

Fundamental Techniques in **Cell Culture** ... a Laboratory Handbook



Laboratory Design
and Equipment

Sourcing of
Cell Lines

Types of
Cell Culture

The Cell
Environment

Quality Control

Basic Techniques
and Protocols

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I.0 Introduction

This laboratory handbook has been produced as a guide for scientists who have recently been introduced to the discipline of cell culture. The book has been written and published by the European Collection of Cell Cultures (ECACC) and Sigma-Aldrich. ECACC is a universally recognised authority on Cell Culture, providing authenticated Cell Lines and Cell Culture services to the scientific community and a wide range of commercial customers. Sigma Aldrich has operations in 33 countries worldwide and manufactures Cell Culture Products in several locations throughout the world.

The handbook contains essential information about the fundamentals of cell culture ranging from the sourcing of cell lines and safety, to aspects of cryopreservation and quality control, which give the reader the essential facts needed when embarking on cell culture for the first time. Additionally there is a series of 10 detailed protocols, which are routinely used in the ECACC laboratories. This handbook is designed to provide scientists with a quick reference guide to cell culture by detailing the more commonly used protocols and methods. However, it is not intended to be an in-depth text book of cell culture and you are encouraged to consult relevant specialised literature to obtain more detailed information.

We hope you like the design of the handbook and that it is small enough to fit in a lab coat pocket, with water resistant paper and not too big to open out on the lab bench. Should your colleagues require their own personal copy then they can complete the business reply card at the back of the handbook to obtain their copy.

ECACC and Human Genetic Research

The European Collection of Cell Cultures (ECACC) was first established within CAMR, Salisbury, UK in 1984 and has grown to be one of the largest animal cell culture collections in the world. ECACC now supplies authenticated cell lines worldwide and provides a wide range of cell culture services.

Whereas the ECACC core business is cryopreservation, cell banking and the supply of authenticated cell lines the operation has progressively diversified and now offers a comprehensive range of services to cell scientists both in the commercial and academic sectors. These services include Patent and Safe deposits, screening for microbiological contaminants, authentication by DNA profiling and a comprehensive programme of training courses. ECACC also undertakes contract cell culture process upscale development and the manufacture of cell products for research use.

The Human Genetic Collection

The fastest-growing service within the ECACC range is its Human Genetic Collection. This is based on processes introduced into, and subsequently developed by ECACC in the late 1980's, for the immortalisation of human lymphocytes. This process essentially comprises the

separation of lymphocytes from samples of human peripheral blood and their transformation into lymphoblastoid cell lines by treatment with the tumourigenic Epstein Barr Virus (EBV). EBV transformation of human lymphocytes to form a cell line that can be continuously propagated provides an indispensable means of expanding the limited amount of cellular material that can be separated from a small volume blood specimen. This expanded cell stock can be banked and cryopreserved as a source of single donor cells and / or extracted DNA that can be made available indefinitely.

Since 1989 a number of major human genetic research programmes, such as the British Diabetes Association-funded Warren I and II studies and the long-running, MRC-funded Human Genome Mapping Project, have depended upon the ECACC EBV transformation service. A cell line manufactured from a phenotypically characterised donor, who possibly has a rare genetic disorder, can be used to provide an almost limitless supply of DNA for distribution to research groups worldwide. Furthermore a cryopreserved bank of these cells can be resuscitated many years later so that investigation may be resumed with the benefit of improved technologies and scientific knowledge.

The ability to expand the amount of cellular DNA available from a small donor sample means that scientific investigations will not be materials limited. It is theoretically possible to achieve this expansion by biochemical means such as "whole genome PCR", but to date this has not proved practicable. Cell line DNA is typically of high quality, and a viable cell offers the opportunity to examine other types of molecule, such as RNA or cellular proteins, if required at a later date.

The ECACC Human Genetic Collection now comprises more than 40,000 single donor lymphocyte stabilates, more than 50% of which are transformed cell lines. This collection represents more than 700 clinical disorders such as breast cancer, Alzheimers disease, schizophrenia and psoriasis.

The ECACC General Collection

This collection comprises approximately 1000 different cell lines originating from over 45 different species and a variety of different tissues. It incorporates over 400 human cell lines with many different cancer cell lines including drug resistant cell lines.

The cells can be shipped either as frozen or growing cultures, and are supplied complete with a specific data sheet together with a technical advisory pamphlet. Batch specific Certificates of Analysis and Authentication Records can be provided on request. ECACC can also supply cell lines on a standing order basis.

All cell lines added to the collection undergo full quality control and authentication procedures. These include testing for mycoplasma by a combination of culture isolation, Hoechst DNA staining and PCR, together with culture for containment bacteria, yeast and fungi.

Isoenzyme analysis is used to verify the species of each cell line deposit while DNA fingerprinting identifies cell line variation and/or cross contamination. ECACC routinely carries out two methods of DNA fingerprinting. Multilocus, applicable to most common species and STR DNA profiling for human cell lines only. The latter has the benefit of producing a digital 'fingerprint' which can be compared to ECACC's extensive range of archived profiles. All HeLa contaminant cell lines are clearly identified in the full catalogue description.

Hybridoma Collection

This collection comprises over 400 monoclonal antibody-secreting hybridomas. The specification of the secreted antibodies covers a wide range of antigens including bacterial and viral; major histocompatibility complex differentiation; oncogenes; plant and immunoglobulin.

ECACC can supply frozen or growing cultures of the hybridomas in the collection. In addition ECACC may also supply, assuming no restrictions are in place, purified antibody on a customised basis. The cost of providing purified antibody is dependent on the antibody production levels of the hybridoma, level of purification and quantity required.

All cell lines added to the collection undergo full quality control and authentication procedures. These include testing for mycoplasma by a combination of culture isolation, Hoechst DNA staining and PCR, together with culture for contaminant bacteria, yeast and fungi.

HLA Defined Human B-Lymphoblastoid Collection

This is a specialised collection of over 350 reference cell lines, originating from laboratories all over the world, to be used in the investigation of the HLA system. The collection was expanded in 1996 with cell lines from the 12th International Histocompatibility Workshop (IHW) being added. A further 60 cell lines from the 13th IHW collection are due to be added later this year, along with a number of recently banked lines that were originally part of the 10th IHW collection.

Both serology and DNA typing data is available for the HLA Class I and II antigens together with information on non-HLA genes within the HLA region. This resource is available both as cell lines or DNA.

Human Random Control Collection

This is a collection of 500 lymphoblastoid cell lines derived from randomly selected Caucasian blood donors whose parents and grandparents were born in the UK or Ireland. DNA from these controls has already been extensively used in genome screening programmes. This resource will be available as extracted DNA in a 96 well array format.

Full information on all the ECACC collections are available from the ECACC Website or in a CDrom format which can be requested by completing the enclosed reply card.

2.0 Design and Equipment for the Cell Culture Laboratory

2.1 Laboratory Design

Perhaps one of the most under-rated 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.

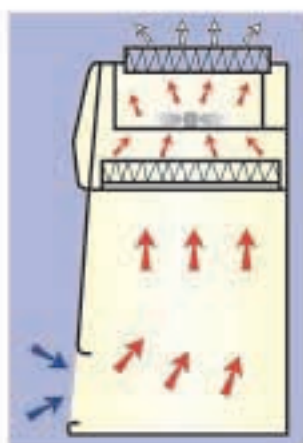
There are several aspects to the design of good tissue culture facilities. Ideally work should be conducted in a single use facility which, if at all possible, should be separated into an area reserved for handling newly received material (quarantine area) and an area for material which is known to be 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 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, 1985)[†]. However, the precise category required is dependent upon the cell line and the 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.

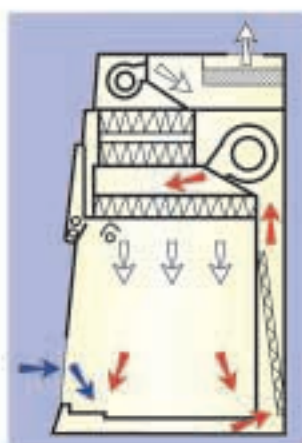
[†]Advisory Committee on Dangerous Pathogens (1985) Categorisation of Biological Agents According to Hazard and Categories of Containment, 4th edition, HSE books, Sudbury, UK.

2.2 Microbiological Safety Cabinets

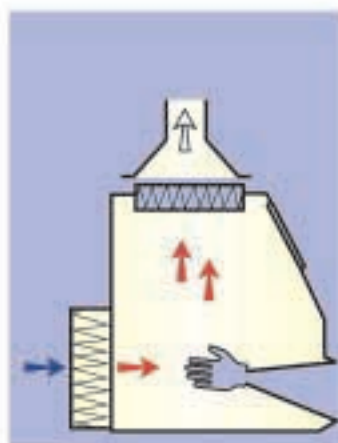
A microbiological safety cabinet is probably the most important piece of equipment since, when operated correctly, it will provide a clean working environment for the product, whilst protecting the operator from aerosols. In these cabinets operator and/or product protection is provided through the use 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.

Figure 1. Diagram of microbiological safety cabinet airflow patterns

Class I






Class II



Class III

KEY

-  Room air
-  Contaminated air
-  Clean air

Environmental monitoring with Tryptose Soya Broth agar settle plates inside the cabinet for a minimum of four hours should be a good indicator of how clean a cabinet is. There should be no growth of bacteria or fungi on such plates.

In most cases a class II 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.

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 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 water bath treatment fluid (Prod. No. S5525) in the incubator water trays will also reduce the risk of bacterial and fungal growth in the water trays. However, there is no substitute for regular cleaning. (Note “Sigma Clean” Prod. No. S5525 is harmful by inhalation, contact with skin or if swallowed and is also a severe irritant.)

2.5 Work Surfaces and Flooring

In order to maintain a clean working environment the laboratory surfaces including benches, 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 7.3 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 packed, plasticware. 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. Obviously all spills should be dealt with immediately. Routine cleaning should also 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 six monthly 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 the temperature adjusted as necessary
- Incubator CO₂ and O₂ levels should also be regularly checked to ensure the levels are being correctly maintained

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. The performance of risk assessment is a legal requirement under the Health and Safety at Work Act, UK. There are other EC 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 representative. Consequently risk assessments must be undertaken prior to starting any activity. The assessment consists of 2 elements:

1. Identifying and evaluating the risks.
2. Defining ways of minimising or avoiding 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 diploid lines of finite lifespan (e.g. MRC-5). |
| Medium risk | ● Poorly characterised mammalian cell lines. |
| High risk | ● Cell lines derived from human/primate tissue or blood. |
| | ● Cell lines with endogenous pathogens (the precise categorisation is dependent upon the pathogen) – refer to ACDP guidelines, 1985, for details [†] . |
| | ● Cell lines used following experimental infection where the categorisation is dependent upon the infecting agent - refer to ACDP guidelines, 1985, for details. |

[†] Advisory Committee on Dangerous Pathogens (1985) Categorisation of Biological Agents According to Hazard and Categories of Containment, 4th edition, HSE books, Sudbury, UK

A culture collection, such as ECACC will recommend a minimum the containment level required for a given cell line based upon its risk assessment. For most cell lines the appropriate level of containment is Category 2. However, this may need to be increased to Category 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 HTLV Category 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 practise 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. Staff training and the use of written standard operating procedures and risk assessments will also reduce the potential for harm. Training courses covering the basics of tissue culture safety are offered by ECACC.

3.2 Disinfection

Methods designed for the disinfection/decontamination of culture waste, work surfaces and equipment represent important means for minimising the risk of harm.

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

Hypochlorites (e.g. Chlorox, Presept)

- 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 washing pipettes, and 10,000ppm for tissue culture waste and spillages

NB: 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 (e.g. Sudol, Hycolin)

- Not active against viruses
- Remains active in the presence of organic matter

Alcohol (e.g. ethanol, isopropanol)

- Effective concentrations 70% for ethanol, 60-70% for isopropanol
- Their mode of activity is by 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. glutaraldehyde, formaldehyde)

- Aldehydes are irritants and their use should be limited due to problems of sensitisation
- Glutaraldehyde may be used in situations where the use of hypochlorites is not suitable e.g. cleaning of centrifuge bowls or materials constructed of stainless steel that may be attacked or corroded by using hypochlorite solutions.

3.3 Waste Disposal

Any 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 overnight 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 autoclaved prior to incineration. These bags are available from Bibby Sterilin and Greiner
- If at all possible waste should be incinerated rather than autoclaved

4.0 Sourcing of Cell Lines

Large numbers of cell lines look identical. 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 significantly change the characteristics of the cells but again such contamination will be inapparent. Cell lines will also change with time in culture, and to add to all these natural hazards it is all too easy to mis-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.

The integrity and biological characteristics of a cell line have to be actively maintained by a well organised system of “husbandry” 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 strongly recommended that new cell lines should only 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 Cell Culture Collection “accessions” a new cell line it will characterise the cell line using techniques such as isoenzyme analysis and DNA profiling so that the identity of the cell line can subsequently 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 these authenticated Working Cell Banks (WCB). Replacement WCB's are manufactured from the original Master Cell Bank (MCB) and the new WCB will again be fully tested.

ECACC supplies its cell lines together with advice on how to maintain the line. A Technical Support team will subsequently assist with any difficulties and can often provide additional technical information about the cell line. 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 “bed rock” of any cell based project.

5.0 Main Types of Cell Culture

5.1 Primary Cultures

Primary cultures are derived directly from excised, normal animal tissue and cultured 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 life span primary cells usually retain many of the differentiated characteristics of the cell *in vivo*.

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.

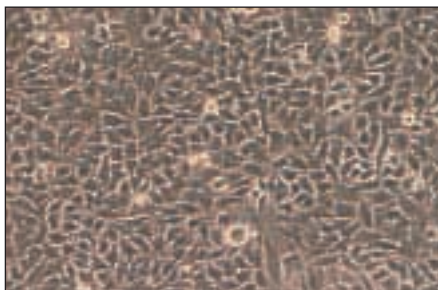
5.3 Culture Morphology

Morphologically 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 (Prod. No. 88031149-1v1), epithelial such as HeLa (Prod. No. 93021013-1v1), neuronal such as SH-SY5Y (Prod. No. 94030304-1v1) or fibroblasts such as MRC-5 (Prod. No. 84101801-1v1) and their morphology reflects the area within the tissue of origin.

Figure 2. Examples of Cell Morphology

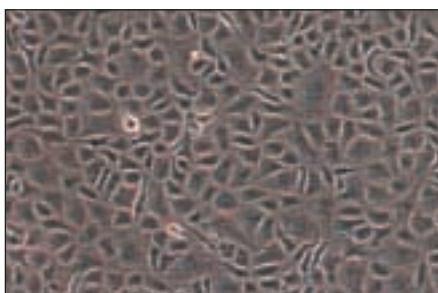
Hela - epithelial

(Prod. No. 93021013-IvI)



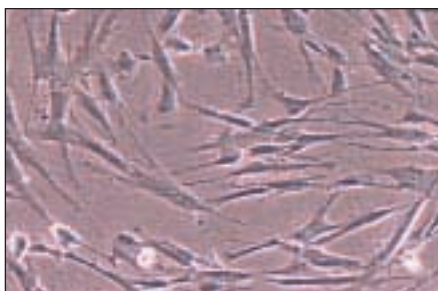
BAE-I - endothelial

(Prod. No. 88031149-IvI)



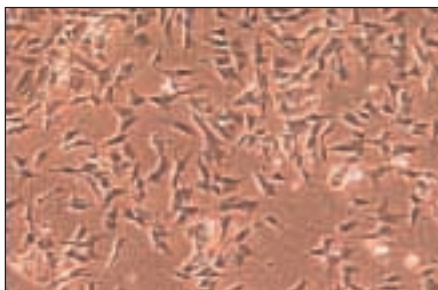
MRC-5 - fibroblast

(Prod. No. 84101801-IvI)



SH-SY5Y - neuronal

(Prod. No. 94030304-IvI)



(magnification x400)

The cell lines most commonly ordered from ECACC are listed in the table below (Table 1). The lines are classified by cell type.

Attached Cell Lines		
<i>Name</i>	<i>Species and tissue of origin</i>	<i>Morphology</i>
MRC-5 (Prod. No. 84101801)	Human lung	Fibroblast
HELA (Prod. No. 93021013)	Human cervix	Epithelial
VERO (Prod. No. 84113001)	African Green Monkey Kidney	Epithelial
NIH 3T3 (Prod. No. 93061524)	Mouse embryo	Fibroblast
L929 (Prod. No. 85011425)	Mouse connective tissue	Fibroblast
CHO (Prod. No. 85050302)	Chinese Hamster Ovary	Fibroblast
BHK-21 (Prod. No. 85011433)	Syrian Hamster Kidney	Fibroblast
HEK 293 (Prod. No. 85120602)	Human Kidney	Epithelial
HEPG2 (Prod. No. 85011430)	Human Liver	Epithelial
BAE-I (Prod. No. 88031149)	Bovine aorta	Endothelial
Suspension Cell Lines		
<i>Name</i>	<i>Species and tissue of origin</i>	<i>Morphology</i>
NSO (Prod. No. 85110503)	Mouse myeloma	Lymphoblastoid-like
U937 (Prod. No. 85011440)	Human Histiocytic Lymphoma	Lymphoblastoid
Namalwa (Prod. No. 87060801)	Human Lymphoma	Lymphoblastoid
HL60 (Prod. No. 98070106)	Human Leukaemia	Lymphoblastoid-like
WEHI 231 (Prod. No. 85022107)	Mouse B-cell Lymphoma	Lymphoblastoid
YAC 1 (Prod. No. 86022801)	Mouse Lymphoma	Lymphoblastoid
U 266B1 (Prod. No. 85051003)	Human Myeloma	Lymphoblastoid
SH-SY5Y (Prod. No. 94030304)	Human neuroblastoma	Neuroblast

Table 1. Commonly used cell lines of each culture type

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.

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 thirty 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 (Prod. No. T2397) 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 the table below (Table 2).

Media type	Examples	Uses
Balanced salt solutions	PBS, Hanks BSS, Earles salts DPBS (Prod. No. D8537/D8662) HBSS (Prod. No. H9269/H9394) EBSS (Prod. No. E2888)	Form the basis of many complex media
Basal media	MEM (Prod. No. M2279)	Primary and diploid cultures.
	DMEM (Prod. No. D5671)	Modification of MEM containing increased level of amino acids and vitamins. Supports a wide range of cell types including hybridomas.
	GMEM (Prod. No. G5154)	Glasgows modified MEM was defined for BHK-21 cells
Complex media	RPMI 1640 (Prod. No. R0883)	Originally derived for human leukaemic cells. It supports a wide range of mammalian cells including hybridomas
	Iscoves DMEM (Prod. No. I3390)	Further enriched modification of DMEM which supports high density growth
	Leibovitz L-15 (Prod. No. L5520, liquid)	Designed for CO ₂ free environments
	TC 100 (Prod. No. T3160) Graces Insect medium (Prod. No. G8142) Schneiders Insect medium (Prod. No. S0146)	Designed for culturing insect cells
Serum Free Media	CHO (Prod. No. C5467) HEK293 (Prod. No. G0791)	For use in serum free applications.
	Ham F10 and derivatives Ham F12 (Prod. No. N4888) DMEM/F12 (Prod. No. D8062)	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	Sf-900 II SFM, SF Insect-Medium-Z (Prod. No. S3902)	Specifically designed for use with Sf9 insect cells

Table 2. Different types of culture medium and their uses

6.1 Basic Constituents of media

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

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 $\text{CO}_3 / \text{HCO}_3$ content of the culture medium and (ii) chemical buffering using a zwitterion called HEPES (Prod. No. H4034).

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 (Prod. No. H4034) 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. HEPES (Prod. No. H4034) buffered cultures do not require a controlled gaseous atmosphere.

Most commercial culture media include phenol red (Prod. No. P3532/P0290) 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.

6.5 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.

6.6 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.

6.7 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.

6.8 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.

Whilst all media may be made from the basic ingredients this is time consuming and may predispose to contamination. For convenience most media are available as ready mixed powders or as 10x and 1x liquid media. All commonly used media are listed in the Sigma-Aldrich Life Science 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 (Prod. No. W3500, water, tissue culture grade). In most cases water prepared by reverse osmosis and resin cartridge purification with a final resistance of 16-18M Ω is suitable. Once prepared the media should be filter sterilised before use. Obviously purchasing 1x liquid media direct from Sigma eliminates the need for this.

6.9 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. 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 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 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.

6.10 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 guidelines on minimising the risk of BSE transmission via medicinal products, CPMP/BWP/877/96, recommends the main

measures to be implemented in order to establish the safety of bovine material versus the BSE risk. Again, 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.

Recent 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.

Similarly the FDA has similar guidelines when accepting regulatory submissions. The FDA regulates all medicinal products for Human use, such as therapeutics, vaccines and diagnostics, and, usually, the United States Department Agriculture (USDA) are not involved.

The USDA regulates all medicinal products for veterinary use or for agricultural use. Similarly, the USDA regulates all products that contain a primary component of animal origin.

It is important that you are aware of the use and restrictions when using serum, for further information contact your local Sigma-Aldrich sales office for specific information on the serum being used (contact information is at the back of this handbook).

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 be allowed into the USA.

6.11 Origin of Serum

USA/Canada, New Zealand, Finland and Denmark - No safety testing required.

Australia, Mexico, Central America - Safety testing may be required, depending on the geographical region where the serum was collected.

Sigma-Aldrich carries out all safety test requirements stipulated by the USDA in USA laboratories. If necessary Sigma-Aldrich will assist customers in obtaining approval from the USDA, on any batch of serum of Australian origin supplied by Sigma-Aldrich.

Sigma-Aldrich sources serum from the states of Victoria and Tasmania. Tasmania will shortly be considered a separate geographical area within Australia eliminating the requirement for any safety testing.

7.0 Cryopreservation and Storage of Cell Lines

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 cell banking section below)
- 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 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 waterbath 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 Prod. No. D2650) or glycerol (Prod. No. G2025) 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. HL60 (Prod. No. 98070106-lv1) where DMSO is used to induce differentiation. In such cases an alternative such as glycerol (Prod. No. G2025) should be used (refer to ECACC data sheet for details of the correct cryoprotectant). Sigma also offers ready made cell freezing media containing DMSO (Prod. No. C6164), glycerol (Prod. No. C6039) and a serum-free formulation containing DMSO (Prod. No. C6295).

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:

Method	Advantages	Disadvantages
Electric (-135°C) Freezer	<ul style="list-style-type: none"> ● Ease of maintenance ● Steady temperature ● Low running costs 	<ul style="list-style-type: none"> ● Requires liquid nitrogen back-up ● Mechanically complex ● Storage temperatures high relative to liquid nitrogen
Liquid Phase Nitrogen	<ul style="list-style-type: none"> ● Steady ultra-low (-196°C) temperature ● Simplicity and mechanical reliability 	<ul style="list-style-type: none"> ● Requires regular supply of liquid nitrogen ● High running costs ● Risk of cross-contamination via the liquid nitrogen
Vapour Phase Nitrogen	<ul style="list-style-type: none"> ● No risk of cross-contamination from liquid nitrogen ● Low temperatures achieved ● Simplicity and reliability 	<ul style="list-style-type: none"> ● Requires regular supply of liquid nitrogen ● High running costs ● Temperature fluctuations between 135°C and -190°C

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

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 and sealed glass ampoules. Both of these requirements create potential hazards. 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 has to 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.

All liquid nitrogen storage vessels should include alarms that at least warn of low liquid nitrogen levels. 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 delivery can be missed without catastrophic consequences.

Inventory Control

All ultra-low temperature storage vessels will 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

7.3 Safety Considerations

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. Proper training and the use of protective equipment will minimise the risk of frostbite and other minor incidents.

Risk of asphyxiation

The single most important safety consideration is the potential risk of asphyxiation due to the high levels of nitrogen that can lead to oxygen depletion. 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. Large volume stores should have low oxygen alarm systems.

Preventative measures

- 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 (~ 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

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 (i.e. surface antigen or monoclonal antibody expression)
- Genetic drift particularly in cells known to have an unstable karyotype (i.e. CHO Prod. No. 85050302-1v1, BHK 21 Prod. No. 85011433-1v1)
- Loss of cell line due to exceeding finite life-span e.g. human diploid cells such as MRC-5 (Prod. No. 84101801-1v1)
- Risk of cross contamination with other cell lines
- Increased consumables and staff costs

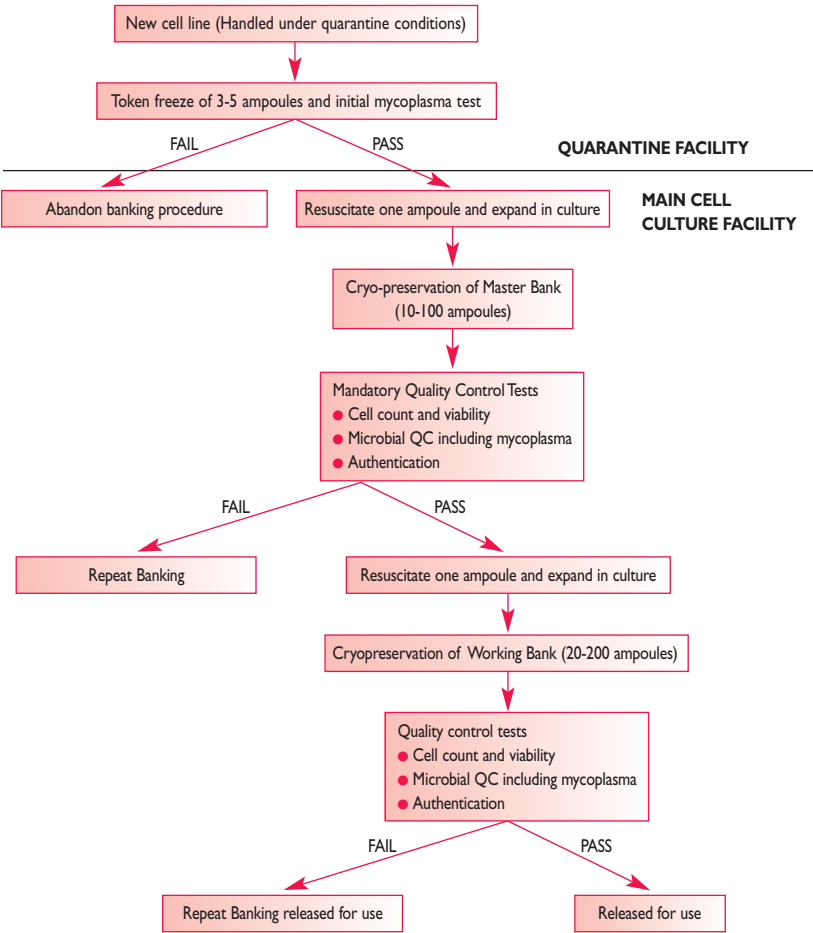
All of these potential risk factors may be minimised by the implementation of a cell banking system as described below. This type of system is known as a tiered banking system or Master Cell Banking system. On initial arrival into the laboratory a new cell culture should be regarded as a potential source of contamination from 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 Bank is prepared. One of the token stock ampoules should then be thawed and expanded to produce a Master Bank of 10-20 ampoules depending upon the anticipated level of use.

Ampoules of this 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 Bank should be thawed and cultured to produce a Working 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 confirm that the Master and Working Banks are genetically identical by DNA profiling techniques.

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



Notes

1. The number of ampoules prepared for Master and Working 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 Bank should be replenished from an ampoule of the Master Bank. This should be done in sufficient time to allow the quality control to be completed.
5. A new Master 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

Introduction

Quality is important in all aspects of tissue culture since the quality of materials used (i.e. media and other reagents) will affect the quality of the cultures 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

9.1 Reagents and Materials

A potential source of contamination is 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 including 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 forms 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 FBS 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 are also supplied with Certificates of Analysis for each batch produced, which should be kept for future reference.

9.2 Provenance and Integrity of Cell Lines

The sourcing of cell lines can have an important effect on quality since 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:

- Contaminant free
- Fully characterised and authenticated in terms of DNA profile and species of origin
- 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.

9.3 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 3 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 (see below). 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).

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 5 major species that are tissue culture contaminants, namely *M. hyorhinis*, *M. arginini*, *M. orale*, *M. fermentans* and *Acholeplasma laidlawii*.

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

- Reduced 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 - Isolation by culture and Protocol 10 – 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. Culture collections such as ECACC are able to test cultures if required. Mycoplasma testing products are available from Sigma, refer to pages 489-490 of Life Sciences Catalogue.

Viral Contamination

Some cell lines contain endogenous viruses and secrete virus particles or express viral antigens on their surface (e.g. 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.

9.4 Environmental Monitoring

It is good practice to monitor the laboratory environment where cell cultures and their products are prepared. Class II microbiology safety cabinets should be checked every 6 months to ensure that they are working efficiently. However it is also advisable to monitor the number of contaminants within the cabinet by periodically placing open settle plates (blood 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 criticality of the work and the levels of cleanliness that can be achieved under normal operating conditions.

9.5 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. However if this is not possible eradication of the contaminant may be attempted with the use of antibiotics. In addition culture collections such as ECACC will attempt to eradicate any contaminants if required. Please contact ECACC for further details.

Viral infections are virtually impossible to remove from cultures since they do not respond to antibiotic treatment. Also, since 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

10.1 Authentication Techniques

Whatever the scope of work to be carried out it is important to know that the work is being conducted using the correct reagents. This is no less important for cell cultures, since if cell cultures are not what they are reported to be then work can be invalidated and resources wasted. There is now considerable evidence of gross cross-contamination of cell lines, in particular with HeLa (Prod. No. 93021013-1v1) where up to 16 lines were offered to ECACC with DNA profiles identical to HeLa. These include Hep 2, WISH, INT 407, Chang liver and Girardi heart. 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 of the cells as part of the banking process.

Tests used to authenticate cell cultures include iso-enzyme analysis, karyotyping/ cytogenetic analysis and more recently molecular techniques of DNA profiling. Whilst most of the techniques above 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.

10.2 Iso-Enzyme Analysis

Iso-enzymes are a series of enzymes present in different species that have similar catalytic properties but differ in their structure. By studying the iso-enzymes present in cell lines it is possible to identify the species from which the cell line was derived. The technique is also used as a means of excluding the possibility of gross cross-contamination of the cell line with another culture of a different species.

The principles upon which iso-enzyme analysis is based are:

- Each iso-enzyme has multiple gene loci coding for different polypeptides with identical enzyme activity (e.g. lactate dehydrogenase, LD)
- Electrophoretic migration rates change dependent on sub-unit composition e.g. LD has five possible iso-forms (LD 1-5)
- Different species have different combinations of these iso-forms
- Using a typical panel of 4 iso-enzymes a composite picture is built up enabling the species of origin to be determined by the use of reference tables

10.3 DNA Fingerprinting

DNA fingerprinting enables the following:

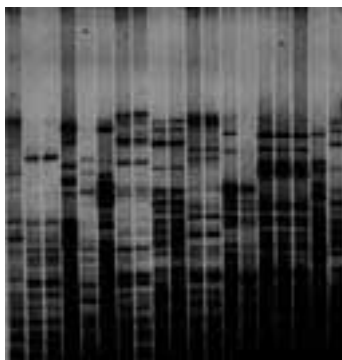
- Identification of individual cell lines from the same species
- Confirmation of the identity of cell banks compared to reference master stocks
- Detection of cross-contamination

Multi locus DNA fingerprinting and multiplex - PCR DNA profiling are the methods used routinely as part of ECACC's routine cell banking procedures.

10.4 Multi Locus DNA Fingerprinting

- Uses multi locus Jeffrey's probes 33.15 or 33.6, along with Southern blotting technology producing a complex banding pattern.
- Probes cross-hybridise with most common species
- Has the disadvantage that the profiles require visual interpretation and comparison with other samples can be subjective

Figure 4. Multi Locus DNA Fingerprinting

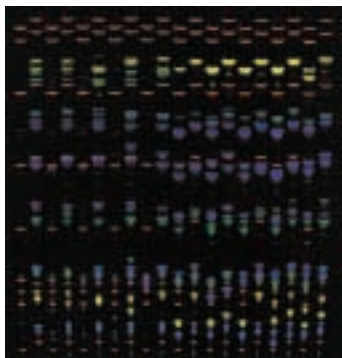


Each lane represents the genotype for one cell line using a series of primers.

10.5 Multiplex - PCR (STR) DNA profiling

- Uses a set of primers (9 used at ECACC) recognising micro-satellites using PCR and automated DNA sequencing techniques
- Primers are species specific and are used only for human cell lines
- Produces a colour-coded banding pattern, that translates into a digital code that can easily be stored on a database and compared to other stored profiles

Figure 5. PCR DNA profiling



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 ranging from 25cm² to 175cm². Typical cell yields from a T175 flask range from 1×10^7 for an attached line to 1×10^8 for a suspension line. However exact yields will vary depending on the cell line. It is not practicable 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 is best left to expert process development scientists.

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 4.

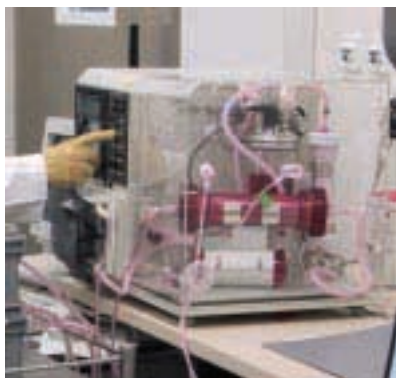
11.2 Scale-up Solutions

Figure 6. Triple Flask



(Prod. No. F8542)

Figure 7. Bioreactor



The example shown is a Cell-Pharm 2000 hollow fibre based bioreactor from Biovest International Inc. and produces grams scale of antibody per month. These reactors are in routine use under cGMP manufacturing conditions at Sigma Aldrich Immunochemical Production facility in Rehovot, Israel.

Technology	Suspension	Attached	Max Vol (ml)	Max S/A (cm ²)	Max cells (susp)	Max cells (att)	Advantages	Disadvantages
T Flask	✓	✓	150	225	1.5×10^8	$\sim 10^7$	Cheap, disposable, no cleaning / sterilisation required	Small scale. Multiples required for larger batches.
Triple Flask	(✓)	✓	150	525	1.5×10^8	3×10^7	Cheap, disposable, no cleaning / sterilisation required	Difficult to harvest attached cells. Multiples required for larger batches
Cell Factory	N/A	✓	8000	25280	N/A	1.5×10^{10}	Disposable – Single batch manufacture	Require additional equipment (vessels etc which may require cleaning). Difficult to harvest cells
Roller Bottles	✓	✓	1000	1700	1×10^9	1×10^8	Cheap, disposable, no cleaning / sterilisation required. Versatile. Automated systems available.	Require "decks" to turn. Multiples required for larger batches. Automation very costly
Expanded Roller Bottles	N/A	✓	(1000)	3400	N/A	2×10^8	As above	As above (no advantage for suspension cells)
Shake Flasks	✓	N/A	1000	N/A	1×10^9	N/A	Some disposables available.	Suspension Only*. Glass vessels to be cleaned & sterilised. Requires Shaker incubator
Spinner Flasks	✓	N/A	1000	N/A	1×10^9	N/A	Some semi-disposables available	Suspension Only* Glass vessels to be cleaned & sterilised. Requires Stirrer-base + incubator

Table 4. "Half-Way House" Solutions to Scale-up - without attempting to adapt cells or the process

A word of caution – although the systems listed in Table 4 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 for the user to adapt to the system as well as the cells!

Figure 8. Shake Flasks**Figure 9. Roller Deck**

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 60 revolutions per hour) which bathes the cells that are attached to the inner surface with medium. Roller bottles are available typically with surface areas of 1050cm² (Prod. No. Z35, 296-9). 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. Recently 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 as some cells lines do not attach evenly. This is a particular problem with epithelial cells. This may be partially overcome a little by optimising the speed of rotation, generally by decreasing the speed, during the period of attachment for cells with low attachment efficiency.

Figure 10. Roller Bottle

11.4 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. Spinner flask systems designed to handle culture volumes of 1-12 litres are available from Techne, Sigma, and Bellco, e.g. (Prod. No's. Z380482-3L capacity and Z380474-1L capacity).

Figure 11. Spinner Flasks

(Prod. No. Z380431)

11.5 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 (Prod. No. C4853 (220v) or C4728 (110v)). 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; in the order of 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, refer to page 356 of Sigma Life Science catalogue for further details.

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, (laboratory coat/gown, gloves and eye protection) at all times. In addition, thermally insulated gloves, full-face visor and splash-proof apron should be worn when handling liquid nitrogen.
2. Always use disposable caps to cover hair.
3. Wear dedicated PPE for tissue culture facility and keep separate from PPE worn in the general laboratory environment. The use of different 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% ethanol) between operations and allow a minimum of 15 minutes between handling different cell lines.
8. Wherever possible maintain separate bottles of media for each cell line in cultivation.
9. Examine cultures and media daily for evidence of gross bacterial or fungal contamination. This includes medium that has been purchased commercially.
10. Quality Control all media and reagents prior to use.
11. Keep cardboard packaging to a minimum in all cell culture areas.
12. Ensure that incubators, cabinet, centrifuges and microscopes are cleaned and serviced at regular intervals.
13. Test cells for mycoplasma on a regular basis.

The Don'ts

1. Do not continuously use antibiotics in culture medium as this will inevitably lead to the appearance of antibiotic resistant strains and may render a cell line useless for commercial purposes.
2. Don't allow waste to accumulate particularly within the microbiological safety cabinet or in the incubators.
3. Don't have too many people in the lab at any one time.
4. Don't handle cells from unauthenticated sources in the main cell culture suite. They should be handled in quarantine until quality control checks are complete.
5. Avoid keeping cell lines continually in culture without returning to frozen stock.
6. Avoid cell culture becoming fully confluent. Always sub-culture at 70-80% confluency or as advised on ECACC's cell culture data sheet.
7. Do not allow media to go out of date. Shelf life is only 6 weeks at +4°C once glutamine and serum is added.
8. Avoid water baths from becoming dirty by using Sigma Clean (Prod. No. S5525).
9. Don't allow essential equipment to become out of calibration. Ensure microbiological safety cabinets are tested regularly.

12.2 Protocol I - 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

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

Equipment

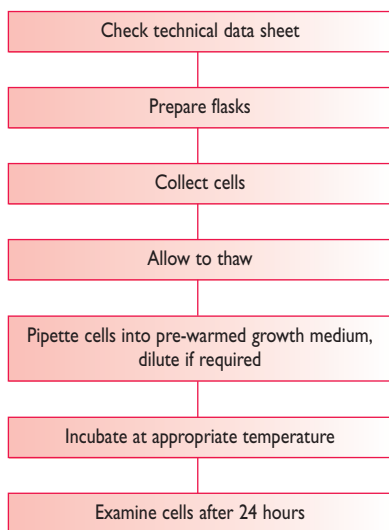
- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Microbiological safety cabinet at appropriate containment level

Procedure

1. Sanitise the cabinet using 70% ethanol before commencing work.
2. Sanitise gloves by washing them in 70% ethanol and allowing to air dry for 30 seconds before commencing work.
3. Put all materials and equipment into the cabinet prior to starting work after sanitising the exterior surfaces with 70% ethanol.
4. Whilst working do not contaminate gloves by touching anything outside the cabinet (especially face and hair). If gloves become contaminated re-sanitise with 70% ethanol as above before proceeding.
5. Discard gloves after handling contaminated cultures and at the end of all cell culture procedures.
6. Equipment in the cabinet or that which will be taken into the cabinet during cell culture procedures (media bottles, pipette tip boxes, pipette aids) should be wiped with tissue soaked with 70% ethanol prior to use.
7. Movement within and immediately outside the cabinet must not be rapid. Slow movement will allow the air within the cabinet to circulate properly.
8. Speech, sneezing and coughing must be directed away from the cabinet so as not to disrupt the airflows.
9. After completing work disinfect all equipment and material before removing from the cabinet. Spray the work surfaces inside the cabinet with 70% ethanol and wipe dry with tissue. Dispose of tissue by autoclaving.

10. Cell culture discard in chlorox (10,000) ppm must be kept in the cabinet for a minimum of two hours (preferably overnight) prior to discarding down the sink with copious amounts of water.
11. Periodically clean the cabinet surfaces with a disinfectant such as Presept, Tegador or Virkon or fumigate the cabinet according to the manufacturers instructions. However you must ensure that it is safe to fumigate your own laboratory environment due to the generation of gaseous formaldehyde, consult your on-site Health and Safety Advisor.

12.3 Protocol 2 - Resuscitation of Frozen Cell Lines



Aim

Many cultures obtained from a culture collection, such as ECACC, will arrive frozen and in order to use them the cells must be thawed and put into culture. It is vital to thaw cells correctly in order to maintain the viability of the culture and enable the culture to recover more quickly. Some cryoprotectants, such as DMSO (Prod. No. D2650), are toxic above 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 size of flask to resuscitation into.)
- 70% ethanol in water (Prod. No. R8382)
- DMSO (Prod. No. D2650)

Equipment

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

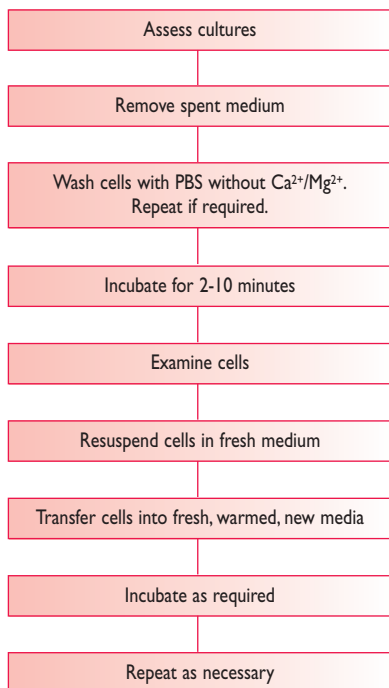
Procedure

1. Read Technical data sheet to establish specific requirements for your cell line.
2. Prepare the flasks as appropriate (information on technical data sheet). Label with cell line name, passage number and date.
3. Collect ampoule of cells from liquid nitrogen storage wearing appropriate protective equipment and transfer to laboratory in a sealed container.
4. Still wearing protective clothing, remove ampoule from container and place in a waterbath at an appropriate temperature for your cell line e.g. 37°C for mammalian cells. Submerge only the lower half of the ampoule. Allow to thaw until a small amount of ice remains in the vial - usually 1-2 minutes. Transfer to class II safety cabinet.
5. Wipe the outside of the ampoule with a tissue moistened (not excessively) with 70% alcohol hold tissue over ampoule to loosen lid.
6. Slowly, dropwise, pipette cells into pre-warmed growth medium to dilute out the DMSO (Prod. No. D2650) (flasks prepared in Step 2).
7. Incubate at the appropriate temperature for species and appropriate concentration of CO₂ in atmosphere.
8. Examine cells microscopically (phase contrast) after 24 hours and sub-culture as necessary.

Key Points

1. Most text books recommend washing the thawed cells in media to remove the cryoprotectant. This is only necessary if the cryoprotectant is known to have an adverse effect on the cells. In such cases the cells should be washed in media before being added to their final culture flasks. See Protocol 7 for further details.
2. Do not use an incubator to thaw cell cultures since the rate of thawing achieved is too slow resulting in a loss of viability.
3. If a CO₂ incubator is not available gas the flasks for 1-2 minutes with 5% CO₂ in 95% air filtered through a 0.2µm filter.
4. For some cultures it is necessary to subculture before confluence is reached in order to maintain their characteristics e.g. the contact inhibition of NIH 3T3 (Prod. No. 93061524) cells is lost if they are allowed to reach confluence repeatedly.

12.4 Protocol 3 - Subculture of Adherent Cell Lines



Aim

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

Materials

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

Equipment

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

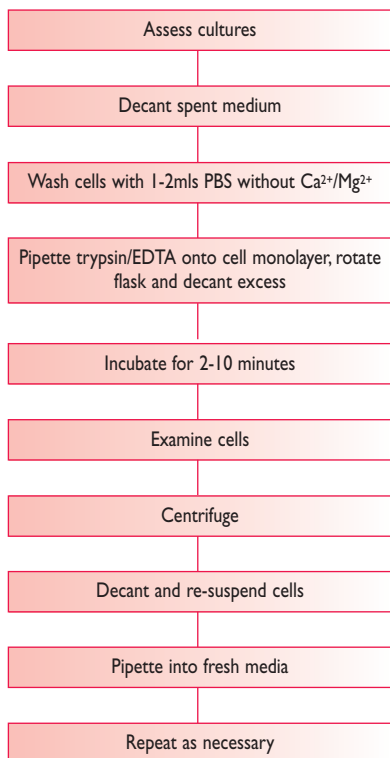
Procedure

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 $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Prod. No. D8537) using a volume equivalent to half the volume of culture medium. Repeat this wash step if the cells are known to adhere strongly.
4. Pipette trypsin/EDTA (Prod. No. T4049) onto the washed cell monolayer using 1 ml per 25 cm² 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 100-200 μl and perform a cell count (Protocol 6- Cell Quantification).
8. Transfer the required number of cells to a new labelled flask containing pre-warmed medium (refer to ECACC Cell Line Data Sheet for the required seeding density).
9. Incubate as appropriate for the cell line.
10. Repeat this process as demanded by the growth characteristics of the cell line.

Key Points

1. Some cultures whilst growing as attached lines adhere only lightly to the flask, thus it is important to ensure that the culture medium is retained and the flasks are handled with care to prevent the cells detaching prematurely.
2. Although most cells will detach in the presence of trypsin alone the EDTA is added to enhance the activity of the enzyme.
3. Trypsin is inactivated in the presence of serum. Therefore, it is essential to remove all traces of serum from the culture medium by washing the monolayer of cells with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Prod. No. D8537).
4. Cells should only be exposed to trypsin/EDTA (Prod. No. T4049) long enough to detach cells. Prolonged exposure could damage surface receptors.
5. Trypsin should be 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 (Prod. No. T6414), 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. This is especially necessary for serum-free cell culture.
7. If a CO_2 incubator is not available gas the flasks for 1-2 min with 5% CO_2 in 95% air filtered through a 0.2 μm filter.

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% ethanol in water (Prod. No. R8382)
- PBS without Ca²⁺/Mg²⁺ (Prod. No. D8537)
- 0.25% trypsin/EDTA in HBSS, without Ca²⁺/Mg²⁺ (Prod. No. T4049)
- Trypsin (Prod. No. T4424)
- Soyabean trypsin Inhibitor (Prod. No. T6414)

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet at the appropriate containment level
- Centrifuge
- Inverted phase contrast microscope
- CO₂ incubator
- Haemocytometer (Bright-line, Prod. No. Z35, 962-9, Improved Neubauer Grid, Camlab CCH.ACI)
- Pre-labelled flasks
- Tissues

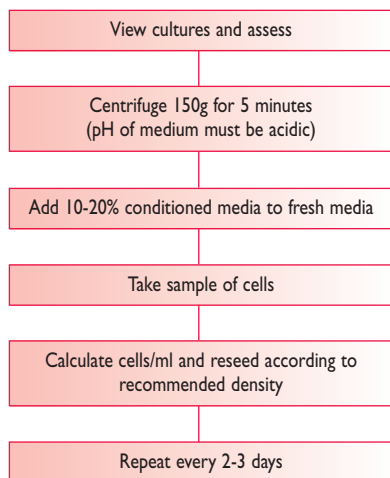
Procedure

1. View cultures using an inverted phase contrast microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants. Give the flask a gentle knock first, this may dislodge the cells from the flask and remove the need for a trypsinisation step with the subsequent loss of some cells due to the washings.
2. Decant spent medium into a sterile centrifuge tube and retain.
3. Wash any remaining attached cells with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Prod. No. D8537) using 1-2ml for each 25cm^2 of surface area. Retain the washings.
4. Pipette trypsin/EDTA (Prod. No. T4049) onto the washed cell monolayer using 1ml per 25cm^2 of surface area. Rotate flask to cover the monolayer with trypsin. Decant the excess trypsin.
5. Return flask to incubator and leave for 2-10 minutes.
6. Examine the cells using an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.
7. Transfer the cells into the centrifuge tube containing the retained spent medium and cells.
8. Centrifuge the remaining cell suspension at 150g for 5 minutes. Also centrifuge the washings from Number 3 above if they contain significant numbers of cells.
9. Decant the supernatants and resuspend the cell pellets in a small volume (10-20mls) of fresh culture medium. Pool the cell suspensions. Count the cells.
10. Pipette the required number of cells to a new 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 is used to enhance the activity of the enzyme.
2. Trypsin is inactivated in the presence of serum. Therefore, it is essential to remove all traces of serum from the culture medium by washing the monolayer of cells with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Prod. No. D8537). Repeated warming to 37°C also inactivates trypsin.
3. Cells should only be exposed to trypsin/EDTA (Prod. No. T4049) long enough to detach cells. Prolonged exposure could damage surface receptors. In general a shorter time of exposure to trypsin is required for semi adherent cell lines.
4. Trypsin should be 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 (Prod. No. T6414), where an equal volume of inhibitor at a concentration of 1mg/ml is added to the trypsinised cells. The cells are then centrifuged, resuspended in fresh culture medium and counted as above.
6. If a CO_2 incubator is not available gas the flasks for 1-2 minutes with 5% CO_2 in 95% air filtered through a $0.2\mu\text{m}$ 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 lines subculture by dilution is relatively easy. But for lines that grow in clumps it may be necessary to bring the cells into a single cell suspension by centrifugation and resuspension by pipetting in a smaller volume before counting.

Materials

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

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- CO₂ incubator
- Inverted phase contrast microscope
- Haemocytometer (Bright-line, Prod. No. Z35, 962-9, Improved Neubauer, Camlab CCH.ACI)
- Pre-labelled flasks

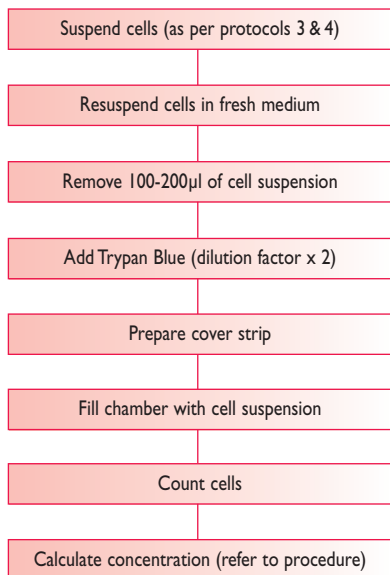
Procedure

1. View cultures using an inverted phase contrast microscope. Cells growing in exponential growth phase should be bright, round and refractile. Hybridomas may be very sticky and require a gentle knock to the flask to detach the cells. EBV transformed cells can grow in very large clumps that are very difficult to count and the 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 150g for 5 minutes, re-seed at a slightly higher cell density and add 10-20% of conditioned medium (supernatant) to the fresh media.
3. Take a small sample of the cells from the cell suspension (100-200µl - Protocol 6 - Cell Quantification). Calculate cells/ml and re-seed the desired number of cells into freshly prepared flasks without centrifugation just by diluting the cells. The data sheet will give the recommended seeding densities
4. Repeat this every 2-3 days.

Key Points

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

12.7 Protocol 6 - Cell Quantification



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.

Materials

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

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- CO₂ incubator
- Haemocytometer (Bright-line, Prod. No. Z35, 962-9, Improved Neubauer, Camlab CCH.AC1)
- Inverted phase contrast microscope
- Pre-labelled flasks

Procedure

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

Where:

- A is the mean number of viable cells counted, i.e. $\frac{\text{Total viable cells counted}}{\text{Number of squares}}$
- B is the mean number of non-viable cell counted, i.e. $\frac{\text{Total non-viable cells counted}}{\text{Number of squares}}$
- C is the dilution factor and
- D is the correction factor (this is provided by the haemocytometer manufacturer).

Concentration of viable cells (cells/ml) = $A \times C \times D$

Concentration of non-viable cells (cells/ml) = $B \times C \times D$

Total number of viable cells = concentration of viable cells x volume

Total number of cells = number of viable + number of dead cells

Percentage Viability = $\frac{\text{No of viable cells}}{\text{Total No of cells}} \times 100$

Key Points

1. Trypan Blue (Prod. No.T8154) is toxic and is a potential carcinogen. Protective clothing, gloves and face/eye protection should be worn. Do not breathe the vapour.
2. The central area of the counting chamber is 1mm^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 0.1mm .
3. The correction factor of 10^4 converts 0.1mm^3 to 1ml ($0.1\text{mm}^3 = 1\text{mm}^2 \times 0.1\text{mm}$)
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, resuspending in a smaller volume and recounting.
 - Too many cells to count. This can be overcome by using a higher dilution factor in trypan blue e.g. 1:10

New Products for Cell Lysis

CellLytic™ M Cell Lysis Reagent

CellLytic™ M is a proprietary detergent solution designed for complete protein extraction from cultured mammalian cells. It enables efficient and rapid cell lysis and solubilization of proteins for both suspension and adherent cells. Use 125µl CellLytic™ - M for 10^6 - 10^7 of suspended cells. For adherent cells use 500-1,000 µl for a 100mm plate; 200-400µl for a 35mm plate.

The extracted proteins can be used for the following:

Reporter Gene Assays: β-gal, CAT, Alkaline Phosphatase

Immunoassays: Western blots, ELISA, Immunoprecipitation

Kinase Assays: Protein Kinase C, Tyrosine Kinase

Phosphatase Assays: General Phosphatases, Tyrosine Phosphatases

DNA-Protein Interaction Assays (Electrophoretic Mobility Shift Assay)

Features and Benefits:

Fast: Cell lysis takes just 5 minutes at room temperature

Efficient: Up to 50% more efficient than freeze-thaw or sonication

Flexible: Compatible with Coomassie Blue® and Silver staining

Convenient: Ready-to-use, no scraping from culture plates required.

Gentle: Non-denaturing and does not interfere in downstream applications.

CellLytic™ MT Cell Lysis Reagent

CellLytic™ MT is an efficient reagent for the extraction of mammalian tissue proteins for analysis. The lysis buffer consists of a dialyzable mild detergent at a low concentration, bicine, and 150mM NaCl, resulting in minimal interference with protein interactions and biological activity, and also acts as a buffer for biological activity. CellLytic™ MT is also used for

extraction of cell-line proteins that require salt in the lysis buffer. 20ml of CellLytic™ MT is sufficient for 1 gram of tissue.

Features and Benefits:

Flexible: Easy-to-remove by dialysis if necessary

Convenient: Ready-to-use.

CellLytic™ Y Cell Lysis Reagent

CellLytic™ Y is versatile, phosphate-free reagent compatible with downstream applications including affinity purification, reporter gene assays, and electrophoretic mobility shift assay (EMSA). An effective method for efficient cell lysis and protein solubilisation, CellLytic™ Y avoids protein degradation and interference with protein immunoreactivity and biological activity.

Use of CellLytic™ Y involves a short procedure performed at room temperature using standard lysis conditions. No extreme conditions or glass beads are required. Use just 2.5-5ml gram yeast cells.

Features and Benefits:

Fast: Cell lysis takes only 20 minutes

Efficient: Three times more efficient than glass beads

Flexible: Extraction efficiency is enhanced by the addition of reducing agents (DTT)

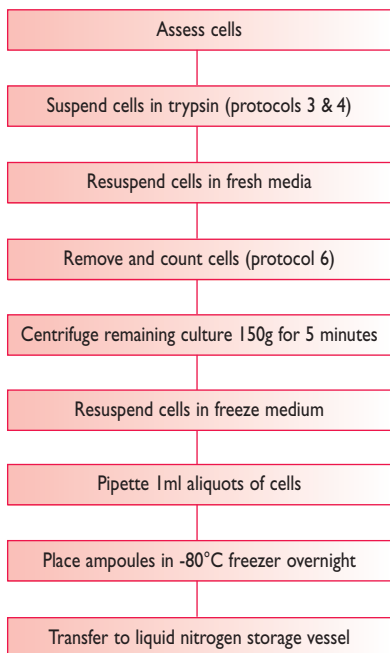
Convenient: Ready-to-use

Gentle: Non-denaturing – preserves biological function.

Product information request

Prod. No.	Description	Pack Size
C2978	CellLytic™ M	250ml
C3228	CellLytic™ MT	500ml
C4482	CellLytic™ Y	500ml
B3553	CellLytic™ B (standard strength)	500ml
B3678	CellLytic™ B11 (2X concentrate)	250ml
N-XTRACT	CellLytic™ Nu-CLEAR™ Extraction Kit	1 kit

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 (Planer Series Two) from Planer Products. This is the most reliable and reproducible way to freeze cells but as the cost of such equipment is beyond the majority of research laboratories the methods below are 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 70% basal medium, 20% FCS, 10% DMSO (Prod. No. D2650) or glycerol, check ECACC data sheets for details).
- 70% ethanol in water (Prod. No. R8382)
- PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Prod. No. D8537)
- 0.25% trypsin/EDTA in HBSS, without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Prod. No. T4049)
- DMSO (Prod. No. D2650)
- Trypsin/EDTA (Prod. No. T4049)
- HL60 (Prod. No. 98070106-1v1)

Equipment

- Personal protective equipment (sterile gloves, Laboratory coat)
- Full-face protective mask/visor
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- Haemocytometer (Sigma Bright- line Prod. No. Z35, 962-9, Improved Neubauer – Camlab CCH.ACI)
- Pre labelled ampoules/cryotubes
- Cell Freezing Device (e.g. Nalgene Mr Frosty Prod. No. C1562)

Procedure

1. View cultures using an inverted microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants.
2. Bring adherent and semi adherent cells into suspension using trypsin/EDTA (Prod. No. T4049) as above (Protocol 3 and 4 – Subculture of adherent/attached and semi-adherent cell lines) and re-suspend in a volume of fresh medium at least equivalent to the volume of trypsin. Suspension cell lines can be used directly.
3. Remove a small aliquot of cells (100-200 μ l) and perform a cell count (Protocol 6 – Cell Quantification). Ideally the cell viability should be in excess of 90% in order to achieve a good recovery after freezing.
4. Centrifuge the remaining culture at 150g for 5 minutes.
5. Re-suspend cells at a concentration of 2-4x10⁶ cells per ml in freeze medium.
6. Pipette 1ml aliquots of cells into cryoprotective 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 (Prod. 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 Prod. No. D2650), however, this is not appropriate for all cell lines e.g. HL60 (Prod. No. 98070106-1v1) where DMSO is used to induce differentiation. In such cases an alternative such as glycerol should be used (refer to ECACC data sheet for details of the correct cryoprotectant).
2. ECACC freeze medium recommended above has been shown to be a good universal medium for most cell types. Another commonly used freeze medium formulation is: 70% basal medium, 20% FCS, 10% DMSO but this may not be suitable for all cell types. Check it works for your cells before using on a regular basis (Prod. No. C6164).
3. It is essential that cultures are healthy and in the log phase of growth. This can be achieved by using pre-confluent cultures (cultures that are below their maximum cell density) and by changing the culture medium 24 hours before freezing.
4. The rate of cooling may vary but as a general guide a rate of between -1°C and -3°C per minute will prove suitable for the majority of cell cultures.
5. An alternative to the Mr Frosty system is the Taylor Wharton passive freezer where ampoules are held in liquid nitrogen vapour in the neck of 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 1 cm 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

Aim

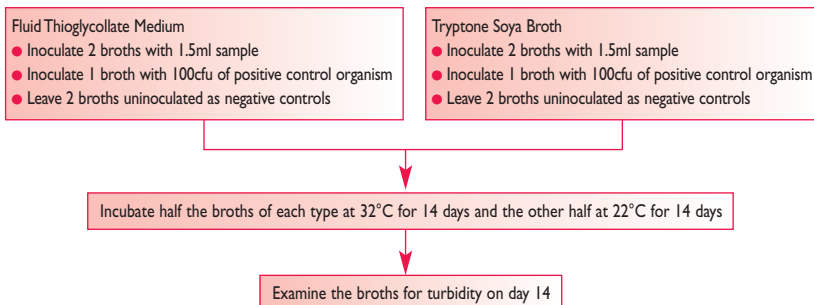
In cases of gross contamination the naked eye may identify the presence of bacteria and fungi. However, it is necessary to detect low-level infections by incubation of cell cultures and/or their products in microbiological broth. Equally these sterility tests can be used to confirm the absence of bacteria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

Materials

- Soyabean Casein Digest (Tryptone Soya Broth, TSB) (15ml aliquots) (Prod. No. S1674) TSB Powder (Prod. No. T8907)
- Fluid Thioglycollate Medium (20ml aliquots) (TGM) (Prod. No. F4797)
- *Bacillus subtilis* NCTC*
- *Candida albicans* NCTC*
- *Clostridium sporogenes* NCTC*

Equipment

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

Figure 12. Flow Scheme for Bacteria and Fungi Testing**Procedure**

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

Criteria for a Valid Result

All positive control broths show evidence of bacteria and fungi within 14 days of incubation and the negative control broths show no evidence of bacteria and fungi.

Criteria for a Positive Result

Test broths containing bacteria or fungi show turbidity.

Criteria for a Negative Result

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

Notes

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

12.10 Protocol 9 - Detection of Mycoplasma by Culture

Aim

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

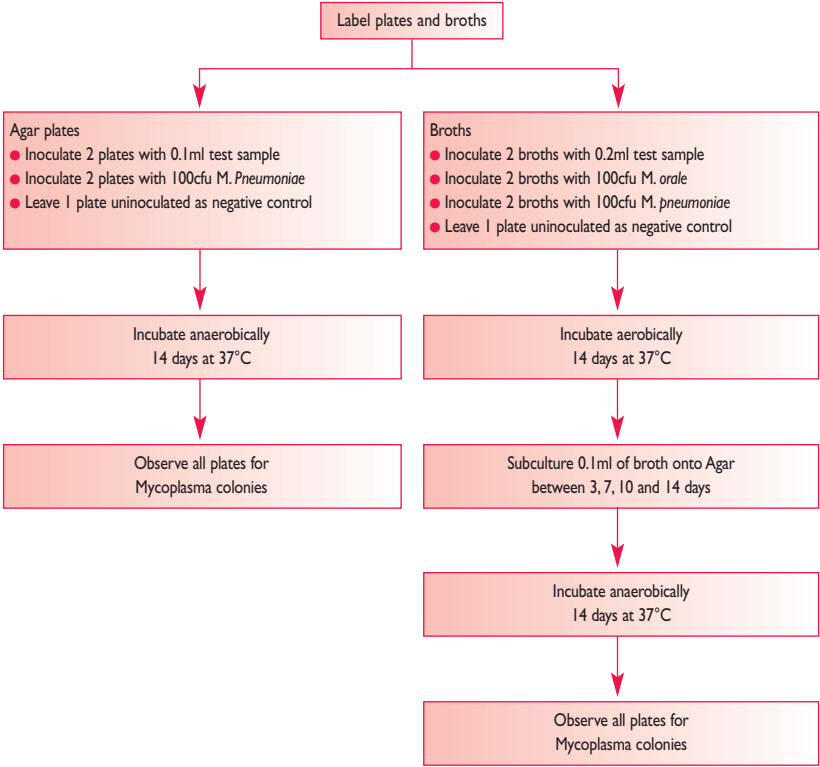
Materials

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

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet at appropriate containment level
- CO₂ Incubator set at 32°C
- Gas Jar (Gallenkamp)
- Gas Pak Anaerobic System (Gallenkamp)
- Gas Pak Catalyst (Gallenkamp)
- Gas Pak Anaerobic Indicator (Gallenkamp)

Figure 13. Flow Scheme for Detection of Mycoplasma by Culture



Procedure

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

Criteria for a Valid Result

All positive control agar plates and broths show evidence of mycoplasma by typical colony formation on agar plates and usually a 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.

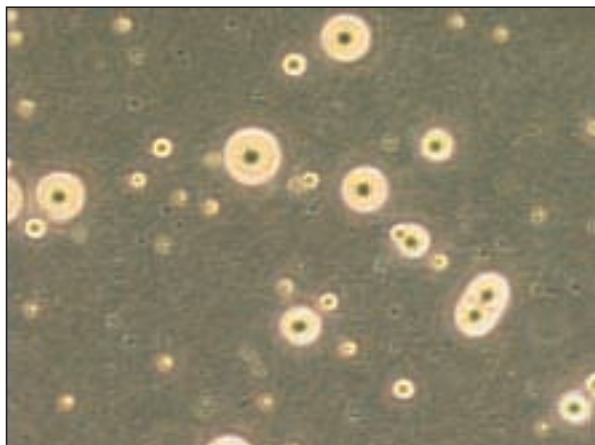
Criteria for a Negative Result

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

Figure 14 - Typical “fried egg colonies” *Mycoplasma pneumoniae*



(magnification x400)

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

Aim

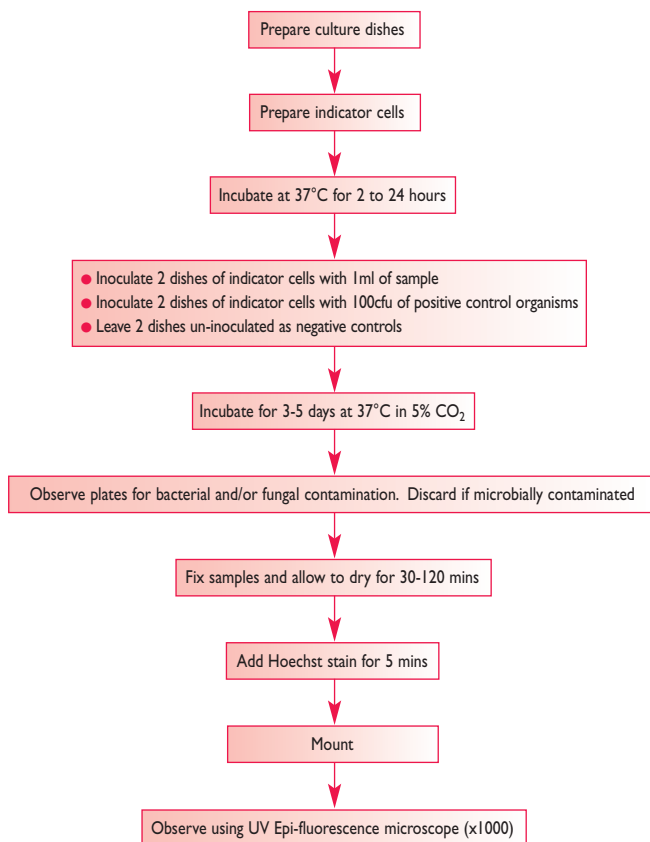
DNA staining methods such as Hoechst staining techniques are quick with results available within 24 hours which compares favourably with 4 weeks for detection by culture. However the staining of cultures directly with a DNA stain, results in a much-reduced sensitivity ($\sim 10^6$ cfu/ml). This may be improved by co-culturing the test cell line in the presence of an indicator cell line such as Vero (Prod. No. 84113001-Iv1). This enrichment step results in a sensitivity of 10^4 cfu/ml of culture. This step also improves sensitivity by increasing the surface area upon which mycoplasma can adhere. Like detection by culture, DNA staining methods are suitable for the detection of mycoplasma from cell cultures or cell culture reagents.

Materials

- Media— pre-warmed to 37°C (refer to the ECACC Cell Line Data Sheet for the correct medium)
- 70% ethanol in water (Prod. No. R8382)
- Methanol (Prod. No. 175)
- Acetic Acid Glacial (Prod. No. A6283)
- Hoechst 33258 stain solution (Prod. No. H6024)
- Vero cells (Prod. No. 84113001-Iv1)
- Mountant (Autoclave 22.2ml 0.2M citric acid with 27.8ml 0.2M disodium phosphate. Add 50ml glycerol. Filter sterilise and store at 4°C) (Prod. No. M1289)
- *Mycoplasma hyorhinis* NCTC* 10112

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to 37°C
- Microbiological safety cabinet of appropriate containment level
- Centrifuge
- CO₂ Incubator set at 37°C
- Microscope (uv Epi-Fluorescent.)
- 35mm plastic tissue culture dishes (Prod. No. C6296)
- Multidish 24 well (Prod. No. M9655)
- Cell scraper
- Microscope slides and 22mm cover slips
- Aluminium foil (Prod. No. Z18514-0)

Figure 15. Testing for Mycoplasma by Indirect DNA Stain**Procedure**

- For each sample and control sterilise 2 cover slips in a hot oven at 180°C for 2 hours or by immersing in 70% ethanol (Prod. No. R8382) and flaming in a blue Bunsen flame until the ethanol has evaporated. Also sterilise 2 cover slips to use as a negative control.
- Place the cover slips in 35mm culture dishes (Prod. No. C6296) (1 per dish).
- Store until needed.
- To prepare the Vero (Prod. No. 84113001-1v1) indicator cells add 2×10^4 cells in 2ml of antibiotic-free growth medium to each tissue culture dish.
- Incubate at 37°C in 5% CO₂ for 2 – 24 hrs to allow the cells to adhere to the cover slips.
- Bring attached test cell lines into suspension using a cell scraper. Suspension cell lines may be tested directly.
- Remove 1ml of culture supernatant from duplicate dishes and add 1ml of test sample to each. Inoculate 2 dishes with 100cfu *M. hyorhinis* and 2 with 100cfu *M. orale*.

8. Leave duplicate tissue culture dishes un-inoculated as negative controls.
9. Incubate dishes at 37°C in 5% CO₂ for 1-3 days.
10. After 1 day observe one dish from each pair for bacterial or fungal infection. If contaminated discard immediately. Leave the remaining dish of each pair for a further 2 days.
11. Fix cells to cover-slip by adding a minimum of 2ml of freshly prepared fixative (1:3 glacial acetic acid: absolute methanol) to the tissue culture dish and leave for 3 to 5 minutes.
12. Decant used fixative to toxic waste bottle. Add another 2ml aliquot of fixative to cover-slip and leave for a further 3 to 5 min. Decant used fixative to toxic waste.
13. Air dry cover-slip by resting it against the tissue culture dish for 30-120 min.
14. Replace cover-slip in dish and add a minimum of 2ml Hoechst stain (Prod. No. H6024). Leave for 5 minutes shielded from direct light by aluminium foil (Prod. No. Z18514-0).
15. Decant used and unused stain to toxic waste.
16. Add 1 drop of mountant to a pre-labelled microscope slide and place cover-slip (cell side down) onto slide.
17. Keep slide covered with aluminium foil (Prod. No. Z18514-0), allowing it to set for at least 15 min at 37°C or for 30 min at room temperature.
18. Observe slide under uv Epi-Fluorescence at $\times 1000$.

Criteria for a Valid Result

Negative controls show no evidence of mycoplasma infection

Positive controls show evidence of mycoplasma infection

Vero cells clearly seen as fluorescing nuclei.

Criteria for a Positive Result

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

Criteria for a Negative Result

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

Notes

1. DNA stains such as Hoechst stain (Prod. No. H6024) bind specifically to DNA. In all cultures cell nuclei will fluoresce. Uncontaminated cultures will show only fluorescent nuclei whereas mycoplasma positive cultures contain small cocci or filaments which may or may not be adsorbed onto the cells (see figure 16).
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. Positives should obviously be handled in a laboratory remote from the main tissue culture laboratory.
5. Control organisms (*M. hyorhinis*) are available from the National Collection of Type Cultures (UK).

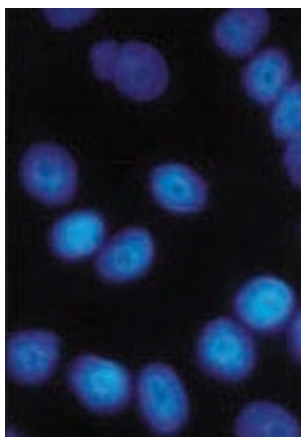
6. In some instances results may be difficult to interpret for the following reasons:
 - Bacterial/yeast/fungal contamination
 - Too much debris in the background
 - Broken nuclei as cells are all dead
 - Too few or no live cells
7. Although this procedure recommends the setting up of positive controls, this may not necessarily be feasible nor desirable in a cell culture facility with limited resources. If positive controls are to be set up they should be done so in a separate laboratory from the main tissue culture facility. If this is not possible then positive slides can be purchased from ECACC. If positive controls are not being used then it is strongly recommended that you get an independent testing laboratory to periodically test your cell lines.

Figure 16. Hoechst Positive Culture



(magnification x400)

Figure 17. Hoechst Negative Culture



(magnification x400)

Glossary of Terms

Abbreviations

A background image of a laboratory setting, showing a person in a white lab coat working at a bench with various pieces of equipment, including a microscope and a pipette.

ACDP	Advisory Committee on Dangerous Pathogens
BSA	Bovine Serum albumin
BSE	Bovine spongiform encephalopathy
BVDV	Bovine viral diarrhoea virus
FBS	Foetal bovine serum
EC	European Community
ECACC	European Collection of Cell Cultures
FDA	Food and Drug Administration
FCS	Foetal Calf Serum
HEPA	High Efficiency Particulate Air
MCB	Master Cell Bank
NAMAS	National Accreditation of Measurement and Sampling
NCTC	National Collection of Type Cultures
PPE	Personal Protective Equipment
USDA	United States Department of Agriculture
WCB	Working Cell Banks

Chemical Symbols

Ca ²⁺	calcium ion
CO ₂	carbon dioxide
DMSO	dimethyl sulphoxide
Mg ²⁺	magnesium ion
O ₂	oxygen
PBS	phosphate buffered saline
TGM	Thioglycollate medium

Notes

Who to Contact

For further details on any of these culture systems please contact ECACC.

ECACC Contact Information

ECACC

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Web www.ecacc.org.uk

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