules.
6. The panel of quality control tests performed depends upon the use intended e.g. regulatory authorities may require additional tests such as viral screening and karyotypic studies.

9.0 Quality Control Considerations

9.1 Introduction

Quality is important in all aspects of tissue culture. The quality of materials used (cell lines, media and other reagents) will affect the quality of the cultures and the subsequent scientific data and products derived from them. The main areas of quality control that are of concern for tissue culture are:

- The quality of the reagents and materials
- The provenance and integrity of the cell lines
- The avoidance of microbial contamination

9.2 Reagents and Materials

Potential sources of contamination are reagents and materials, in particular bovine serum which has been identified as a source of bovine viral diarrhoea virus (BVDV). Porcine trypsin is also a potential source of Mycoplasma hyorhinis. Good quality reagents and materials are available from numerous manufacturers of tissue culture media and supplements. In addition, manufacturers such as Sigma will carry out a range of quality control tests including screening for mycoplasma and BVDV and supply a Certificate of Analysis with their products. These state the product and lot numbers and form a vital part of record keeping and tracking of reagents used in the production of cell stocks. It is advisable to further test key reagents such as foetal bovine serum to ensure that they are 'fit for purpose' due to batch-to-batch variation.

Manufacturers of sterile plastic ware (flasks, centrifuge tubes, pipettes) designed for tissue culture use also supply Certificates of Analysis for each batch produced, which should be kept for future reference.

9.3 Provenance and Integrity of Cell Lines

The sourcing of cell lines can have an important effect on quality; freshly imported cell lines are a major source of contamination. The advantages of obtaining cell lines from a recognised source such as a culture collection are that the cultures will be:

- Contaminant free
- Fully characterised and authenticated by use of short tandem repeat profiling for human cell lines, and determination of the species of origin by DNA bar-coding for other species
- Supplied with a detailed data sheet

Once cell lines have been obtained from a reputable source it is important to implement master and working cell banking procedures and the associated quality control steps such as routine testing for microbial contaminants and confirming the identity of cultures.



9.4 Avoidance of Microbial Contamination

Potential sources of contamination include other cell lines, laboratory conditions and staff poorly trained in core areas such as aseptic techniques and good laboratory practice. Thus the use of cells and reagents of known origin and quality alone is not sufficient to guarantee quality of product (cell stock or culture products); it is necessary to demonstrate quality throughout the production process and also in the final product. Routine screening aids the early detection of contamination since all manipulations are a potential source of contamination.

The three main types of microbial contaminants in tissue culture are:

- Bacteria and Fungi
- Mycoplasma
- Viruses

Bacterial and Fungal Contamination

Bacterial contamination is generally visible to the naked eye and detected by a sudden increase in turbidity and colour change of the culture medium as the result of a change in pH. The cell culture may survive for a short time but the cells will eventually die. Daily microscopic observation of cultures will ensure early detection of contamination and enable appropriate action to be taken as soon as the first signs of contamination become apparent. In addition, specific tests for the detection of bacteria and fungi should be used as part of a routine and regular quality control screening procedure (see Protocol 8 on page 55).

Mycoplasma Contamination

Mycoplasmas are the smallest free-living self-replicating prokaryotes. They lack a cell wall and lack the ability to synthesize one. They are 0.3µm in diameter and can be observed as filamentous or coccal forms. There are 6 major species that are tissue culture contaminants, namely *M. hyorhinis*, *M. arginini*, *M. orale*, *M. fermentans*, *M. hominis* and *Acholeplasma laidlawii*.

Did You Know?

Mycoplasmas are the smallest free-living self-replicating prokaryotes. They lack a cell wall and do not have the ability to synthesize one.

The effects of mycoplasma infection are more insidious than those of bacteria and fungi, inducing several long term effects in cell cultures. These include:

- Altered growth rate
- Morphological changes
- Chromosome aberrations
- Alterations in amino acid and nucleic acid metabolism

However, despite these well-documented effects the presence of mycoplasma is often not tested for with the consequence that in such laboratories the majority of cell lines are positive for mycoplasma. Mycoplasma contamination is difficult to detect requiring the use of specialist techniques (see Protocol 9 on page 57 - Isolation by Culture and Protocol 10 on page 59 – Detection by DNA Staining). In the past only specialist laboratories, such as culture collections, have performed these tests. However, a variety of commercial kits are now available although the performance characteristics of these kits can be extremely variable. A combination of these should be used as part of a routine and regular quality control screening procedure. ECACC tests cultures for the presence of mycoplasma on a routine basis and offers a mycoplasma testing service.

Viral Contamination

Some cell lines contain endogenous viruses and secrete virus particles or express viral antigens on their surface (e.g. Epstein-Barr Virus (EBV) transformed lines). These cell lines are not considered contaminated. However, bovine serum is a potential source of bovine viral diarrhoea virus (BVDV) contamination. Use of infected serum will lead to contamination of cell lines with the virus. Contamination of cell lines with BVDV may cause slight changes in growth rate but since this virus is non-cytopathic macroscopic and microscopic changes in the culture will not be detected. Suppliers of bovine serum are aware of this and screen sera accordingly and generally serum is sold as BVDV tested. Having said this you should be always check carefully to ensure that you understand the results of any testing that is performed on serum. Is the serum indicated as BVDV tested and none detected? What is the sensitivity of detection? BVDV tested serum is not necessarily BVDV free.

9.5 Environmental Monitoring

It is good practice to monitor the laboratory environment where cell cultures and their products are prepared. Class 2 microbiology safety cabinets, fitted with HEPA filters, should be tested every 6 months to ensure that they are working efficiently, i.e. the level of airflow across the filter should be tested. However, it is also advisable to monitor the level of ingress of contaminants into the cabinet by periodically placing open settle plates (Tryptone Soya Bean Agar bacteriological culture plates) on the cabinet work surfaces. In addition, settle plates should be used to assess airborne microbial burden at selected points around the laboratory. Plates should be left open for a period of 4 hours. After this time they should be covered, placed in sealed boxes and incubated at 32°C and 22°C for up to 7 days. At the end of this period the plates should be examined for the presence of microbial growth. The position of each plate in the cabinet should be recorded and results stored for trend analysis.

Acceptable limits should be defined in terms of “alert” levels and “action” levels, the actual values being dependent on the containment classification of the work area, the criticality of the work and the levels of cleanliness that can be achieved under normal operating conditions.

9.6 Aseptic Technique and Contamination Control

Personal Hygiene

When entering the laboratory it is important to wash hands since this will remove dry skin and loosely adherent microorganisms which could potentially contaminate cell cultures. Gowns and surgical gloves must be worn. Gloves should be frequently swabbed with 70% (v/v) sterile isopropanol. Other personal protective equipment includes head caps and face masks, but these are not always necessary, particularly when a class 2 microbiological safety cabinet is being used. Long hair should be tied back to remove obstruction and reduce the risk of contamination.

Working within the Microbiological Safety Cabinet

When working within the cabinet the operator should remember that the air-flow does not make the environment sterile but keeps it clean. Before any practical procedure is conducted the cabinet should be stocked with all the materials required for the experiment. In doing so the operator restricts the number of times that their hand/arm is removed from the cabinet into a non-clean environment. When stocking up the cabinet it is essential that a clutter free condition is maintained. Each item within the cabinet should be positioned to minimise movement and traffic over the area where cell culture operations are performed. Both the rear and front of the cabinet should be cleared to achieve maximum airflow. Flasks and dishes should be the last items to enter the cabinet. All items that enter the cabinet must be sprayed with 70% (v/v) sterile isopropanol to prevent dust and particulates from entering the cabinet. Twenty minutes should elapse before any tops or containers are opened to allow the airflow to purge the work area of particulates that may have been introduced.

Pipetting and Prevention of Aerosols

Disposable plastic pipettes (1ml, 2ml, 5ml, 10ml and 25ml) are the easiest forms to use for cell culture. Microbial and cellular contamination can arise through pipetting errors such as spillage of material. Adherence to the following guidance can minimise contamination and safety risks associated with pipetting:

- Never mouth pipette
- Use automatic pipette aids, with one pipette aid designated to each cabinet. Ensure the pipette fits into the pipette aid without any force. To avoid contamination disinfect the pipette aid regularly and ensure that filters are changed regularly (weekly)
- Use plugged pipettes when transferring medium
- Avoid drawing liquid into the pipette plug. Use an individual pipette once only
- To avoid generating aerosols do not create bubbles in the medium or pipette. Aerosols can spread contaminating microorganisms and by

Did You Know?

The main source of microbial contamination in the cell culture laboratory is the operator.





introducing cells into the air increases the potential risk of cross-contamination

- Clean spills that arise immediately with 70% (v/v) sterile isopropanol

9.7 What to do in the Event of Contamination

One hugely under-estimated problem in tissue culture is the routine use of antibiotics. Continuous use of antibiotics is unnecessary and can lead to the development of resistant strains that are difficult to eradicate and may require the use of more exotic antibiotics that may be toxic to the cell cultures. In addition, the use of antibiotics may mask a low level of contamination.

Once a contamination has been detected, whether it is due to bacteria, fungi or mycoplasma, the recommended course of action is to discard the culture and continue the work with earlier stocks that are known to be free of contaminants or obtain fresh stocks from a recognised source.

Viral infections are virtually impossible to remove from cultures since they do not respond to antibiotic treatment. Also, as they are intra-cellular parasites it is not possible to remove them by centrifugation or other separation techniques. If virus free stocks or a virus free alternative is not available, then a thorough risk assessment should be undertaken prior to continuing work with the infected cell line.

10.0 Authentication of Cell Lines

When culturing cells it is important to know that you are working with the correct reagents and this includes confirming the identity of the cells that you are working with. If the cells that you are growing are not what they are reported to be then work can be invalidated and resources wasted. A cell line is misidentified when its DNA profile is no longer consistent with the donor, from which it was originally established. This may be caused by a laboratory error such as mislabelling, confusion of similarly named cells lines, or it may be as the result of cross contamination from another, faster growing cell line being accidentally introduced into the culture. The exchange of cell cultures between research groups, without additional authentication has also contributed to the spread of this problem. There is now considerable evidence for widespread cross-contamination and misidentification of cell lines, in particular with HeLa cells, but there are many examples of other contaminating cells. The problem of misidentified and cross-contaminated cell lines has had a significant impact on the reproducibility of research, with one paper suggesting that 70-90% of preclinical research is not reproducible. Some studies have suggested that up to 20% of cancer research publications are based on work using misidentified cell lines. The financial implications of this problem are also considerable with the results of many research projects being invalidated and so the research funding wasted.

In 2012 ECACC scientists together with colleagues from other international

biological resource centres, and independent experts in the cell culture community established the International Cell Line Authentication Committee (ICLAC). ICLAC aims to make the issue of cell line misidentification more visible to the scientific community, promoting awareness and raising the profile of authentication testing as an effective way to combat the problem. ICLAC publishes a regularly updated database of misidentified cell lines which have been cross-contaminated or overgrown by other cells, and encourages researchers to submit additional information regarding any misidentified cell lines that are not listed on the ICLAC database. View the list at www.phe-culturecollections.org.uk/ICLAC. In 2015 more than 450 cell lines were listed as misidentified or cross-contaminated, many of these with no known authentic stock available. Worryingly many of these misidentified cell lines continue to be widely used and reported in the scientific literature. Many scientific journals are however now requiring some evidence of cell line authentication, as a pre-requisite to publishing, and many grant awarding bodies are making funds available for cell line authentication to be included in any projects involving cell culture, that they support.

To minimise the risk of working with contaminated cell lines it is advisable to obtain cells from a recognised source such as a culture collection that will have confirmed the identity and purity of the cells as part of the banking process. Tests used to authenticate cell cultures include isoenzyme analysis, karyotyping/cytogenetic analysis, molecular techniques including DNA profiling and, more recently short tandem repeat (STR) profiling for human cell lines and determination of the species of origin by DNA barcoding for other species. The use of single nucleotide polymorphisms (SNP's) for authentication may offer a cheaper method for 'in-house' testing that is more compatible with laboratory work-flows. While most of these techniques are generalised tests and are applicable to all cell lines additional specific tests may also be required to confirm the presence of a product or antigen of interest. ECACC offers a cell line identity verification service.

Tips for keeping your cells authentic

1. Check if the cell line you have chosen is on the ICLAC list of misidentified cells.
2. Obtain evidence of authenticity from your supplier.
3. If you need to prepare stocks of your chosen cell line use good cell culture techniques.
4. Confirm that your stocks are free from mycoplasma and bacterial contamination - Use a third party service if you are not sure how to undertake these tests.
5. Re-test the identity of the cells at the end of the project.
6. If you are establishing a new cell line keep a sample of the donor material (blood, biopsy etc); determine the profile of the donor material and the cell line using the STR profiling method for human cells or another suitable method for non-human cells. Use third party services if necessary.

Introduction to 3D Cell Culture

For decades, three-dimensional (3D) cell culture has been employed by tissue engineers, stem cell scientists, cancer researchers and cell biologists, largely in university settings. The development of new materials or methods has been driven by the desire of these scientists to incorporate experimental systems that better represent the *in vivo* environment into their research. Early adopters of 3D cell culture technology have reaped the benefits of better data with groundbreaking knowledge of tissue and cancer behavior. 3D cell culture methods were once expensive, messy, laborious, and difficult to adapt to existing procedures. Today, researchers can pick among an array of 3D cell culture tools, ranging from simple to complex, to fit their specific needs. Segmentation of the 3D cell culture choices into discrete categories demonstrates a new maturation in the market.

Option	Advantages	Concerns	Applications
Scaffold-free systems	No added materials; Consistent spheroid formation; Co-cultures possible; Transparent HTS capable; compatible with liquid handling tools	No support or porosity; Limited flexibility; Size of spheroid limiting	Basic research; Drug discovery; Personalized medicine
<i>In vitro</i> 3D scaffolds for laboratory applications	Large variety of materials possible for desired properties; Customizable Co-cultures possible	Possible scaffold-to-scaffold variation; May not be transparent; Cell removal may be difficult; HTS options limited	Basic research; Drug discovery; Cell expansion
Gels	Large variety of natural or synthetic materials; Customizable Co-cultures possible	Gelling mechanism Gel-to-gel variation and Structural changes over time; Undefined constituents in natural gels; May not be transparent; HTS options limited	Basic research; Drug discovery
Bioreactors	Several options available; High volume cell production Customizable	Cost; HTS options limited	Basic research; Tissue engineering; Cell expansion
Microchips	<i>In vitro</i> organ specific systems; High gas permeability; Transparent	Commercial availability; Required expertise; Cost; HTS options limited	Basic research; Drug discovery

Table 4. Different existing options for 3D cell culture approach

11.0 Alternative Culture Systems

11.1 Cell Culture Scale-up Systems

Most tissue culture is performed on a small scale where relatively small numbers of cells are required for experiments. At this scale cells are usually grown in T flasks (see image on page 7) ranging from 25cm² to 175cm². Typical cell yields from a T175 flask range from 1x10⁷ for an attached line to 1x10⁸ for a suspension line. However, exact yields will vary depending on the cell line. It is not practical to produce much larger quantities of cells using standard T flasks, due to the amount of time required for repeated passaging of the cells, demand on incubator space and cost.

When considering scaling up a cell culture process there are a whole range of parameters to consider which will need to be developed and optimised if scale-up is to be successful. These include problems associated with nutrient depletion, gaseous exchange, particularly oxygen depletion, and the build up of toxic by-products such as ammonia and lactic acid. To optimise such a process for quantities beyond 1L volumes it is best left to expert process development scientists. Note, in these cases often a “scale-down” approach is adopted to allow many parameters to be evaluated on many replicates.

However, there are many commercially available systems that attempt to provide a “half-way house” solution to scale-up which do not necessarily require expert process development services. A selected list of some of the systems available along with a brief summary of their potential yields, advantages and disadvantages is provided in Table 5 on page 34.

11.2 Scale-up Solutions

Please refer to the following sub-sections for a variety of alternative scale-up solutions.

A word of caution – although the systems listed in Table 5 are often described as “off-the-shelf” solutions to scale-up they are not universally applicable to all cell types and often require a period of familiarisation and optimisation.

11.3 Roller Bottle Culture

This is the method most commonly used for initial scale-up of attached cells also known as anchorage dependent cell lines. Roller bottles are cylindrical vessels that revolve slowly (between 5 and 300 revolutions per hour) which bathe the cells that are attached to the inner surface with medium. Roller bottles are available typically with surface areas of 1050cm². The size of some of the roller bottles presents problems since they are difficult to handle in the confined space of a microbiological safety cabinet. Roller bottles with expanded inner surfaces have become available which has made handling large surface area bottles more manageable, but repeated manipulations and subculture with roller bottles should be avoided if possible. A further problem with roller bottles is with the attachment of cells since some cell lines do not attach evenly. This is a particular problem with epithelial cells. This may be partially overcome by optimising the speed of rotation, generally by decreasing the speed, during the period of attachment for cells with low attachment efficiency. The RC40 from Cellaon provides a semi-automated multiple roller bottle platform.



Figure 4. Roller Deck



Figure 5. Roller Bottles



Figure 6.
Disposable Spinner Flasks

Table 5. 'Half-Way House' Solutions to Scale-up - without attempting to adapt cells or the process.

Technology	Sus	Att	Vol (ml)	S/A (cm ²)	Max cells (sus)	Max cells (att)	Advantages	Disadvantages
T Flask	✓	✓	150	225	1.5x10 ⁶	~10 ⁷	Cheap, disposable, no cleaning/sterilisation required	Small scale. Multiples required for larger batches
Triple Flask	✓	✓	150	525	1.5x10 ⁶	3x10 ⁷	Cheap, disposable, no cleaning/sterilisation required. Save space.	Difficult to harvest attached cells. Multiples required for larger batches
Cell Stack™ (10 layers)	N/A	✓	2000		N/A	1.5x10 ¹⁰	Disposable – single batch manufacture.	Require additional equipment (vessels etc. may require cleaning). Difficult to harvest cells
Roller Bottles	✓	✓	1000	1700	1x10 ⁹	1x10 ⁸	Cheap, disposable, no cleaning/sterilisation required. Automated systems available.	Require "decks" to turn. Multiples required for larger batches. Automation very costly
Expanded Roller Bottles	N/A	✓	(1000)	3400	N/A	2x10 ⁸	As above	As above (no advantage for suspension cells)
Shake Flasks	✓	N/A	1000	N/A	1x10 ⁹	N/A	Some disposables available.	Suspension only. Glass vessels to be cleaned & sterilised. Requires shaker incubator.
Spinner Flasks	✓	N/A	1000	N/A	1x10 ⁹	N/A	Some semi-disposables available	Suspension only. Glass vessels to be cleaned & sterilised. Requires Stirrer-base and incubator.
HyperFlask™	N/A	✓	560	1720	N/A	2x10 ⁸	Disposable, Small footprint	Revolutionary Design
Sus = suspension, Att = attached, Max Vol = maximum volume, Max cells = maximum cells, S/A = surface area								

11.4 Multilayer Vessels

A variety of disposable multi-layer vessels are now available for simple and rapid scale-up of anchorage dependent cells with little or no process optimisation. These include triple layer flasks which are useful for maximising incubator space, however it is vessels such as CellStacks and Hyperflasks that offer the greatest advantages. CellStacks™ provide multiples of 1, 2, 5, 10 and 40 layers, each layer offering 636cm² (therefore a 10 layer Cellstack™ provides 6,360cm² for cell growth in a single vessel). Cell stacks are in effect giant cell culture flasks but with two vented caps for filling, harvest and gas exchange rather than a single cap. Some familiarisation, validation of cell growth and care and attention to manual handling is required as these vessels can only be practically employed if pouring techniques are used for filling and harvesting (although filling connectors can be exchanged for the caps) but in essence most cell growth in T flasks can be directly translated to CellStacks™. Forty layer CellStacks™ are too large to be handled manually and require specialised trolleys for manipulations. Originally designed for robotic systems Hyperflasks™ are another multilayer system, however, rather more revolutionary in their design. Consisting of 10 multiple “flasklets” each with the same approximate footprint as a T175 flask the HyperFlask™ is entirely filled with medium and cell inoculum. Gaseous exchange in this case is achieved by diffusion of gases directly through the thin surfaces of the flasklets.

11.5 Disposable solutions for anchorage independent (suspension) cells

In the last few years many new disposable systems for growth of cells in suspension have emerged. These include disposable stirrer vessels, single use disposable bioreactors and Wave/Cultibag (GE Healthcare®/Sartorius Stedim®) technology. The Wave™/Cultibag™ systems allow cell growth in large (10s of litres) sterile, disposable bags which are filled with cells, medium and an air/gas headspace and then gently agitated on a temperature controlled rocking platform. These bags are either “off the shelf” or custom made with most of the connectors for seed, harvest and sampling built in. Recent advances in disposable sensors has now also meant that pH and dissolved oxygen sensors can be built into the bags making them efficient bioreactors suitable for GMP production and seed vessels for larger bioreactors.

11.6 Spinner Flask Culture

This is the method of choice for suspension lines including hybridomas and attached lines that have been adapted to growth in suspension e.g. HeLa S3. Spinner flasks are either plastic or glass bottles with a central magnetic stirrer shaft and side arms for the addition and removal of cells and medium, and gassing with CO₂ enriched air. Inoculated spinner flasks are placed on a stirrer and incubated under the culture conditions appropriate for the cell line. Cultures should be stirred at 100-250 revolutions per minute.

Footnote: Information on commercial scale-up systems is valid on the date of last revision, but such systems may be improved or supplanted subsequently.

11.7 Other Scale-up Options

The next stage of scale-up for both suspension and attached cell lines is the bioreactor that is used for large culture volumes (in the range 100-10,000 litres). For suspension cell lines the cells are kept in suspension by either a propeller in the base of the chamber vessel or by air bubbling through the culture vessel. However, both of these methods of agitation give rise to mechanical stresses. A further problem with suspension lines is that the density obtained is relatively low e.g. 2×10^6 cells/ml.

For attached cell lines the cell densities obtained are increased by the addition of micro-carrier beads. These small beads are 30-100µm in diameter and can be made of dextran, cellulose, gelatin, glass or silica, and increase the surface area available for cell attachment considerably. The range of micro-carriers available means that it is possible to grow most cell types in this system. A recent advance has been the development of porous micro-carriers which has increased the surface area available for cell attachment by a further 10-100 fold. The surface area on 2g of beads is equivalent to 15 small roller bottles.

Cell Lines available from ECACC

ECACC has a range of cell line collections grouped according to the type of cell line and the data available. We have an active program to acquire new cell lines and therefore it is always worth checking our website for new lines. ECACC is accredited to ISO 9001:2008. The collections include:

General Collection

More than 1200 cell lines originating from over 45 different species and a wide variety of tissues. Over 600 human cell lines including many different cancer and drug resistant cell lines are available.

Primary Cells & Media Collection

A range of normal primary cells from a diverse selection of human and animal tissues. These cells are usually not more than 6 passages from the primary culture. They are more representative of the main functional component of the tissue from which they are derived when compared to continuous (tumour or artificially immortalised) cell lines. The recommended culture media is also available to order.

Hybridoma Collection

Over 400 monoclonal antibody-secreting hybridomas with a diverse range of antigen specificities.

Chromosomal Abnormality Collection

Approximately 3000 cell lines (the majority are B-lymphoblastoid cell lines) from individuals with defined chromosomal abnormalities.

HLA-Typed Collection

A specialised collection of over 430 reference B-lymphoblastoid cell lines, originating from laboratories all over the world, for which Human Leukocyte Antigen (HLA) typing data is available.

Human Random Control Collection

A collection of more than 700 B-lymphoblastoid cell lines derived from randomly selected Caucasian blood donors whose parents and grandparents were born in the UK or Ireland.

Supply Formats

Cell lines can be supplied as frozen or growing cultures. Genomic DNA, RNA and cDNA, extracted from the cell lines are also available.

Quality Control & Authentication

All cell lines supplied by ECACC undergo full quality control and authentication procedures. These include testing for mycoplasma by culture isolation, Hoescht DNA staining and PCR, together with culture testing for contaminant bacteria, yeast and fungi. Authentication procedures used include species verification by isoenzyme analysis and identity verification by DNA profiling. Classical DNA fingerprinting using multi-locus probes is carried out for non-human cell lines. Human cell lines are analysed by PCR of short tandem repeat sequences within chromosomal microsatellite DNA (STR-PCR). Certificates of Analysis are available for each cell line lot no. supplied which report the cell count, identity of the cell line and the absence of bacteria, fungi and mycoplasma.

European Bank of Induced Pluripotent Stem Cells (EBiSC) Collection

This growing collection is supplied by EBiSC a large European public-private partnership project supported jointly by the Innovative Medicines Initiative (IMI) and members of the European Federation of Pharmaceutical Industries and Associations (EFPIA). It contains disease representative and control human induced pluripotent stem cells (iPSCs) for medical researchers both studying diseases and developing new treatments.

The Human Induced Pluripotent Stem Cells Initiative (HipSci) Collection

A large, high-quality reference panel of human induced pluripotent stem cell (iPSC) lines. The iPSC lines are generated from hundreds of healthy donors, plus several cohorts of donors with inherited genetic diseases. Cell lines from phenotypically normal donors allows the study of how common genetic variations affect the cellular phenotypes.

Visit www.phe-culturecollections.org.uk to view the collections.

ECACC cell lines can be ordered via **SIGMA**
Life Science



12.0 Cell Culture Protocols

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 (PPE), (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. Use disposable head caps to cover hair.
3. Wear dedicated PPE for the tissue culture facility and keep separate from PPE worn in the general laboratory environment. The use of different coloured 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 ampoules 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 mislabelling 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% isopropanol) between operations and allow a minimum of 15 minutes between handling different cell lines.
8. Maintain separate bottles of media for each cell line in culture.
9. Examine cultures and media daily for evidence of gross bacterial or fungal contamination. This includes medium that has been purchased commercially.
10. Check quality control information for all media and reagents and ideally test performance prior to use.
11. Keep cardboard packaging to a minimum in all cell culture areas.
12. Ensure that incubators, cabinets, centrifuges and microscopes are cleaned and serviced at regular intervals.
13. Test cells for the presence of mycoplasma on a regular basis.

The Don'ts

1. Do not use antibiotics continuously in culture medium as this can lead to the appearance of antibiotic resistant strains and may mask underlying contamination.
2. Do not allow waste to accumulate particularly within the microbiological safety cabinet or in the incubators.
3. Do not have too many people in the lab at any one time.
4. Do not 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 cultures from becoming fully confluent. Always sub-culture at 70-80% confluency or as advised on the ECACC cell culture data sheet.
7. Do not allow media to go out of date. The shelf life is only 4 - 6 weeks at +4°C once glutamine and serum are added.
8. Avoid water baths from becoming dirty by regular cleaning.
9. Do not 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

- 70% (v/v) isopropanol in sterile water
- Sodium Hypochlorite (also known as 'Chlorox')
- Appropriate disinfectant

Equipment

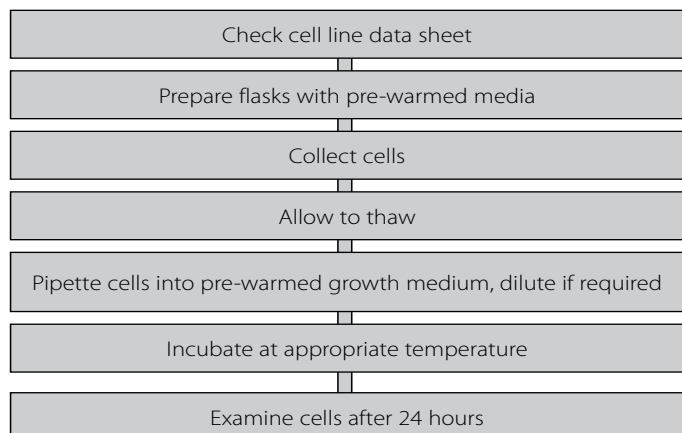
- Personal protective equipment (sterile gloves, laboratory coat, safety visor, overshoes, head cap)
- Microbiological safety cabinet at appropriate containment level

Procedure

1. Sanitise the cabinet using 70% isopropanol before commencing work.
2. Sanitise gloves by spraying them with 70% isopropanol and allowing to air dry for 30 seconds before commencing work.

3. Put all materials and equipment into the cabinet prior to starting work. 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% isopropanol prior to use.
4. Whilst working do not contaminate gloves by touching anything outside the cabinet (especially face and hair). If gloves become contaminated re-spray with 70% isopropanol as above before proceeding.
5. Discard gloves after handling contaminated cultures and at the end of all cell culture procedures.
6. Movement within and immediately outside the cabinet must not be rapid. Slow movement will allow the air within the cabinet to circulate properly.
7. Speech, sneezing and coughing must be directed away from the cabinet so as not to disrupt the airflows.
8. After completing work disinfect all equipment and material before removing from the cabinet. Spray the work surfaces inside the cabinet with 70% isopropanol and wipe dry with tissue. Dispose of tissue by incineration.
9. Liquid cell culture waste should be discarded in sodium hypochlorite (10,000 ppm) and must be kept in the cabinet for a minimum of two hours (preferably overnight) prior to discarding to the drain with copious amounts of water.
10. Periodically clean the cabinet surfaces with a disinfectant 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 the cells they 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, are toxic above 4°C therefore it is essential that cultures are thawed quickly and diluted in culture medium to minimise the toxic effects.

Materials

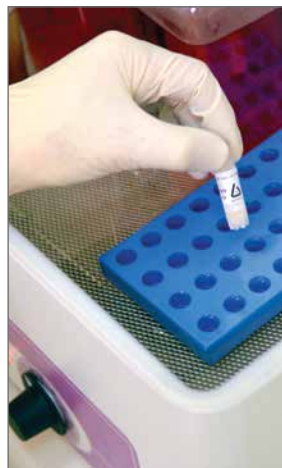
- Media– pre-warmed to the appropriate temperature (refer to the ECACC Cell Line Data Sheet for the correct medium and temperature.)
- 70% (v/v) isopropanol in sterile water
- DMSO
- Trypan blue (vital stain)

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Incubator
- Pre-labelled flasks
- Inverted phase contrast microscope
- Haemocytometer
- Centrifuge
- Marker Pen
- Pipettes
- Ampoule Rack
- Tissue
- Container to transport frozen ampoules e.g. box of dry ice or liquid nitrogen Dewar
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at appropriate containment level

Procedure

1. Read the cell line data sheet to establish specific requirements for your cell line.
2. Prepare the flasks - label with cell line name, passage number and date.
3. Collect an ampoule of cells from liquid nitrogen storage wearing appropriate personal protective equipment and transfer to the laboratory in a container of liquid nitrogen or on dry ice. It is important to handle the ampoules with care: on rare occasions ampoules may explode on warming due to expansion of trapped residual liquid nitrogen.
4. In a microbiological safety cabinet, hold a tissue soaked in 70% alcohol around the cap of the frozen ampoule and turn the cap a quarter turn to release any residual liquid nitrogen that may be trapped. Re-tighten the cap. Quickly transfer the ampoule to a 37°C waterbath until only one or two small ice crystals, if any, remain (1-2 minutes). It is important to thaw rapidly to minimise any damage to the cell membranes. Note: Do not totally immerse the ampoule as this may increase the risk of contamination.
5. Wipe ampoule with a tissue soaked in 70% alcohol prior to opening.
6. Pipette the whole content of the ampoule into a sterile tube (e.g. 15 ml capacity). Then slowly add 5ml pre-warmed medium that has already been supplemented with the appropriate constituents. Determine the viable



cell density (see Protocol 6 – Cell Quantification on page 50). Transfer the appropriate volume of cell suspension to a flask to achieve the cell seeding density recommended on the cell line data sheet.

For adherent cell lines: Adjust the volume of the medium, and if necessary the flask size, to achieve the cell seeding density recommended on the cell line data sheet. A pre-centrifugation step to remove cryoprotectant is not normally necessary as the first media change will remove residual cryoprotectant. If it is, then this will be specified on the data sheet. If the cells are to be used immediately (e.g. for a cell based assay), rather than subcultured, it may be advisable to perform a pre-centrifugation step to remove cryoprotectant.

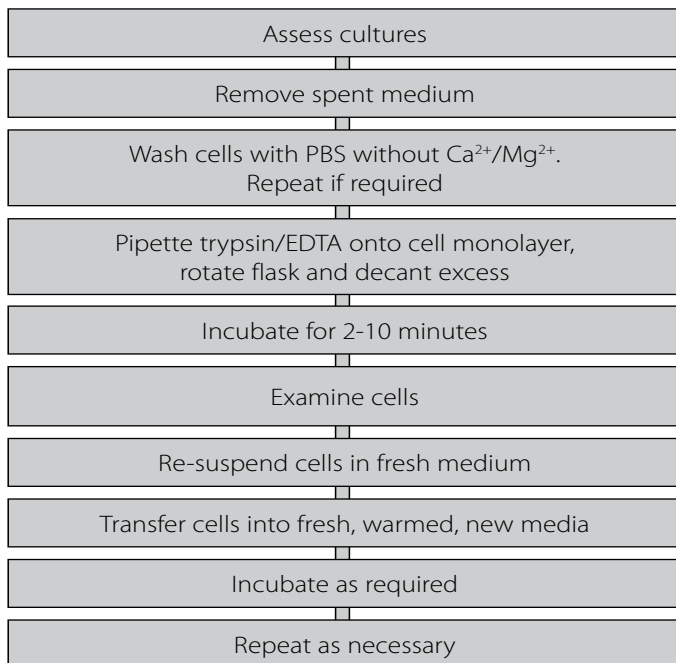
For suspension cell lines: A pre-centrifugation step to remove cryoprotectant is recommended i.e. pellet the cells by centrifugation at 150 x g for 5 minutes and resuspend the cell pellet in fresh medium using the appropriate volume to achieve the correct seeding density.

7. Incubate at the temperature and CO₂ level recommended on the data sheet. If a CO₂ fed incubator is used the flask should have a vented cap to allow gaseous exchange.
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 particular cell type. For example, some cell types are known to differentiate in the presence of DMSO. In such cases the cells should be washed in media before being added to their final culture flasks.
2. The addition of the thawed cell suspension to culture medium effectively dilutes the cryoprotectant (e.g. DMSO) reducing the toxicity of the cryoprotectant. That is why it is important to add the thawed cell suspension to a larger volume of culture medium immediately after the ampoule has thawed; do not allow thawed ampoules to sit at room temperature for long periods.
3. Do not use an incubator or the palm of your hand to thaw cell cultures since the rate of thawing achieved is too slow resulting in a loss of viability. Use a water bath as described in the protocol above.
4. If a CO₂ incubator is not available gas the flasks for 1-2 minutes with 5% CO₂ in 95% air filtered through a 0.2µm filter.
5. For most cultures it is best practice to subculture before confluence is reached so that the cells are harvested during their log phase of growth and are at optimum viability ready for seeding into new flasks. Furthermore there are some specific cell types that must be subcultured before confluence is reached in order to maintain their characteristics e.g. the contact inhibition of NIH 3T3 cells is lost if they are allowed to reach confluence repeatedly.
6. Some hybridomas may be slow to recover post resuscitation therefore start in 20% (v/v) FBS and 10% (v/v) hybridoma enhancing supplement in the appropriate medium.

12.4 Protocol 3 - 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 subcultured 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% (v/v) isopropanol in sterile water
- PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$
- 0.25% trypsin/EDTA in HBSS, without $\text{Ca}^{2+}/\text{Mg}^{2+}$
- Soyabean Trypsin Inhibitor
- Trypan blue (vital stain)



Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at appropriate containment level
- Incubator
- Pre-labelled flasks
- Inverted phase contrast microscope
- Centrifuge
- Haemocytometer
- Marker Pen
- Pipettes
- Ampoule 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.
3. Wash the cell monolayer with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ 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 onto the washed cell monolayer using 1ml per 25cm² 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 100-200µl and perform a cell count (see Protocol 6 - Cell Quantification, p.50). In the case of cells cultured in serum-free media, use a trypsin inhibitor e.g. soyabean trypsin inhibitor to inactivate the trypsin.
8. Transfer the required number of cells to a new labelled flask containing pre-warmed medium (refer to the appropriate 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.

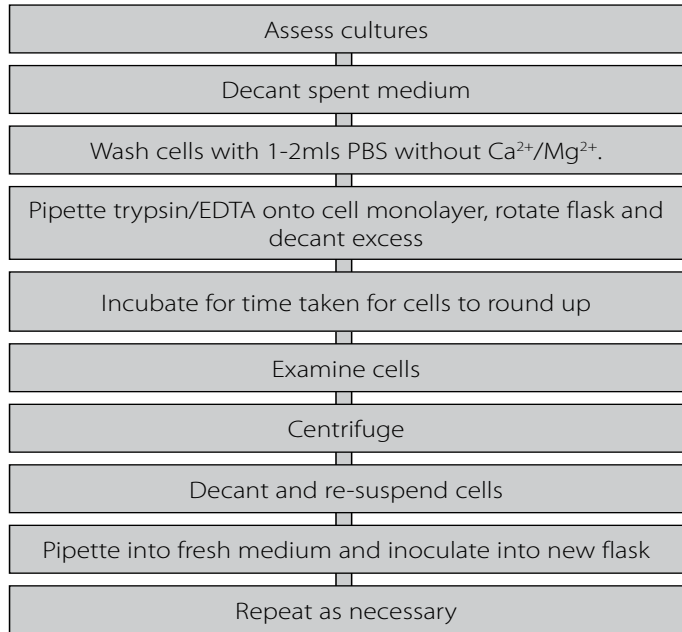
Key Points

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 EDTA enhances the activity of the enzyme by removing inhibitory cations.
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 $\text{Ca}^{2+}/\text{Mg}^{2+}$.
4. Cells should only be exposed to trypsin/EDTA long enough to detach cells. Prolonged exposure could damage cell surface receptors.
5. Trypsin should be neutralised with serum prior to seeding cells into new flasks otherwise cells will not attach.
6. Trypsin may also be neutralised by the addition of soyabean trypsin inhibitor, 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 cultures.
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 $0.2\mu\text{m}$ filter.
8. If the cells harvested are at too low a cell density to re-seed at the appropriate cell density into fresh flasks it may be necessary to centrifuge the cells e.g. 5 mins at $150 \times g$, and resuspend in a smaller volume of medium.

Did You Know?

Serum-free media will not inactivate trypsin. A trypsin inhibitor, e.g. soyabean trypsin inhibitor, must be used.

12.5 Protocol 4 - 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% (v/v) isopropanol in sterile water
- PBS without Ca²⁺/Mg²⁺
- 0.25% trypsin/EDTA in HBSS, without Ca²⁺/Mg²⁺
- Soyabean Trypsin Inhibitor
- Trypan blue (vital stain)

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at the appropriate containment level
- Centrifuge
- Inverted phase contrast microscope
- Incubator
- Haemocytometer
- Pre-labelled flasks
- Marker Pen
- Pipettes

Procedure

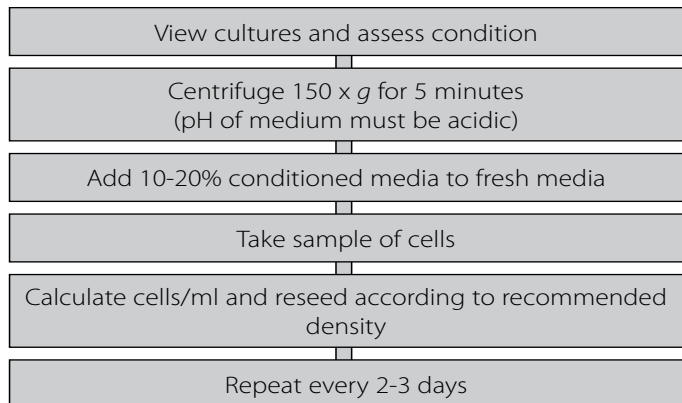
1. View cultures using an inverted phase contrast microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants. Give the flask a gentle knock first, this may dislodge the cells from the flask and remove the need for a trypsinisation step loss of some cells due to associated washes.
2. Decant medium containing non-adherent cells into a sterile centrifuge tube and retain.
3. Wash any remaining attached cells with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ using 1-2ml for each 25cm^2 of surface area. Retain the washings.
4. Pipette trypsin/EDTA 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 (see Protocol 6 - Cell Quantification).
7. Transfer the cells into the centrifuge tube containing the retained spent medium and cells.
8. Centrifuge the entire cell suspension at $150 \times g$ for 5 minutes.
9. Remove the supernatant and re-suspend the cell pellet in a small volume (10-20ml) of fresh culture medium. Count the cells.
10. Pipette the required number of cells into a new labelled 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 enhances the activity of the enzyme by removing inhibitory cations.
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 $\text{Ca}^{2+}/\text{Mg}^{2+}$. Repeated warming to 37°C also inactivates trypsin.
3. Cells should only be exposed to trypsin/EDTA long enough to detach cells. Prolonged exposure could damage cell surface receptors. In general, a shorter time of exposure to trypsin is required for semi-adherent cell lines in comparison to adherent cell lines.
4. Trypsin should be neutralised with serum prior to seeding cells into new flasks otherwise cells will not attach.
5. Trypsin may also be neutralised by the addition of Soyabean Trypsin Inhibitor, where an equal volume of inhibitor at a concentration of 1 mg/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 $5\% \text{CO}_2$ in 95% air filtered through a $0.2\mu\text{m}$ filter.

12.6 Protocol 5 - 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 cell lines subculture by dilution is relatively easy. However, for cell lines that grow in clumps it may be necessary to bring the cells into a single cell suspension by centrifugation and re-suspension 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% (v/v) isopropanol in sterile water
- Trypan blue (vital stain)

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- Incubator
- Inverted phase contrast microscope
- Haemocytometer
- Pre-labelled flasks
- Marker Pen
- Pipettes



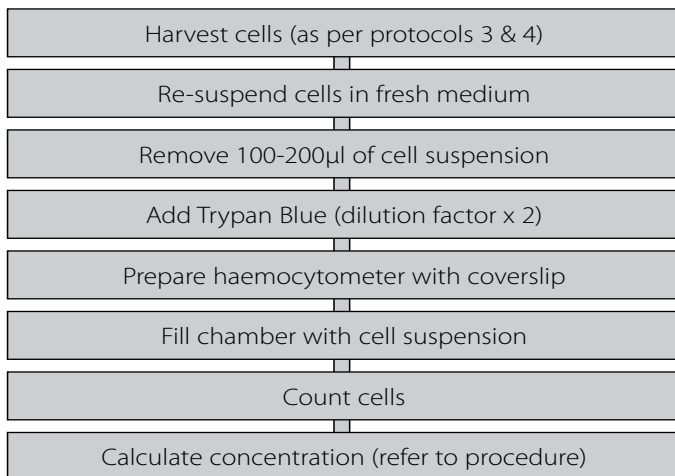
Procedure

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 centre 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 150 x g 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 (100-200µl) of the cells from the cell suspension and count the cells (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. Refer to the data sheet supplied with the cell line for the recommended seeding density.
4. Repeat this every 2-3 days.

Key Points

1. If the cell line is a hybridoma or another 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



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 standardise procedures using cell cultures. This in turn gives results with better reproducibility.

Equipment

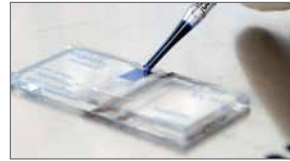
- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- CO₂ incubator
- Haemocytometer
- Inverted phase contrast microscope
- Pre-labelled flasks

Materials

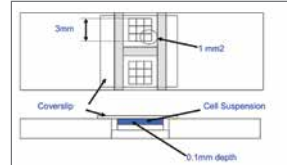
- Media– pre-warmed to appropriate temperature (refer to the ECACC Cell Line Data Sheet for the correct medium and temperature)
- 70% (v/v) isopropanol in sterile water
- 0.4% Trypan Blue Solution
- Trypsin/EDTA

Procedure

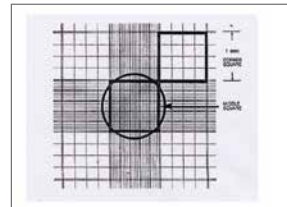
1. Bring adherent and semi-adherent cells into suspension using trypsin/EDTA as described previously (Protocol 3 and 4) and resuspend in a volume of fresh medium at least equivalent to the volume of trypsin. For cells that grow in clumps centrifuge and resuspend in a small volume and gently pipette to break up clumps.
2. Under sterile conditions remove 100-200µl of cell suspension.
3. Add an equal volume of Trypan Blue (dilution factor =2) and mix by gentle pipetting.
4. Clean the haemocytometer.
5. Moisten the coverslip with water or exhaled breath. Slide the coverslip 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 coverslip).
6. Fill both sides of the chamber with cell suspension (approximately 5-10µl) and view under an inverted phase contrast microscope using x20 magnification.
7. Count the number of viable (seen as bright cells) and non-viable cells (stained blue). 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.



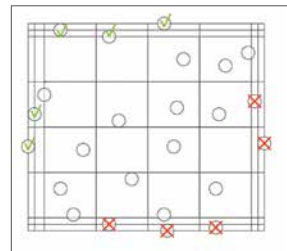
A haemocytometer being filled with cell suspension



Dimensions of a haemocytometer



Grid on a haemocytometer



Guide to which cells to count on grid i.e. include cells in middle of grid and cells that overlap edge on two sides (ticked), exclude cells that overlap edge on other two sides (crossed)

Where:

$$\text{Viable Cell Count (live cells per millilitre)} = \frac{\text{Number Live Cells Counted}}{\text{Number of large corner Squares counted}} \times \text{Dilution factor (in this example 2) } \times 10,000$$

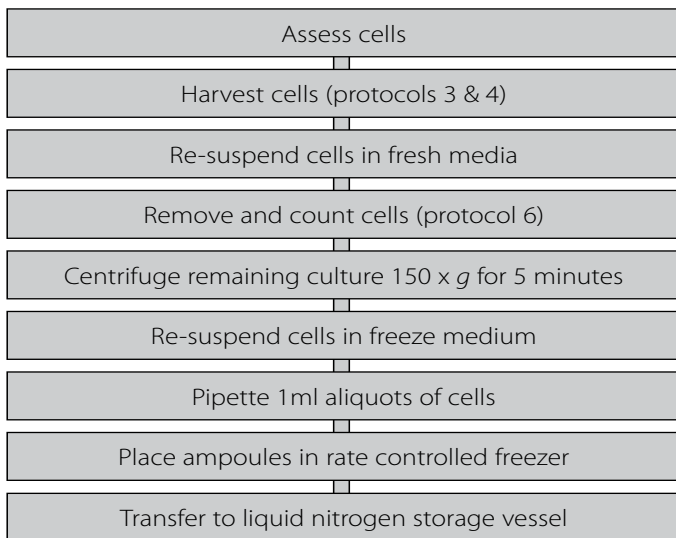
$$\text{Non-viable Cell Count (dead cells per millilitre)} = \frac{\text{Number Dead Cells Counted}}{\text{Number of large corner Squares counted}} \times \text{Dilution factor (in this example 2) } \times 10,000$$

$$\text{Percentage Viability} = \frac{\text{No of Viable Cells}}{\text{Total No. of Cells}} \times 100$$

Key Points

1. Trypan Blue is toxic and is a potential carcinogen. Protective clothing, gloves and face/eye protection should be worn. Do not breathe the vapour.
2. The central area of the counting chamber is 1 mm^2 . This area is subdivided into 25 smaller squares ($1/25\text{ mm}^2$). Each of these is surrounded by triple lines and is then further divided into 16 ($1/400\text{ mm}^2$). The depth of the chamber is 0.1 mm .
3. The correction factor of 10^4 converts 0.1 mm^3 to 1 ml ($0.1\text{ mm}^3 = 1\text{ mm}^2 \times 0.1\text{ mm}$)
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, re-suspending 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
5. The use of a haemocytometer can be time consuming, susceptible to subjective judgements by the operator and some cell types, such as those that form clusters, are particularly difficult to count using this method. Equipment that counts cell nuclei, such as the NucleoCounter (Chemometec), is available offering an alternative cell quantification method. Unlike other cell quantification methods, automated nuclei counters eliminate manual counting and do not rely on the retention of physical and/or morphological properties of the cells. Low volumes, e.g. $200\mu\text{l}$, of prepared samples are drawn into a cassette which is inserted into the nuclei counter providing a cell count in approximately thirty seconds.

12.8 Protocol 7 - 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. 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 described in detail. If large numbers of cell cultures are regularly being frozen then a programmable rate controlled freezer is recommended.

Materials

- Freeze medium (commonly 90% FBS, 10% DMSO or glycerol, check ECACC data sheets for details)
- 70% (v/v) isopropanol in sterile water
- PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$
- 0.25% trypsin/EDTA in HBSS, without $\text{Ca}^{2+}/\text{Mg}^{2+}$
- DMSO

Equipment

- Personal protective equipment (sterile gloves, laboratory coat)
- Microbiological safety cabinet at appropriate containment level
- Full-face protective mask/visor
- Haemocytometer
- Waterbath set to appropriate temperature
- Pre-labelled ampoules/cryotubes
- Centrifuge
- Cell Freezing Device

Procedure

1. View cultures using an inverted microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants. Harvest cells in the log phase of growth. For adherent cell lines harvest cells as close to 80 - 90% confluency as possible.
2. Bring adherent and semi adherent cells into suspension using trypsin/EDTA as described previously (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 (100-200µl) 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 150 x g for 5 minutes.
5. Re-suspend cells at a concentration of $2-4 \times 10^6$ cells per ml in freeze medium.
6. Pipette 1ml aliquots of cells into cryoprotective ampoules that have been labelled with the cell line name, passage number, cell concentration and date.
7. Place ampoules inside a passive freezer e.g. Nalgene Mr Frosty box Sigma cat no. C1562. Fill freezer with isopropyl alcohol and place at -80°C overnight.
8. Frozen ampoules should be transferred to the vapour phase of a liquid nitrogen storage vessel and the locations recorded.

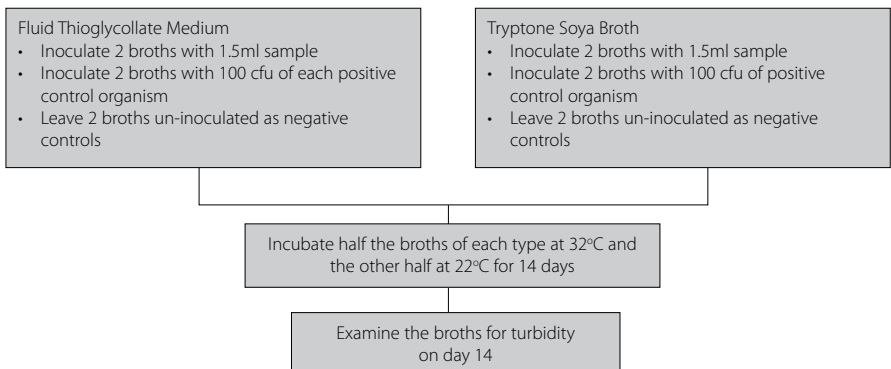
Key Points

1. The most commonly used cryoprotectant is dimethyl sulphoxide (DMSO), however, this is not appropriate for all cell lines e.g. HL60 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% FBS, 10% DMSO but this may not be suitable for all cell types. Check if it works for your cells before using on a regular basis.
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 ampoules are held in liquid nitrogen vapour in the neck of a Dewar. The system allows the ampoules to be gradually lowered thereby reducing the temperature. Rate controlled freezers are also available and are particularly useful if large numbers of ampoules are frozen on a regular basis.

6. As a last resort, if no other devices are available, ampoules 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, ampoules should be transferred to the vapour 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.

12.9 Protocol 8 - Testing for Bacteria and Fungi

Figure 7. Flow Scheme for Bacteria and Fungi Testing



Aim

In cases of gross contamination the naked eye may identify the presence of bacteria and fungi. However, in order to detect low-level infections the method above is recommended.

Materials

- Aerobic nutrient broth e.g. Soyabean Casein Digest (Tryptone Soya Broth, TSB) (15ml aliquots)
- Anaerobic nutrient broth Fluid Thioglycollate Medium (20ml aliquots) (TGM)
- Positive control organisms e.g.
- *Bacillus subtilis* NCTC*
- *Candida albicans* NCTC*
- *Clostridium sporogenes* NCTC*

* Available from the National Collection of Type Cultures (NCTC)

Equipment

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

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 aerobic broths and 2 x anaerobic broths with 1.5ml test sample.
4. For each of the positive control organisms inoculate 2 x aerobic broths and 2 x anaerobic broths (e.g. 0.1ml control at 100 cfu per broth).
5. Leave 2 aerobic and 2 anaerobic broths un-inoculated as negative controls.
6. Incubate broths as follows:
 - Incubate one broth of each pair at 32°C the other at 22°C for 14 days
7. 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

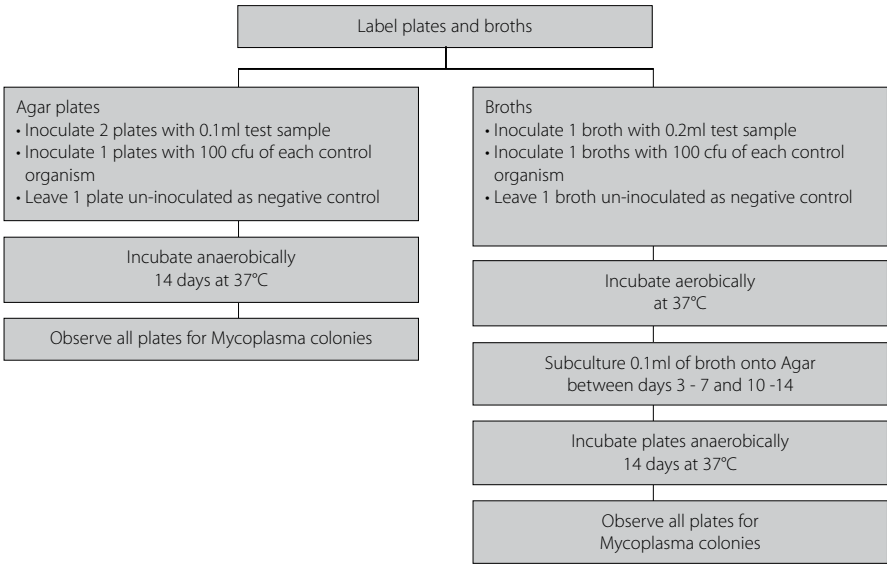
Test broths should be clear and show no evidence of turbidity.

Notes

1. Control organisms (*Bacillus subtilis*, *Clostridium sporogenes* and *Candida albicans*) are available from the National Collection of Type Cultures (NCTC), UK.
2. This test procedure should be carried out in a microbiology laboratory away from the cell culture laboratory.

12.10 Protocol 9 - Testing for Mycoplasma by Culture Isolation

Figure 8. Flow Scheme for 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 9).

Materials

- 70% (v/v) isopropanol in sterile water
- Mycoplasma plates (in 5cm petri dishes)
- Mycoplasma horse serum broths (in 1.8ml aliquots)
- *M. orale* (NCTC* 10112)
- *M. pneumoniae* (NCTC* 10119)

*Available from the National Collection of Type Cultures (NCTC)

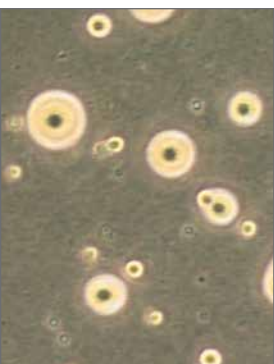


Figure 9. Typical “fried egg colonies” *Mycoplasma pneumoniae* (magnification x400)

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- Incubator set at 37°C
- Anaerobic jar system

Procedure

1. Inoculate 2 agar plates with 0.1 ml of test sample.
2. Inoculate an agar plate with 100 cfu of each control organism.
4. Leave 1 agar plate un-inoculated as a negative control.
5. Inoculate 1 broth with 0.2 ml of test sample.
6. Inoculate a broth with 100 cfu of each control organism.
7. Leave 1 broth un-inoculated as a negative control.
8. Incubate agar plates anaerobically for 14 days at 37°C.
9. Incubate broths aerobically for 14 days at 37°C.
10. Between days 3 - 7 and 10 - 14 of incubation, subculture 0.1 ml of test broth onto an agar plate and incubate plate anaerobically as above.
11. Observe agar plates after 14 days incubation at x400 magnification using an inverted microscope for the presence of mycoplasma colonies (see Figure 9).

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 colour 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.

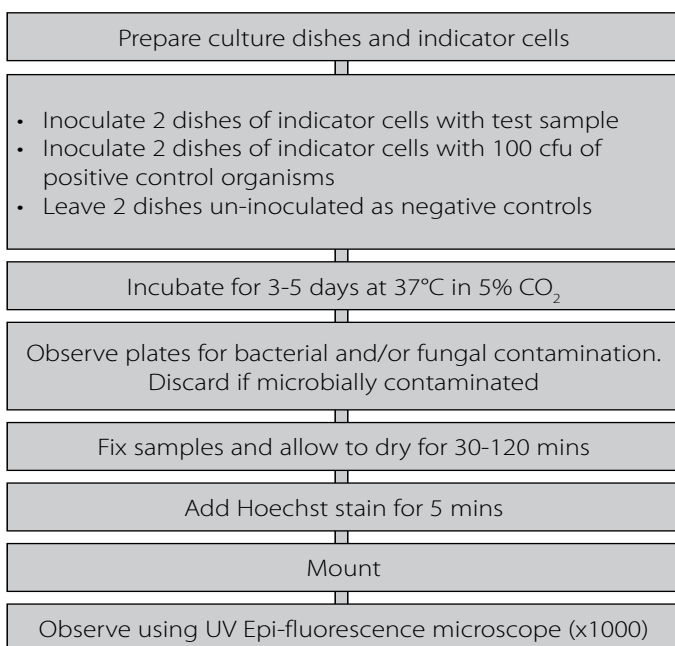
Notes

1. Mycoplasma colonies have a typical colony formation commonly described as “fried egg” (see figure 9) due to the opaque granular central zone of growth penetrating the agar surrounded by a flat translucent peripheral zone on the surface.
2. Mycoplasma pneumoniae is a potential pathogen and must be handled in a class 2 microbiological safety cabinet operating to ACDP Category 2 Conditions.
3. This test procedure should be carried out in a microbiology laboratory away from the cell culture laboratory.

4. ECACC recommends that samples are tested for mycoplasma using at least two detection methods (e.g. indirect DNA stain and culture isolation) for a more reliable result. This is due to the varying detection sensitivities of the methods for different species of mycoplasma.
5. ECACC also offers a mycoplasma screening PCR assay which can return a result within 24 hours.

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

Figure 10. Flow Scheme for Detection of Mycoplasma by Indirect DNA Stain



Aim

DNA staining methods such as indirect Hoechst staining techniques are quick with results available within 72 hours which compares favourably with 4 weeks for detection by culture. Staining of cultures directly with a DNA stain can provide results in 24 hours, however, in a much-reduced sensitivity ($\sim 10^6$ cfu/ml). This may be improved by co-culturing the test cell line in the presence of an indicator cell line such as Vero. This enrichment step results in a sensitivity of 10^4 cfu/ml of culture and is the preferred method used by ECACC. This step also improves sensitivity by increasing the surface area upon which mycoplasma can adhere. As with 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 37°C (refer to the ECACC Cell Line Data Sheet for the correct medium)
- Methanol
- Acetic Acid Glacial
- Hoechst 33258 stain solution
- Indicator cells e.g. Vero cells (ECACC catalogue no. 84113001)
- Mycoplasma hyorhinis NCTC10112

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet of appropriate containment level
- CO₂ Incubator set at 37°C
- Microscope (UV Epi-Fluorescence.)
- Tissue culture dishes
- Multi-dish 24 well
- Microscope slides and 22mm coverslips
- Aluminium foil

Procedure

1. Place sterile coverslips in tissue culture dishes/plates e.g. 12 well plates.
2. Inoculate indicator cells into the prepared dishes e.g. 2×10^4 Vero cells per well of 12 well plate.
3. Incubate at 37°C in 5% CO₂ for 2 – 24 hrs to allow the cells to adhere to the cover slips.
4. Bring attached test cell lines into suspension using a cell scraper. Suspension cell lines may be tested directly.
5. Remove 1ml of culture supernatant from duplicate dishes and add 1ml of test sample to each. Inoculate 2 dishes with 100 cfu of each positive control organism.
6. Leave duplicate tissue culture dishes un-inoculated as negative controls.
7. Incubate dishes at 37°C in 5% CO₂ for 1-3 days.
8. After 3 - 5 days fix cells to cover-slip by adding a minimum of 2 ml of freshly prepared Carnoy's fixative (1:3 glacial acetic acid: absolute methanol) to each dish and leave for 3 to 5 minutes. Then decant to toxic waste bottle. Repeat once more. Add a minimum of 2ml Hoechst stain 0.4µg/nl. Leave for 5 minutes shielded from direct light e.g. by covering with aluminium foil.
9. Decant used and unused stain to toxic waste.
10. Add 1 drop of mountant to a pre-labelled microscope slide and place coverslip (cell side down) onto slide.
11. Keep slide covered with aluminium foil, allowing it to set for at least 15 minutes at 37°C or for 30 minutes at room temperature.
12. 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

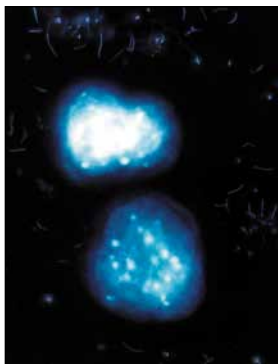
Uninfected samples are seen as fluorescing nuclei against a dark background. There should be no evidence of mycoplasma i.e. extra-nuclear fluorescence of mycoplasma DNA.

Notes

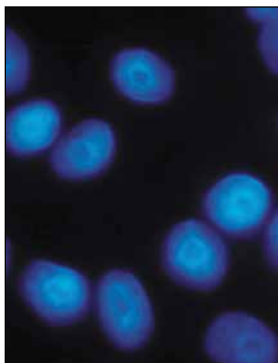
1. DNA stains such as Hoechst stain bind specifically to DNA. In all cultures cell nuclei will fluoresce. In theory uncontaminated cultures will show only fluorescent nuclei whereas mycoplasma positive cultures contain small cocci or filaments which may or may not be adsorbed onto the cells (see figure 11). However, please note that any extraneous DNA will also fluoresce e.g. cell debris - which is sometimes mistaken for mycoplasma contamination.
 2. Hoechst stain is toxic and should be handled and discarded with care.
 3. Culture dishes should be placed in a sealed box or cultured in large petri dishes to reduce evaporation.
 4. In some instances results may be difficult to interpret for the following reasons:
 - Bacterial/yeast/fungal contamination
 - Too much debris in the background (as in the case of hybridoma cell lines)
 - Broken nuclei as cells are all dead
 - Too few or no live cells
 5. Although this procedure recommends the use of positive controls, this may not necessarily be feasible or desirable in a cell culture facility with limited resources. If positive controls are to be used they should be done so in a separate laboratory from the main tissue culture facility. If this is not possible then positive control 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.
6. ECACC recommends that samples are tested for mycoplasma using at least two detection methods (e.g. indirect DNA stain and culture isolation) for a more reliable result. This is due to the varying detection sensitivities of the methods for different species of mycoplasma.

ECACC offers a mycoplasma testing service where three different methods of detection are available i.e. PCR, indirect DNA stain and culture isolation.

Figure 11. Testing for Mycoplasma by Indirect DNA Stain

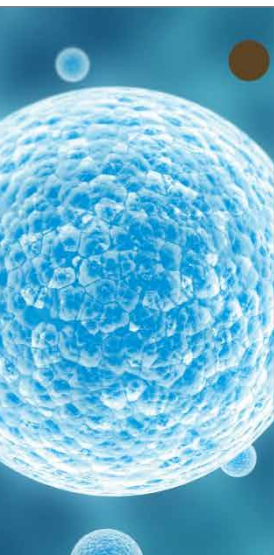


(a) Hoechst Positive Culture (magnification x 1000)



(b) Hoechst Negative Culture (magnification x 1000)

13.0 Protocol for the use of EBiSC Induced Pluripotent Stem Cells



Human induced pluripotent cell (iPSC) lines are different to any other established cell line. If you are not familiar with culturing iPSCs make sure you carefully read these protocols.

Key points of success

- Carefully read these instructions, including the sections on required reagents, thawing and passaging with ROCK inhibitor and precautions & troubleshooting tips
- Make sure all necessary reagents are available prior to thawing the cells. iPSCs do not grow in standard media such as DMEM
- Take care that you use the correct media and matrix combination (information found on CoA)
- Make sure your equipment is calibrated regularly and no reagents have expired

13.1 General Guidelines of Handling Human iPSCs

These protocols provides guidance on how to resuscitate, culture and cryopreserve human induced pluripotent stem cells (iPSCs) supplied by the European Bank of induced pluripotent Stem Cells (EBiSC). All recommendations refer to the culture of iPSCs in one well of a 6-well plate.

All cell manipulations, tissue culture vessel preparations and medium preparations should be performed under aseptic conditions within a Class II Microbiology Safety Cabinet (MSC). The cabinet should be cleaned thoroughly before use and after processing each cell line by wiping all base surfaces with 70% alcohol. No more than one cell line should be handled in an MSC at any one time to avoid mislabeling or cross-contamination between cell lines. It is advisable that a small number of vials are cryopreserved as a master stock. It is recommended to perform regular checks on equipment used to culture iPSCs or to store reagents to ensure they are working within specifications.

For recommendations on culture systems refer to the accompanying Certificate of Analysis (CoA).

The EBiSC - European Bank for induced pluripotent Stem Cells project has received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement n° 115582, resources of which are composed of a financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution.

www.imi.europa.eu

Materials & Reagents

- 6 well tissue culture treated plate (Corning Cat. No. CLS 3736 or similar)
- Culture matrix & Dilutants
 - Matrigel (BD Biosciences) and DMEM (Sigma-Aldrich Cat. No. D6546-500ML) or
 - ECM Gel (Sigma-Aldrich Cat. No. E1270-10ML) and DMEM (Sigma-Aldrich Cat. No. D6546-500ML) or
 - Geltrex (Life Technologies) and DMEM/F12 (Sigma-Aldrich Cat. No. D8062-500ML) or
 - Vitronectin (Sigma-Aldrich Cat. No. SRP3186-250UG 1ml at 0.5mg/ml) and PBS (-/-) (Sigma-Aldrich Cat. No. D8537-500ML)
- Culture medium
 - mTeSR1 culture media (StemCell Technologies) or
 - Essential 8 media kit (Life Technologies)
- DMEM (Sigma-Aldrich Cat. No. D6546-500ML)
- DMEM/F12 (Sigma-Aldrich Cat. No. D8062-500ML)
- PBS (-/-) (Sigma-Aldrich Cat. No. D8537-500ML)
- EDTA – (Sigma-Aldrich Cat. No. E8008-100ML)
- Cryostor Cryopreservation Medium (Sigma-Aldrich Cat. No. C2874)
- DMSO (Sigma-Aldrich Cat. No. D2438 (50ml))
- FBS of US or Australian origin (Sigma-Aldrich Cat. No. F2442 (500ml))
- ROCK inhibitor (Sigma-Aldrich Cat. No. Y0503-1mg)

13.2 Matrix Preparation

Stock vials of Matrigel and Geltrex should be thawed overnight on ice or within a refrigerator (5°C) prior to use. Culture vessels, tubes and stripettes™ should be pre-chilled prior to making aliquots or coating. All manipulations of Matrigel and Geltrex must be carried out on ice-packs to avoid premature gelling. Do not repeatedly freeze thaw stock or working vials of Matrigel or Geltrex.

Preparation of Matrigel

1. Upon receipt, store Matrigel at -20°C.
2. Protein concentration within the Matrigel stock vial is batch dependent and must be obtained from the accompanying certificate of analysis. Use this concentration to calculate the volume of Matrigel required for 2mg protein / working vial.
3. Transfer 2mg of protein into pre-chilled 15ml tubes. These tubes are working aliquots and should be store at -20°C until required.
4. When required, thaw the working Matrigel aliquot in the fridge (5°C) overnight. Add 6ml of cold (5°C) DMEM to the vial and mix by pipetting up and down thoroughly. This is enough for a whole 6-well plate as 1 ml of Matrigel is required for one well of a 6-well plate. Chilled, diluted Matrigel must be used immediately to coat ice-cold tissue culture vessels. The vessel should be incubated at 37.0°C / 5.0% CO₂ for 1 hour and equilibrated to room temperature 30 minutes prior to use. Alternatively, vessels can be sealed with Parafilm™ after the incubation at 37.0°C / 5.0% CO₂ stored at 5°C for a maximum of one week. Ensure stored vessels are equilibrated to room temperature 30 minutes prior to use.
5. Prior to use aspirate Matrigel from the vessel using a stripette™ or similar, wash with 1ml of DMEM and replace with an appropriate volume of culture medium (i.e. 2ml of medium per well in a 6-well plate). Vessels are now ready for cell culture use.

Preparation of Geltrex

1. Upon receipt Geltrex should be stored at -80°C. After thawing, 5ml of Geltrex stock solution should be mixed with 5ml of ice-cold DMEM-F12. Make 180µl aliquots of diluted Geltrex in pre-chilled 15ml tubes. These tubes are working stocks and should be stored at -20°C.
2. When required, thaw the working Geltrex aliquot at 5°C overnight. To dilute the working stock for use, add 8.82ml of ice-cold DMEM-F12 to the vial using a chilled stripette™ and mix by pipetting up and down thoroughly. This is enough for a whole 6-well plate as 1.5 ml of chilled, diluted Geltrex is required for one well of a 6-well plate.
3. After adding the diluted Geltrex to the required wells the vessel should be incubated at 37.0°C / 5.0% CO₂ for 1 hour and equilibrated to room temperature 30 minutes prior to use. Alternatively, vessels can be sealed with Parafilm™ after the incubation at 37.0°C / 5.0% CO₂ stored at 5°C for a maximum of two weeks. Ensure stored vessels are equilibrated to room temperature 30 minutes prior to use.

4. Prior to use aspirate the solidified Geltrex from the vessel, wash the vessel with 1 ml of DMEM-F12 and replace with an appropriate volume of culture medium (i.e. 2 ml per well of a 6 well plate). Vessels are now ready for tissue culture use.

Preparation of Vitronectin

1. Upon receipt, store Vitronectin at -80°C. Prior to use, thaw the stock vial of Vitronectin at room temperature and prepare 60µL aliquots in sterile polypropylene tubes and freeze the aliquots at -80°C or use immediately. One aliquot is sufficient for coating all wells of a 6-well plate.
2. To prepare Vitronectin at a working concentration of 0.5µg/cm², dilute the Vitronectin 1:100 by gently mixing 6ml of room temperature PBS (-/-) with 60µl of Vitronectin. Add 1ml of diluted Vitronectin to each well of a 6-well.
3. Incubate the coated culture vessels at room temperature for 1 hour. If storage is required, vessels can be sealed with Parafilm™ and stored at 5°C for up to 3 days. Allow the vessel to equilibrate to room temperature for 1 hour prior to use.
4. To prepare the vessel for culture, remove the excess Vitronectin from the culture vessel and discard. It is not necessary to wash the culture vessel after the removal of Vitronectin.

13.3 Medium Preparation

mTeSR1

1. When required, remove the mTeSR1 supplement (5x) from the freezer and thaw overnight at 5°C prior to use. Do not thaw at 37°C.
2. Aseptically add 100ml of mTeSR1 supplement (5x) to 400ml of cold (5°C) basal medium.
3. Aliquot medium into volumes required for 1 week of culture work.
4. Complete mTeSR1 may be stored at 5°C for 1 week or at -20°C for 6 months. Frozen complete mTeSR1 may be thawed once. Do not repeatedly freeze thaw medium. Prior to use, warm mTeSR1 to room temperature, do not leave medium at room temperature for longer than 2 hours per day and avoid exposure to light to avoid degradation of medium components.

Essential 8 (E8)

1. When required, remove the E8 supplement (50x) from the freezer and thaw overnight at 5°C prior to use. Do not thaw at 37°C.
2. Aseptically remove 10ml of E8 basal medium to leave 490ml.
3. Add 10ml of E8 supplement (50x) to the 490ml of basal cold (5°C) medium.
4. Aliquot medium into volumes required for 1 week of culture work.
5. Complete E8 may be stored at 5°C for 1 week or at -20°C for 6 months. Frozen complete E8 may be thawed once. Do not repeatedly freeze thaw medium. Prior to use, warm E8 to room temperature, do not leave medium at room temperature for longer than 2 hours per day and avoid exposure to light to avoid degradation of medium components.

Thawing of human iPSCs

1. Cells should be thawed rapidly by placing the cryovial in a water bath set to maintain 37.0°C. Swirl the cryovial gently in the water bath to ensure rapid thaw but do not submerge the cap of the cryovial. Disinfect with 70% alcohol or an equivalent disinfectant before opening.
2. Using a 5ml sterile stripette™, transfer the cryoprotectant/cells mix from the cryovial into a 15ml centrifuge tube. Care should be taken not to physically damage cells.
3. Slowly, drop by drop, add 10ml of appropriate medium at room temperature to the cells within the 15ml centrifuge tube. Gently rock the 15ml centrifuge tube back and forth while adding drops to minimise osmotic shock to the cells. This is a crucial step and cells should be treated as gently as possible.
4. Check tube to ensure all cell contents are removed and if not, rinse with 1ml of appropriate medium.
5. A small amount of cells can be used for performing a cell count. A single cell suspension should be created using trypsin or similar. As a general guideline the seeding density range for one well of a 6-well plate is between 2×10^5 - 1×10^6 . Refer to CoA for guidelines for a specific EBiSC cell line lot number.
6. Centrifuge the cells at 200 g for 2 min. Remove and discard the supernatant.
7. Prepare culture vessels by adding an appropriate amount of medium (i.e. 1.5 - 2ml per one well of a 6-well plate)
8. Gently tap the 15ml centrifuge tube to dislodge the cell pellet then gently add 1ml of appropriate medium and seed into 2-wells of a coated 6-well plate (adjust if using other culture formats or if advised differently in the Certificate of Analysis). Do not over aspirate the cells as this will lead to decreased viability due to generation of a single cell suspension.
9. Gently rock plate side to side, back and forth to spread the cells evenly across the well.
10. It is advisable to record images of cells immediately post-thaw, at 48 hours and at approximately 70-80% of confluence.

13.4 Culturing of human iPSCs

1. It is good practice to observe iPSC lines daily under phase contrast microscope (4x, 10x, 20x and 40x magnification) to check for iPSC-like morphology, the presence of differentiated cells and confluence. A typical scoring method could look like this (for examples of iPSC colonies and different differentiation levels see figures 12 and 13):

A	Optimal, compacted iPSC colonies with defined edges; morphology uniform across colonies
B	Acceptable iPSC colonies with some differentiation around the edges, cells more loosely packed within colonies
C	Good adherence with iPSC colonies emerging
D	Poor adherence and no obvious iPSC

2. Cells are fed by removing 95% of the medium from the wells using an aspirator pipette. Do not completely remove the medium; a thin film of medium should cover the cell layer to avoid drying out the cells.
3. Aseptically add 2ml of fresh medium per 1 well of a 6-well plate by gently adding to the side of the well. Incubate cells at 37°C / 5 % CO₂.
4. Typically, medium exchanges occur daily on six of seven days with increased volume of media (1.5x - 2x the normal amount; cell density dependent) if cells need to be left for longer periods between media changing. Do not leave more than two days between medium exchanges.

Figure 12. Examples of scoring of iPSC colonies

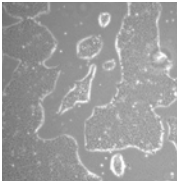
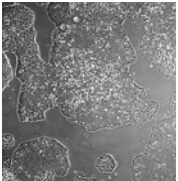
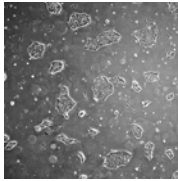
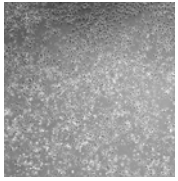
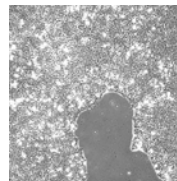
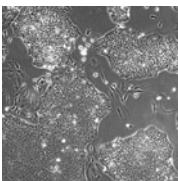
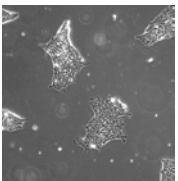
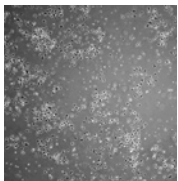
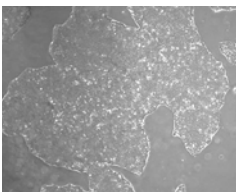
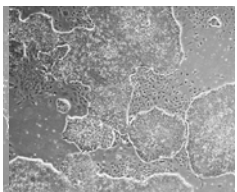
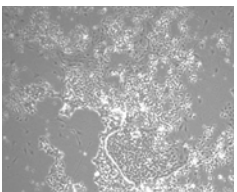
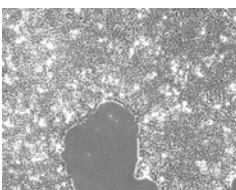
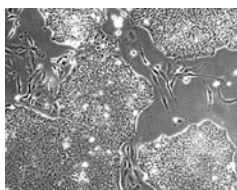
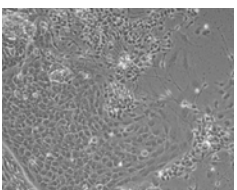
	A	B	C	D
	Optimal, compacted iPSC colonies with defined edges; morphology uniform across colonies	Acceptable iPSC colonies with some differentiation around the edges, cells more loosely packed within colonies	Good adherence with iPSC colonies emerging	Poor adherence and no obvious iPSC
X4				
X10				

Figure 13. Examples of different levels of differentiated cells present in culture

	L	M	H
	Low levels of differentiated cells in culture	Medium levels of differentiated cells in culture	High levels of differentiated cells in culture
X4			
X10			

13.5 Passaging of human iPSCs using EDTA

NOTE: Passaging method is determined by growth matrix. Read explanation below carefully.

Typically established cultures can be split 1:4 to 1:8 (i.e. transferring all colonies from one well to four or up to eight) but exact split ratio can vary for each cell line. Refer to Certificate of Analysis for recommended split ratios for your cell line. Split ratios can be adjusted to ensure cells are passaged within 4-5 days of culture in order to maintain log phase of growth. Cell lines should be passaged when the cells are approximately 70-80 % confluent or if colonies have been growing on the same plate for more than 7 days. Colonies must not become too dense or display enhanced differentiation.

1. Passage cells with 0.5mM EDTA by first removing spent medium from each vessel requiring passage.
2. Cells growing on Geltrex or Matrigel should be washed with 1ml of 0.5mM EDTA per well. For cells growing on Vitronectin, wash each well with 1ml of PBS (-/-). Quickly aspirate the EDTA or PBS from the well and repeat the wash step.
3. Add 1ml of 0.5mM EDTA to each culture vessel and incubate at 37.0 °C / 5.0 % CO₂ for 4 minutes with Geltrex or Matrigel and for 5 min at room temperature with Vitronectin. Ambient temperature can influence EDTA efficacy and incubation time should be optimized
4. On completion of incubation time, check the cell layer under an inverted microscope to see if the colonies have feathered edges and holes throughout. This indicates that cells have detached from each other but not from the culture vessel and are ready to be removed from the vessel. Remove the 0.5mM EDTA from the vessel by tilting the vessel forward slightly to collect the EDTA in the bottom edge of the vessel. Take care as the cells are loosely attached. Immediately add 1ml of desired media to 1 well in order to neutralise the EDTA.
5. Gently wash the cells from the culture vessel using a 1ml sterile pipette. Three gentle aspirations of the well or flask should suffice. These aspirations will dislodge cell clusters, ideally leaving a proportion of differentiated cells attached. Excessive aspiration of the cells will result in single cell suspension rather than cell clusters which will decrease cell viability. It can be beneficial to leave some cell material behind rather than trying to remove all cells and compromising clump size.
6. Seed the cells at an appropriate cell density by transferring the required volume of cells (in accordance with your desired split ratio) to a new plate coated with the matrix of choice containing an appropriate volume of desired medium.
7. To ensure even distribution of cell clusters, gently disperse the clusters by carefully moving the vessel side to side, back and forth several times before placing the vessel in an incubator maintained at 37.0 °C/ 5.0 % CO₂.

13.6 Thawing & Passaging with ROCK inhibitor

1. Normally we do not recommend the use of selective Rho-associated kinase (ROCK) inhibitor (Y27632) inhibitor. However, in a case of poor cell survival after dissociation ROCK inhibitor can be used to offset the effect of apoptosis. Additionally Rock inhibitor enhances cell survival after cryopreservation and can therefore be used during the initial thaw. This could be an option for inexperienced users to enhance their chances of success.
2. When thawing or passaging cells supplement appropriate medium with ROCK inhibitor to maintain 10 μ M final concentration. To obtain the required concentration dilute 10mM of stock ROCK inhibitor 1:1000 in cell culture medium, e.g. for 1ml of medium add 1 μ l of 10mM ROCK inhibitor.

Reconstituting ROCK inhibitor

1. ROCK inhibitor is light sensitive hence special care should be taken to avoid and minimize potential exposure to light.
2. Centrifuge stock vial for few seconds to bring down the powder to the bottom of the vial, spray and wipe the vial with 70% ethanol and place it in the microbiological safety cabinet.
3. Add appropriate volume of sterile molecular grade water to lyophilized ROCK inhibitor. Calculate the required volume as per formula below
4. $\text{Mass [g]} = \text{Molarity [mol/L]} \times \text{Volume [L]} \times \text{Molecular Weight [g/mol]}$
5. Mix thoroughly, incubate for 3 minutes at room temperature and dispense into appropriate size aliquots.
6. Store aliquots at -20 °C and avoid repeated thawing and freezing.
7. Once thawed aliquots may be kept at 2-8°C for a week.

13.7 Cryopreservation of human iPSCs

1. Keep reagents and freezing container (e.g. Mr. Frosty™) chilled during the cryopreservation procedure.
2. Cells must be cryopreserved when in their log phase of growth to enhance survival upon thaw. The optimal time for harvest is normally when cells are approximately 70-80% confluent
3. The type of cryoprotectant medium used depends on culture conditions and laboratory preferences. Use either commercially available Cryostor CS10 or DMSO based freeze mix (10% DMSO in FBS and culture medium). Cryostor is supplied ready to use and is stored at 2-8°C. To prepare DMSO based cryoprotectant, mix 40% FBS with 10% DMSO, then mix with 50% appropriate medium.
4. Remove spent medium from the tissue and wash the vessel twice with the recommended volume of wash buffer depending on culture conditions (Wash buffer for Geltrex/Matrigel is 0.5mM EDTA, wash buffer for Vitronectin is PBS -/-).
5. To lift the cells from the tissue culture plastic, add 1ml of 0.5mM EDTA to the tissue culture vessel. Incubate the cells for the recommended time and temperature, depending on matrix used. Aspirate the EDTA from the well. Care must be taken as the colonies are very loosely attached to the plastic.
6. Afterwards add 1 ml of cryoprotectant per 1 well. Gently wash the cryoprotectant over the vessel with a ml sterile pipette to dislodge the cells from the plastic. Do not aspirate more than 3 times to avoid breaking the cell clumps into single cells. Place the cryoprotectant and cell mix into an appropriately labelled cryovial.
7. If cryopreservation of more wells is desired, cells from the same passage number and culture condition should be pooled together. An aliquot of pooled cells can be used for a cell count. Centrifuge harvested cells at 200xg for 2min, aspirate spent medium and gently re-suspend the cell pellet in an appropriate volume of cryoprotectant. One well of a 6-well plate gives rise to approximately $1-2 \times 10^6$ cells. It is recommended to freeze around $1-2 \times 10^6$ cells per cryovial. Use 1ml of cryoprotectant-cell mix per cryovial.
8. Immediately place the cryovials into a pre-chilled Mr Frosty tub (2-8°C) then immediately transfer the Mr Frosty tub to a -80°C freezer. Allow the cells to remain at -80°C overnight (16-36 hours). Once frozen transfer the cells, on dry ice, to an ultra-low temperature storage vessel (LN₂ or -150°C freezer).

13.8 Adaptation of EBiSC cell lines to alternative matrices and media

Where required, the matrix and media used for a specific cell line can be changed to an appropriate alternative during passage or at thaw. This process is carried out in one step by simply passaging or thawing into the new tissue culture system. No guarantees can be given regarding cell viability or quality where the advised tissue culture system is not used.

13.9 Precautions & Troubleshooting Tips

Problem	Observation	Possible Solution/Precaution
Low viability of iPSCs after thaw	Little to no colonies visible within 4 days after recovery	- Ensure that cryovials are thawed quickly and that medium is added to the cells very slowly (drop-wise while gently swirling the tube) - Add 10µm ROCK inhibitor at thaw but do not use routinely - Ensure that cells were banked at log phase of growth with low levels of differentiation - Let small colonies grow until robust and passage with low split ratio (1:1 or 1:2)
Low viability after Passage	- Cells do not attach properly - Non-typical morphology - High levels of cell death - Cells do not proliferate	- Use lower split ratio and maintain a more confluent culture - Ensure cells are in log phase of growth at passaging - Work quickly or reduce incubation time of EDTA as clump size could be influenced by too long an exposure to EDTA - Increase incubation time of EDTA if cells do not come off easily. This is to avoid having to harshly rinse cells off thereby creating too small aggregates/ single cell suspension - Check if plates were coated correctly, matrix is within expiry date and check batch with manufacturer if this issue occurs on a regular basis and other reasons have been excluded
Spontaneous differentiation	- Colonies do not have defined edges - Cells within the colonies are less compact - Cells appear flattened and bigger or fibroblastic	- Ensure cells are being cultured using recommendations given here (i.e. daily feeding of cells) - Ensure that reagents are freshly prepared (i.e. used within two weeks) - Avoid leaving plates outside the incubator to decrease temperature fluctuation and exposure to light - Decrease colony density by plating fewer cell aggregates per cm² during passaging - Removal of differentiated cells by scraping the differentiation away with a pipette tip leaving iPSC colonies intact can be considered. Care must be taken not to disturb the iPSC colonies and not to scrape away too much of the matrix layer in this process - If good iPSC colonies persist between differentiated areas manual picking of colonies with good iPSC morphology using a pipette tip can be considered. It is recommended to select several colonies and cut them in pieces with a pipette tip, lift them, aspirate them and pass them to a fresh 1:6 well
Non-uniform distribution of colonies within plate	Areas with too high density of iPSC cells and where cells start to differentiate from the middle. Additionally to areas with hardly any colonies	- Make sure that the whole surface area of the tissue culture vessel is coated with the appropriate matrix - Ensure that the cell aggregates are evenly distributed by gently rocking the plate back and forth and side to side - Take care when placing plate into the incubator and leave undisturbed for 24h
Significant scraping is required to dislodge cells	Colonies do not come off the plate with 2- 3 rinses with a 1ml pipette	- Ensure that incubation time and temperature of EDTA are in accordance with matrix - Increase incubation time of EDTA - Do not let cells become more than 70% confluent - Do not let colonies to be overgrown in the centers, sometimes it is necessary to passage a less confluent plate with fewer but robust colonies, using lower split ratio
Poor attachment and significant increase in cell death post-passage	Cells start to lift off even though they seemed to attach after passage	- Rather than exchanging medium top up wells with fresh medium to ensure sufficient amount of nutrients and leave cell undisturbed for an additional 24h to allow aggregates to fully attach - Exchange medium very gently, do not subject colonies to excessive shear forces by rapid addition of medium

Contact Details

Further information about EBiSC may be found at www.ebisc.org.



All EBiSC lines are distributed through ECACC.



14.0 Perfecta3D® Hanging Drop Plates protocol

Improving Compactness of Spheroids

Some cell lines establish only weak intercellular interactions, forming aggregates with less tightly associated cells. These aggregates are not the best in vitro models since they lack diffusion barriers, as well as a stratified cellular composition with proliferating cells in the rim and quiescent and necrotic cells in the core. With the addition of exogenous extracellular matrix (ECM), these aggregates are able to form compact spheroids, which resemble more closely the morphology and diffusion properties of xenografts derived from these tumor cell lines.

Required chemicals/solutions:

- Media soluble ECM, individual matrix components (such as laminin, collagen, or fibronectin), or methylcellulose. The cell specific matrix requirements and final concentration should be determined experimentally for each cell type and downstream application. We suggest a starting range of 0.1% to up to 10% depending on the recommendations of the manufacturer

Example cell lines that form compact spheroids with the addition of ECM:

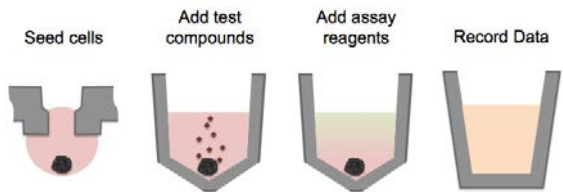
- MDA-MB-231 human breast adenocarcinoma cells
- HepG2 human hepatocellular carcinoma cells
- SK-OV-3 human ovarian adenocarcinoma cells

Steps:

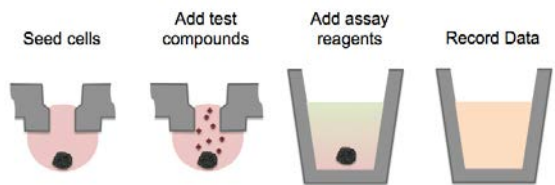
1. Prepare and handle ECM or methylcellulose as described by the manufacturer.
2. Detach cells from flask and centrifuge to collect cell pellet.
3. Resuspend cell pellet in fresh medium and count cells.
4. Prepare cell suspension for seeding by diluting it to the desired concentration, taking into consideration the addition of the matrix in the next step.
5. Add ECM or methylcellulose to the cell suspension as directed by the manufacturer to the experimentally predetermined final concentration.
6. Seed cells by forming hanging drops of the ECM- or methylcellulose-containing cell suspension in a Perfecta3D® Hanging Drop Plate.

14.1 Assay Work Flow and Considerations Overview

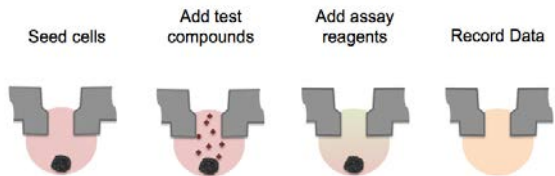
Scheme A – Two transfers, convenient optical imaging, for assays with mixing/shaking steps



Scheme B – One transfer, for assays with mixing/shaking step



Scheme C – No transfer, for colorimetric and fluorometric assays without mixing/shaking step



14.2 Scheme A – Two transfers, convenient optical imaging, for assays with mixing/shaking steps

Spheroid Formation 4 days	1. Seed cells. - Establish optimal seeding densities such that spheroids fell within a size range of 300 to 500 μm in diameter on day 4 - Depending on cell types, cells may form tight, compact, or loose spheroids. The 3D structure of compact and loose spheroids can be improved by the addition of 2.5% Matrigel™ to the cell seeding solution Day 4 is an appropriate time for initiating the experiment as cells have been given sufficient time to reorganize and form spheroids and to establish cell-cell interactions and produce extracellular matrix.
Compound Exposure 3 days	2. Transfer spheroids to a standard multi-well plate. A V-bottom plate is recommended because the V-shaped wells help to prevent inadvertent aspiration of spheroids during media changes and also helps to center spheroids for ease of optical imaging 3. Add test compounds. Cells are usually less sensitive to treatments when cultured in 3D, so a 72- hour exposure is generally necessary to ensure toxic effects are detected.
Assay Incubation 10 minutes to 24 hours	4. Add assay reagents. Determine optimal incubation times by incubating day 4 spheroids with assay reagents for various times. For example, 10, 30 and 60 minutes with Promega CellTiter-Glo® reagents.

5. Transfer assay mixture to an appropriate flat-bottom plate.

- Select an appropriate flat-bottom plate for optimal measurements. To maximize signal-to-noise ratio, typically black plates are used for fluorometric assays and white plates for luminescent assays

6. Record data.

14.3 Scheme B – One transfer, for assays with mixing/shaking step

1. Seed cells.

- Establish optimal seeding densities such that spheroids fell within a size range of 300 to 500 μm in diameter on day 4
- Depending on cell types, cells may form tight, compact, or loose spheroids. The 3D structure of compact and loose spheroids can be improved by the addition of 2.5% Matrigel™ to the cell seeding solution

Day 4 is an appropriate time for initiating the experiment as cells have been given sufficient time to reorganize and form spheroids and to establish cell-cell interactions and produce extracellular matrix.

2. Add test compounds.

Cells are usually less sensitive to treatments when cultured in 3D, so a 72- hour exposure is generally necessary to ensure toxic effects are detected.

3. Transfer spheroids to an appropriate flat-bottom plate.

- Select an appropriate flat-bottom plate for optimal measurements. To maximize signal-to-noise ratio, typically black plates are used for fluorometric assays and white plates for luminescent assays.

4. Add assay reagents.

Determine optimal incubation times by incubating day 4 spheroids with assay reagents for various times. For example, 10, 30 and 60 minutes with Promega CellTiter-Glo® reagents.

5. Record data.

14.4 Scheme C – No transfer, for colorimetric and fluorometric assays without mixing/shaking step

Spheroid Formation 4 days	1. Seed cells. • Establish optimal seeding densities such that spheroids fell within a size range of 300 to 500 µm in diameter on day 4 • Depending on cell types, cells may form tight, compact, or loose spheroids. The 3D structure of compact and loose spheroids can be improved by the addition of 2.5% Matrigel™ to the cell seeding solution Day 4 is an appropriate time for initiating the experiment as cells have been given sufficient time to reorganize and form spheroids and to establish cell-cell interactions and produce extracellular matrix.
Compound Exposure 3 days	2. Add test compounds. Cells are usually less sensitive to treatments when cultured in 3D, so a 72-hour exposure is generally necessary to ensure toxic effects are detected.
Assay Incubation 10 minutes to 24 hours	3. Add assay reagents. Determine optimal incubation times by incubating day 4 spheroids with assay reagents for various times. For example, 10, 30 and 60 minutes with Promega CellTiter-Glo® reagents. 4. Record data.

14.5 Example cell seeding densities:

A431.H9 human epithelial carcinoma cells	3,750 cells per well
HCT-116 human colorectal carcinoma cells	3,750 cells per well
HT-29 human colorectal adenocarcinoma cells	7,500 cells per well
T47D human breast ductal carcinoma cells	3,750 cells per well

- Seed cells in 25 μL hanging drops in Perfecta3D® Hanging Drop Plates. Prepare enough samples for treatments to be conducted in triplicates
- Culture cells for 4 days (or until spheroids have formed). Exchange cell culture media as necessary

Example test compound treatment concentrations:

5-Fluorouracil	Doxorubicin	Irinotecan	Levofloxacin	Taxol
0.1 μM	0.1 μM	0.01 μM	1 μM	1 nM
1 μM	1 μM	1 μM	10 μM	10 nM
10 μM	5 μM	10 μM	25 μM	50 nM
100 μM	10 μM	50 μM	100 μM	100 nM
500 μM	25 μM	100 μM	250 μM	500 nM
2,000 μM	50 μM	200 μM	1,000 μM	1000 nM

- Prepare working solutions of the test compound at 5 times the final treatment concentrations
- Remove 5 μL of culture medium from each well to obtain a drop volume of 20 μL
- Initiate test compound treatment by adding 5 μL of working solution to each well. Treat spheroids with empty vehicle (e.g. 0.2% DMSO) as a control (0 μM)
- Expose spheroids to treatments for 48 to 72 hours. Replenish hanging drops with fresh media as necessary to compensate for volume lost due to evaporation

Table 6. Cell Culture Reagents

The following list of products, available from Sigma-Aldrich, is provided to aid acquisition of reagents commonly used by ECACC for the culture of cell lines.

Cat No.	Product	Description
M6250	2ME	2 – Mercaptoethanol
M4526	ALPHA MEM	Minimum Essential Medium Eagle's – Alpha Modification
B1522	BME	Basal Medium Eagle
B9285	Budr	Bromo-deoxy Uridine
C5467	CHO PF Media	CHO Protein Free Medium - Animal Component Free
C8919	Collagen Type I	Collagen Type I
C5533	Collagen Type IV	Collagen Type IV
F6636	Coon's F12	Nutrient Mixture F-12 Ham Coon's Modification
D6546	DMEM	DME, Dulbecco's Modified Eagle's Medium
D8062	DMEM:F12 (1:1)	DMEM:Nutrient Mix F12 Ham (1:1) ratio mix
D2650	DMSO	Dimethyl Sulphoxide
M2279	EMEM (EBSS)	Minimum Essential Medium Eagle's (Earle's Balanced Salt Solution)
M5775	EMEM (HBSS)	Minimum Essential Medium Eagle's (Hank's Balanced Salt Solution)
F2442	FBS / FCS	Foetal Bovine Serum / Foetal Calf Serum (USA Origin)
F4135	FBS HI	Foetal Bovine Serum – Heat Inactivated (USA Origin)
F4759	Fibronectin	Fibronectin
C2639	Freeze Media SF	Cell Freezing Medium – Serum free
G9391	Gelatin	Gelatin
G7513	Glutamine	L-Glutamine
G2025	Glycerol	Glycerol
G5154	GMEM	Glasgow Minimum Essential Medium
N6013	Ham's F10	Nutrient Mixture F-10 Ham's
N4888	Ham's F12	Nutrient Mixture F-12 Ham's
H0887	HEPES	HEPES
H8142	HES	Hybridoma Enhancing Supplement
H1270	HS	Horse Serum
I3390	IDMEM	IMDM / Iscove's Modified Dulbecco's Medium
I3146	ITS	Insulin – Transferrin – Selenium
M8028	JMEM	Minimum Essential Medium Eagle's – Joklik Modification
N3520	Kaighn's F12	Nutrient Mixture F-12 Ham Kaighn's Modification
L5520	L15	L-15 Medium (Leibovitz)
A5006	L-Arginine	L-Arginine
L9010	LEH	Lactalbumin Enzymatic Hydrolysate
M2154	M199	Medium 199
M8403	McCoy's 5A	McCoy's 5A Medium Modified
M6395	MCDB 105	MCDB 105 Medium
S8761	NaHCO ₃	Sodium Bicarbonate
S8636	NaP	Sodium Pyruvate
N4637	NBCS	Newborn Calf Serum
N1140	NCTC 109	NCTC 109 Medium
M7145	NEAA	Non Essential Amino Acids
D8537	DPBS	Dulbecco's Phosphate Buffered Saline
P8139	PMA	Phorbol Myristate Acetate
R0883	RPMI 1640	Roswell Memorial Park Institute 1640 Medium
S9895	SDM	Schneider's Drosophila Medium
M8167	SMEM	Minimum Essential Medium Eagle's - Spinner Modification
T8159	TPB	Tryptose Phosphate Broth
T8154	Trypan Blue	Trypan Blue
T4424	Trypsin	Trypsin
T6522	Trypsin Inhibitor	(Soybean) Trypsin Inhibitor
T4049	Trypsin/EDTA	0.25% Trypsin / EDTA
M6895	VITS (MEM)	MEM Vitamins
R7256	VITS (RPMI)	RPMI Vitamins
W1625	WMB 752/1	Waymouth Medium MB 752/1

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