Standard Operating Procedure **SOP036**

Title: MAINTENANCE OF A QUALITY LABORATORY ENVIRONMENT

Location: CBE Laboratories

1. PURPOSE

To provide a quality framework for the monitoring and control of a clean working environment to ensure an appropriate level of performance is maintained in the Centre for Biological Engineering (CBE) Laboratories.

2. SCOPE

The CBE comprises (1) a self-contained, Containment Level 2 Laboratory Unit, located in Holywell Park, comprising 7 laboratories with ancillary rooms such as changing rooms, store rooms and an autoclave room; (2l) CBE Tissue Engineering Laboratory (T208B), located in the Wolfson School. The CBE Laboratory Unit and T208B represent a shared multi-user facility. The primary purpose of the CBE laboratories is translational research aimed at the generation of new medical therapies, healthcare technologies and associated enabling technologies with a particular focus on manufacturing and bioprocessing. Much of the work in the CBE laboratories involves biological material. The CBE Laboratories operate under a Quality Management System to both be compliant to the necessary regulations, to ensure research quality and relevance and to protect research materials.

This SOP applies mainly to cell culture activities within the CBE Containment Level 2 Laboratories. Quality is important in all aspects of cell/tissue culture since the quality of materials used (i.e. media and other reagents) and the operating environment in which they are used will affect the quality of the cultures and products derived from them. According to Good Cell Culture Practice Guidelines, the value of studies on cell cultures is endangered by contamination and/or infection. Based on an assessment of these risks, this SOP in conjunction with these guidelines describes a strategy to minimize the potential for contamination and/or infection in order to maintain the integrity, validity, and reproducibility of any work conducted in the CBE laboratories.

The areas of quality control covered in this SOP that are of concern for cell/tissue culture are: (1) assurance of the quality of reagents and materials, (2) the provenance and integrity of the cell lines and (3) tests for frequent contaminations. GCCP sets the minimum standards for any work involving cell and tissue cultures. However, its detailed implementation depends on the nature of the work involved. Whilst details in this SOP are considered important for the preparation and maintenance of cell cultures, deviations from its specific elements may be necessary under certain conditions, in which case they should be justified.

3. RESPONSIBILITES

(i) Laboratory Staff shall:

- Ensure that the potential biological contamination status (bacterial, mycoplasma, viral, fungal, transmissible encephalopathies (TSEs)) of cell culture reagents is constantly monitored.

- Ensure that all Quality Assurance measures described in this document are applied wherever practicable.

(ii) Laboratory/Quality Manager shall:

- Ensure that compliance to the recommended Quality assurance procedures is maintained.

4. EQUIPMENT AND MATERIALS

See individual SOPs for required materials.

5. PROCEDURE

5.1. Quality Assurance for Incoming Biological Materials

All CBE laboratory personnel should refer to specific SOPs for the receipt of new or incoming cells and tissues (SOP008), and for the handling, maintenance and storage of all cells and tissues (SOP005).

5.1.1 Cell Line Acquisition: Risk Assessment

- A risk assessment form should be completed **before** acquiring the material and must be completed and approved **before** commencing the work. This risk assessment form should be completed for **all** work activities and should be filed in the laboratory along with any supporting documentation e.g. a written protocol. Although the risk assessment process may be delegated, the person managing the work area or activities remains responsible for the findings and for ensuring that the conclusions relating to any remedial action are implemented. The assessment form requires that the responsible person to confirm their acceptance of any delegated assessments.
- In analysing the risks arising from any particular activity each of the hazards involved needs to be considered separately against the precautions, which are already provided. These may or may not be satisfactory. Where the precautions are dealt with by other documents (such as local rules and procedures (e.g. Standard Operating Procedures) for working with hazardous substances, the use and maintenance of specialist items of equipment etc) then it is only necessary to refer to these documents and conclude whether the detailed contained therein is sufficient to control the hazards.
- Risk assessments required by the Genetic Modification (Contained Use) Regulations must always be recorded, must be considered and approved by a competent GM Safety Committee in advance of the work starting, be regularly reviewed and, for higher risk activities, be forwarded as part of a notification to the Health and Safety Executive. These risk assessments must also incorporate consideration of the potential impact upon the environment of any deliberate, or accidental, release of genetically modified material likely to result from the work activity - a requirement of the Environmental Protection Act that is repeatedly highlighted by HSE Inspectors. Details on how to complete this risk assessment are given in the local CBE Code of Practice.

- Blank templates of the Risk Assessment Forms are available on the CBE website or the University Health, Safety and Environment (HS&E) website. These model forms serve to keep information in a consistent manner and assist the CBE to comply with the detailed requirements for risk assessment and classification as set out in the COSHH and GM (Contained Use) Regulations.

5.1.2 Cell Line Acquisition: Notifications

- Certain types of work involving Hazard Group 2 (and above) organisms (or where doubt exists concerning the classification) or GMOs may require notification to the HSE **prior to the commencement** of the work. The University HS&E Department must be informed of plans to work with Hazard Group 2 organisms (or where doubt exists concerning the classification) or GMOs.
- The CBE Code of Practice must be consulted to confirm the notification requirements for the storage and Use of Biological Agents

5.1.3 Cell Line Acquisition: Handling and Assessment

Appropriate measures should be taken when a cell line is introduced into the CBE Laboratories to ensure that no infection/contamination of cell lines already present can occur. Tests for biological contamination (such as Mycoplasma) should be performed on a regular basis, and results should be discarded in the event of any evidence of contamination of materials. If an infection has been eradicated, the regimen should be defined and reported.

- The receiving laboratory should carry out a risk assessment before placing any reliance on historic data obtained with a cell line.
- The conformity of each cell line should be documented by certificates of analysis or other documentation that address specific safety issues, such as an evaluation of the risk of cross-contamination, contamination with micro-organisms, mycoplasma etc.

  NOTE: Check the list of cross contaminated or misidentified cell lines available from HPA website (http://www.hpacultures.org.uk/media/E50/3B/Cell_Line_Cross_Contaminations_v6_0.pdf) to exclude the use of suspect cell lines.

- If insufficient evidence is available, the receiving laboratory should always carry out its own testing procedures before accepting an incoming cell line into general use.
- For critical work, it is recommended that measures are taken to assess the status of cells before any experiment is undertaken. Characterisation is essential not only when deriving new lines, but also when a cell line is obtained from a cell bank or other laboratory. A published description of a cell line with a certain property is no guarantee that it is still the same line or has the same properties.

NOTE: A number of "culture collections" or "cell banks" have been established by either academic or commercial bodies. Cell lines from these sources are unlikely to be contaminated with microrganisms, unless so stated in the accompanying literature.

(i) Quarantine

Unless cells and tissues new to the laboratory have been authenticated they should be handled, if practicable, under a strict quarantine procedure. To prevent the spread of potential contamination, the general guidance given below should be followed, with additional controls, as necessary.

- If the cells or tissues originate from a certified source, such as a recognised cell bank, which provides certification of freedom from certain contaminants, this documentation may suffice for risk assessment, provided that the cells have not been exposed to potential sources of contamination since leaving the bank.

- It is recommended that, as a minimum and where advisable, mycoplasma testing should be carried out on all samples received.

- If quarantine is required - keep cell lines new to a laboratory separate from existing cell line stocks. If a Class II Biological Safety Cabinet (BSC) and an incubator dedicated for quarantine are not available, other steps should be taken to minimise the risk of contamination, including:

    - cells in quarantine should be handled only after all other cell culture activities have been completed that day,
    - the new cultures should be placed in a dedicated incubator or a sealed container before going into a general incubator,
    - the BSC should be cleaned after use with a suitable disinfecting agent and run for at least another 5 minutes prior to shutdown.

(ii) Cryopreservation

- Cell stocks should be stored at temperatures below -130^{o}C as viability may be progressively lost within a few months at - 80^{o}C. Cells should be frozen down according to standard procedures.

- Every time a batch of cells is frozen down, it is recommended that one vial is resuscitated immediately to check viability. Vials removed from the bank should be thawed rapidly according to standard procedures.

- For liquid nitrogen storage it is a legal requirement in the UK to store potentially infectious material in the vapour phase. This reduces the risk of transfer of contaminating organisms and eliminates the hazard of liquid nitrogen penetrating ampoules which may then explode on warming. For security, divide important material (e.g. Master Cell Banks) into more than one storage vessel.

- Access to these storage vessels should be strictly controlled.
- Controlled temperature storage units such as freezers should be monitored on a regular basis.
- The level of liquid nitrogen in the storage vessels should be checked regularly.
- The Automatic controlled-rate cooling apparatus should be used to obtain the most reproducible cryopreservation, although home made devices (e.g. expanded polystyrene boxes packed with paper towelling) placed in a -80^{o}C freezer overnight can also be used successfully.
- Dimethyl sulphoxide (DMSO) at 5-10% v/v is the most common cryoprotectant used for mammalian cells. However, it can be toxic and may cause differentiation in some cultures (e.g. HL-60). Glycerol (10-15% v/v) should be used as a suitable alternative.

(iii) Storage and Banking: Strategic Use of a Frozen Cell Repository

- To ensure a reliable and reproducible supply of cells, a Master Cell Bank of 10-20 ampoules should be established. One ampoule from the Master Cell Bank should be used to generate a Working Cell Bank (or Distribution Cell Bank). The Working Cell Bank should contain sufficient ampoules to provide at least one ampoule for every 3 months of the proposed experimental period plus sufficient ampoules for contingencies (and distribution).

  NOTE: Incorrect or serial banking (as occurs for cultures passed from one laboratory to another in a chain) results in a progressive increase in the population doubling number and additional risk of contamination or loss of key characteristics.

- Ensure that the cultures contained in the cell repository are been properly tested, labelled and stored.

  NOTE: A cryogenic cell repository is commonly used in laboratories to reduce the need to carry large numbers of cultures and to provide replacements for cultures lost to contamination or accidents. The cell repository is used strategically to convert continuously carried cultures into a series of short-term cultures, thereby greatly reducing both the amount of quality control testing required and potential problems from cryptic contaminants.

- Cultures that are carried for long periods in the CBE Laboratories should be tested regularly for contaminants (i.e. every six months is recommended for critical applications).

  NOTE: If they are not tested regularly, then when a cryptic contaminant, such as a mycoplasma or another cell line, is finally uncovered, it is impossible to determine how long it has been in the culture and how much research has been invalidated by its presence. In addition, if the contaminant is mycoplasma, it is likely to have spread by then to other cultures.

- To avoid the need for regular testing (which can require considerable effort, especially in laboratories using multiple cell lines) cultures should be discarded every three months; replacing them from the repository with cultures from the same lot or batch that have been previously tested to ensure their integrity.

- If necessary, tested stocks should be set-up in the cell repository for each culture that is routinely used in the CBE Laboratories. The cultures should be grown for at least two weeks in antibiotic-free media, and then thoroughly tested to check their viability, ensure they are free of contamination, and confirm their identity and presence of any important characteristics. Testing should be done both immediately before and after freezing or accepting some added risk, testing can be left until after freezing.

  NOTE: Assuming a consumption rate of five vials per year, each working stock will be good for four years, with the seed stock lasting for 40 to 80 years. This approach reduces the amount of routine testing to practical levels since only newly introduced cultures will require testing. Equally important, discarding cultures after growing them for three months also destroys any undiscovered biological contaminants that may have gained access to the cultures, limiting both their damage to the integrity of the research and their spread to other cultures.

- Do not transfer cell lines to another laboratory without proper validation. The whole provenance, or an abbreviated form of it, should be sent with the cells. Even well validated cell lines run the risk of losing this validation by cross contamination or microbial contamination, or by undergoing phenotypic or genotypic alteration, if passed on from one laboratory to another. If you do transfer cells to another laboratory, insist that they do not pass it on, but refer any future requests to you, the originator, or to your nominated cell bank.

- Once a newly established or newly acquired cell line has been properly validated it is advisable to submit it to a recognised cell bank. Distribution of this line can be agreed with the depositor, and the cells need not be distributed, other than to the laboratory of the originator, if so desired. Lodging cells in a cell bank, not only protects the depositor against accidental loss, but also provides authenticated stock for future reference.

(iv) Cell Characterisation and Quality Control

Characterisation of a cell line, using the correct parameters, will confirm its identity and may enable confirmation of its origin. It will also reveal any signs of instability and variation occurring in early passage finite cell lines due to selection and overgrowth, and in continuous cell lines due to genetic instability. The procedures used will depend on the type of technology accessible to the user, but certain general recommendations can be made. The following characterization methods are recommended for monitoring cell cultures - at least one of these methods should be used as part of their monitoring program:

- Karyotyping, a relatively simple method used to determine the modal chromosome number and presence of any unique marker chromosomes.

- Electrophoresis and isoenzyme analysis to generate a protein 'fingerprint' that can be used to determine species or for future comparisons.
- Immunological or biochemical techniques to detect markers that are unique to the tissue, cell line or the species from which it is derived.
- DNA fingerprinting can be used to detect both intra- and interspecies contamination. DNA fingerprinting or profiling are the recommended methods for confirming the origin of a cell line and to check for cross-contamination between cells. Ideally it will be possible to compare the DNA with that of the tissue of origin. Unfortunately this is only possible in a minority of the cell lines already available. Nevertheless, it is desirable that a DNA fingerprint or profile is defined before the cell line is used, so that at least it can be distinguished from other cell lines in the same laboratory and other common cross-contaminants and can, therefore, be tracked through subsequent transfers.

The results from these characterization tests can serve as an important baseline against which any future changes can be compared.

- Genetic Instability And Phenotypic Drift

There are several other important quality control tests that should be used to both identify and characterize the cell cultures. Besides the serious and widespread problem of cross-contamination by other cell lines described earlier, cells are also continually evolving in culture: important characteristics can be lost, mutations can occur, or chromosomes can undergo rearrangements or changes in number. Monitoring these changes is important because altered cell cultures can have a significant impact on the reproducibility of your research.

Since genetic instability and phenotypic drift can affect the utility of cell lines, it is recommended that records are kept of the length of time a cell line has been kept in culture. Record passage numbers from the time of initial establishment. The use of continuous cell lines at low passage number (less than 50 passages from the time of immortalisation) is recommended.

To minimise genotypic and phenotypic variation of a cell line within and between laboratories, it should be expanded and frozen, and used to provide the seed stock for future work. Cells should be replaced from frozen stocks after a maximum of 10 passages or 3 months continuous culture (whichever is the shorter). It is important and probably essential for comparative purposes that different laboratories using the same cell line should match their culture conditions as closely as possible.

(v) Cell misidentification and cross-contamination

- STR DNA profiling is a useful identification technique – the HPA offer this as a service.
- Where cell identification confirmation is required the CBE typically uses use karyology (e.g. hESCs) which is commonly used to back-up STR profiling. Karyology only identifies gross chromosomal changes and has a 4 week lead time (in-house capability requires specialist training or should be outsourced).

- NOTE: It is the ambition of some journals to request profiling prior to publishing material on certain cell types to prevent fraudulent publications.
- Buyer beware – suppliers should be challenged to provide not only a CoA for the cells but also the methods used to qualify the cell type, and the level of distinction achievable with the methods used for the particular cell type.
- List of misidentified cell lines is available from HPA website (http://www.hpacultures.org.uk/media/E50/3B/Cell_Line_Cross_Contaminations_v6_0.pdf) If a problematic cell type is identified the user should provide the route of provenance back to the originator of the cell line and CoA (incl identification method).

5.2 Acquisition of other Materials: In Vitro Culture Conditions

The quality control of media, supplements and additives is both time-consuming and expensive. Since most of these materials are obtained commercially, the supplier should be expected to operate according to standards appropriate to their supply and use, and to provide the relevant quality control documentation (see Coecke et al, 2005). The impact of variation of these materials should be monitored and documented.

5.2.1 Material specifications, Certificates of Analysis and Source of Materials

For all primary raw materials, processing materials, reagents and culture media, including cell cultures, the laboratory should establish or verify appropriate measures and/or systems adopted by suppliers to ensure the safety and quality of raw materials.

- Ensure wherever possible that the material suppliers operate to a relevant quality standard audited by a recognised independent qualified body.
- Ensure that the conformity of each material is documented by certificates of analysis
- In cases where there is a need to address specific safety issues, such as materials of animal origin, verification of source and preparation of the material is required
- Obtain documentation that demonstrates the application of appropriate quality assurance measures by suppliers of biological material, including origins and veterinary certificates for the animals used in the preparation of the material (e.g. bovine serum albumin).

(i) Media and Reagents

- Evaluate culture media, reagents and processing materials derived from animals for the risk of contamination with micro-organisms, particularly viruses and agents of transmissible spongiform encephalopathies e.g. products of animal origin at risk of TSEs, e.g. foetal calf serum, should originate from countries of class I Geographical BSE-Risk, e.g. Australia and New Zealand.

- Verify that all primary raw materials of animal origin originated from animals that had been subject to veterinary inspection, certification, an effective surveillance system and comprehensive sourcing controls.

- Obtain reagents, such as trypsin and collagenase, used for cell disaggregation and passage and are potential vectors for microbial contamination, from a source with full documentation of supplementary information. Perform microbiological testing of such reagents if required.

- Verify reagents that are subject to inter-batch variation in quality and/or performance and that are significant to the characteristics or performance of final product before being used.

- Specify, confirm and monitor the quality of all raw materials and equipment used in the laboratory. Special attention should be paid to all raw materials and equipment to which cells are or may be exposed. The quality assurance measures adopted need to take account of the inherent variability of biological systems while ensuring that characteristics which are critical to quality are reliably identified and that, in respect of these, appropriate quality control measures are clearly specified and implemented.

(ii) Culture reagents

- Whenever possible purchase reagents and sera from suppliers who issue certificates of analysis or results of quality control (QC) testing with each batch of products.

- Whenever possible, purchased reagents in bulk to avoid variation between batches, depending on shelf life.

- Store serum should at -20^{o}C, but not in frost-free freezers as temperature cycling may crack the bottles. The shelf life of serum is 12-18 months and longer term storage is not recommended as any advantages gained by a single batch may be offset by deterioration. The shelf life of single strength medium is approximately 9-12 months, concentrated medium (10X) approximately 12-24 months and powdered medium approximately 24-36 months.

- In the case of critical reagents, the user should define a specification to include general details of the reagent, such as quality controls for identity (composition), purity and activity and stability.

- Regularly monitor all other working materials which come into direct contact with cell and tissue cultures, this includes procedures for ensuring; the quality of culture vessels and surface coatings; the cleanliness and sterility of any re-used equipment (for example, glassware); and lack of toxicity (for example, plastic, absence of detergents, and rubber components).

(iii) Media Production

The production of media from individual ingredients is complex and time consuming. Most commercial suppliers offer a custom media service for specialised formulations. Most basic media formulations are offered both as single strength and as 10x concentrated liquids by suppliers. Considerable cost savings can be achieved by using the 10x concentrates. If concentrate is used, this is diluted into bottles containing sterile high quality water. Sterile L-glutamine and sodium bicarbonate are then added and finally the pH is adjusted. The advantage of this system is that it is quick and technically undemanding.

However, several points should be borne in mind.

- Media concentrates have changes made to their basic formulations, mainly to overcome problems of solubility.
- Precipitate is often seen on storage. If the concentrate is aliquotted the precipitate can cause variation between bottles.
- Suppliers acidify the medium to improve solubility. This in turn requires significant amounts of base to neutralise the medium.
- Powdered media produce more stable uniform products with longer shelf lives than concentrates but the process does require specialised equipment for filtration and bottling.

  The following general points should be noted:
    - The powder should be free flowing and white to off-white in colour, with no sign of dampness.
    - The medium should be stirred until all the powder is dissolved. The presence of fine particulate matter may require pre-filtration or a change of supplier.
    - Medium should always be prepared and filtered on the same day.

- Sterilisation requires filtration to a pore size of 0.22um. cellulose filters are most common but PVDF filters should be used when protein is present in the medium. Although a 0.22um filter will prevent the passage of bacteria and fungi and fungi, mycoplasma can pass through the pores of greater than 0.1 um diameter. The following basic points should be noted:

- The equipment should be dedicated for media production only.

- A Class II BSC should be dedicated to media and supplement production. If this is not possible then the cabinet should not have been used for cell culture for at least one hour. It must also be cleared of all equipment and thoroughly cleaned with 70% alcohol or non-corrosive disinfectant.

- All tubing should be clean and autoclaved before use and connections should be securely in place.

- Sterile bottles and caps should be stacked outside the cabinet and introduced one at a time to receive medium. Stacking bottles within the flow cabinet will seriously compromise the airflow, and consequently sterility.

- During bottling, representative samples should be drawn off at regular intervals. These samples should then be incubated at 37°C for at least 10 days to check for contamination. If any sample shows contamination, the whole batch of medium should be discarded.

- Most bottled media should be stored at 4°C in the dark.

(iv) Serum Batch Testing

Simple preliminary tests can help avoid the disastrous consequences of using media, sera or supplements which do not adequately support cell growth.

Large batches of serum should be purchased when possible and retested before use.

(v) Equipment

Standard procedures must be in place to ensure that equipment and instruments have been properly maintained and calibrated; for example, control of fridge and freezer temperature, CO2 levels of incubators, laminar air flow and safety cabinets and other sterile work areas; automatic pipettes and pipettors; autoclaves and other analytical or production equipment.

It should also be born in mind that other environmental conditions critical to the quality of stem cell lines, such as incubation and cold storage, will need to be controlled through careful monitoring of equipment and appropriate staff training.

(vi) Record Keeping

- In cell and tissue culture, as in any practical science, clear documentation of the systems used and procedures followed is mandatory, in order to permit the traceability, interpretation and repetition of the work. Therefore, accurate records of cell type, origin, authentication and characterisation, and of the materials used and the culture techniques performed, are essential.

- Record details of all routine and experimental procedures as they are generated. Follow SOP002 since good practice requires that records must be dated, legible, clear in content and made in ink directly into a bound laboratory notebook or onto a standard form. Record enough detail to enable the work to be reproduced exactly. Reference Standard Operating Procedures (see below) wherever possible.

- Attach graphs, figures and photographs to the notebook and ensure that they are signed, dated and identified in such a way that, should they become detached, they could be re-assigned to their correct place. If large numbers of print-outs or other documents are generated, these should be annotated and stored in a dedicated file.

- A certificate of analysis should be requested from the supplier for each batch of material, and this should be stored securely for future reference with the date received.

- The originals of all experimental records remain the property of the funding agency or CBE, and must be lodged with them when an experimenter leaves that CBE or changes funding agencies. Such records should be securely archived, with systems in place to permit easy retrieval along with protection against tampering.

5.3 Management and Control of Cell Culture Contamination

Culture contaminants may be biological or chemical, seen or unseen, destructive or seemingly benign, but in all cases they adversely affect both the use of your cell cultures and the quality of your research. Contamination problems can be divided into three grades:

- **Low grade:** when up to several flasks are occasionally lost to contamination;
- **Medium grade:** when contamination frequency increases or entire experiments or cell cultures are lost;
- **High grade:** contaminants are discovered that call into doubt the validity of your past or current work.

The seriousness of any culture contaminant is usually directly proportional to the difficulty of detecting it; those that go undetected the longest have the most serious consequences. Cultures containing non-lethal (but not harmless) biological contaminants are sometimes used in research for months or even years before being uncovered; during this time the quality and validity of all research done with those cultures is compromised, as is the reputation of the researchers using them.

The most obvious consequence of cell culture contamination is the loss of your time, money and effort spent developing cultures and setting up experiments. However, the less obvious consequences are often more serious. First there are the adverse effects on cultures suffering from undetected biological contaminants. These hidden (cryptic) contaminants can achieve high densities altering the growth and characteristics of the cultures. Worse yet are the potentially inaccurate or erroneous results obtained by unknowingly working with these cryptically contaminated cultures.

NOTE: It is impractical, if not impossible, to prevent all cell culture contamination but it must be managed to reduce both the frequency of its occurrence and the seriousness of its consequences. Cell cultures should be managed to reduce and control both the frequency and seriousness of culture-related problems, especially contamination.

A cell culture contaminant can be defined as some element in the culture system that is undesirable because of its possible adverse effects on either the system or its use. These elements can be divided into two main categories: chemical contaminants (refer to Annex I) and biological contaminants.

5.3.1 Biological Contamination Monitoring Programme

To reduce the frequency of biological contamination and effectively prevent and contend with cell line problems, it is important to understand not only the nature and identity of the contaminants but also

where they come from and how they gain entry into cultures. Biological contaminants can be subdivided into two groups based on the difficulty of detecting them in cultures:

- those that are easy to detect — bacteria, moulds and yeast;
- those that are difficult to detect, and as a result potentially more serious culture problems, - viruses, protozoa, mycoplasmas and other cell lines.

Ultimately, it is the length of time that a culture contaminant escapes detection that will determine the extent of damage it creates in a laboratory or research project.

The following section describes the routine procedures that should be carried out in the CBE Laboratories to monitor incoming raw materials and subsequent biological contamination events. Recommended steps for preventing or reducing biological contamination are described in Annex II of this SOP

As described in Section 5.1 and 5.2 the conformity of each cell line and associated cell culture reagents where applicable, should be documented by certificates of analysis or other documentation that address specific safety issues related to the risk of cross-contamination (see Annex II), contamination with micro-organisms, mycoplasma etc. However, if insufficient evidence is available, the receiving laboratory should carry out the following:

5.3.1.1 Mycoplasma Contamination

Mycoplasmas represent the most serious, widespread, and devastating culture contaminants. Mycoplasmas have the ability to alter their host culture's cell function, growth, metabolism, morphology, attachment, membranes, cause chromosomal aberrations and damage, and cytopathic effects including plaque formation.

NOTE: The presence of mycoplasma may invalidate the results obtained with that culture. The presence of mycoplasma-infected cultures can result in the shut-down of the entire laboratory until the infection can be eliminated, whereupon complete restocking is required.

NOTE: Regulatory bodies now insist that cell cultures used for the production of reagents for diagnostic kits or therapeutic agents are free from mycoplasma infection.

NOTE: Some scientific journals have the policy of requiring statements from authors that the culture work reported in those journals is carried out with mycoplasma-free cells.

See Annex II for guidance on prevention of mycoplasma contamination.

(i) Mycoplasma testing prior to use

If no mycoplasma testing has been performed by the supplier or provider (i.e. non-commercial supplier), incoming cells and applicable cell culture reagents, particularly media, sera* and other animal-derived products (historically a common source of mycoplasma contamination – see

Annex II for other sources) should be quarantined and tested (as below) before commencing the work activity.

NOTE: It is recommended that this forms part of the QC of every new cell line as it enters the laboratory. In addition, all Master and Working Cell Banks should be tested at the time of freezing.

NOTE: It is recommended that cultures brought into the laboratory are quarantined until they have tested free from mycoplasma contamination because high levels of contamination are common and because of the proven ease with which mycoplasmas can be spread from contaminated cultures. As a minimum safeguard against microbial contamination, screening for mycoplasma is essential before the cell line leaves quarantine.

***NOTE:** Sera and other biologicals should only be purchased from sources that have a good reputation and that use currently acceptable filtration (0.1 µm or smaller) and testing procedures.

(ii) Regular Mycoplasma testing

Routine mycoplasma testing should be carried out every six months for growing cell cultures in the CBE Laboratories (or any time they behave suspiciously). This may be contracted out (i.e. by supplying actively growing cells and conditioned media) to an appropriate testing company.

- If any contamination is detected, all cell culture work should cease. All cells and reagents contaminated or suspected to be contaminated must be disposed of.
- Equipment and work surfaces should be decontaminated and if necessary tested for exposure to mycoplasma.
- All cell culture results of contaminated cultures within the previous testing window (i.e. 6 months) should be carefully examined to identify potentially abnormal results due to contamination.
- In addition, mycoplasma testing should continue on a 3-monthly basis until all cells and reagents have been determined as mycoplasma-free.

5.3.1.2 Contamination by Bacteria, Moulds, and Yeasts

Bacteria, Moulds, and Yeasts are found virtually everywhere and are able to quickly colonize and flourish in the rich and relatively undefended environment provided by cell cultures. Because of their size and fast growth rates, these microbes are the most commonly encountered cell culture contaminants. The common contaminants, fungi, yeasts, and bacteria and will usually become apparent by naked eye or microscopic examination, (increase in turbidity of the medium and/or a decrease in pH (yellow in media containing phenol red as a pH indicator)), if the cells are cultured in the absence of antibiotics.

- It is recommended that cells are inspected daily, and must always be examined under an inverted phase microscope before use in an experiment.

  NOTE: For most purposes, it is sufficient to grow the cells antibiotic-free to demonstrate that they are free from microbial contamination. Incubate 10 mL of test cell culture reagents, e.g. basal media with serum, at 37°C/CO_2 for 24 hours prior to use followed by a visual inspection for contamination.

  NOTE: Cell culture media, especially unopened bottles of media that are outdated or no longer used in the laboratory (as long as they do not contain any antibiotics) can provide a very rich, readily available and useful substitute for standard microbiological media. A small amount of serum (3 to 5% — again outdated or unwanted sera can be used) should be added to promote growth. The medium can be dispensed in 10 mL amounts into sterile 16 mm by 125 mm glass or plastic screw cap culture tubes or clear 15 mL plastic centrifuge tubes and be stored at 4°C until needed. The sterility of either filtered solutions or cultures and products suspected of being contaminated can be routinely and easily checked by placing a small sample into each of two tubes and incubating one at 30°C and the other at 37°C for at least two weeks.

- If a microbial contamination is suspected but not evident on visual examination, or more stringent quality control is required, it may be necessary to culture a sample of the cells and/or medium in nutrient broth or on nutrient agar. Please see ANNEX III for methodology on agar culture.

  NOTE: The direct culture tests and the indirect DNA fluorochrome test for mycoplasma, although not designed for this purpose, will also detect most bacteria, yeasts and fungi, including intracellular forms, reducing the need for the traditional tests.

- If a cell culture is contaminated with bacteria or fungi, then the best method of elimination is to discard the culture and obtain fresh stock cultures or new supplies. In the case of irreplaceable stocks, it will be necessary to use antibiotics. The more antibiotics that are tested, the more chance there is of finding one that eliminates the infection. However, if the cells have been routinely grown in media supplemented with antibiotics (which is not recommended), it is almost certain that the contamination will be with organisms that are already resistant to this and some other antibiotics.

- To eliminate infection, the cells should be cultured in the presence of the antibiotic for at least three passages. If the contamination appears to be eradicated, then the cells should be cultured in antibiotic free medium for one month before re-testing.

5.3.1.3 Contamination by Viruses

Due to their extremely small size, are the most difficult cell culture contaminants to detect in culture, requiring methods that are impractical for most research laboratories? Their small size also makes

them very difficult to remove from media, sera, and other solutions of biological origin (refer to Annex II).

5.3.1.4 Contamination by Protozoa

Both parasitic and free-living, single-celled protozoa, such as amoebas, have occasionally been identified as cell culture contaminants. Usually of soil origin, amoebas can form spores and are readily isolated from the air, occasionally from tissues, as well as throat and nose swabs of laboratory personnel. They can cause cytopathic effects resembling viral damage and completely destroy a culture within ten days. Because of their slow growth and morphological similarities to cultured cells, amoebas are somewhat difficult to detect in culture, unless already suspected as contaminants. Fortunately, reported cases of this class of contaminants are rare, but it is important to be alert to the possibility of their occurrence.

5.3.1.5 Contamination by Invertebrates

Insects and arachnids commonly found in laboratory areas, especially flies, ants, cockroaches and mites, can both be culture contaminants as well as important sources of microbial contamination. While not nearly as common as other culture contaminants, it is important to be alert to the presence of these invertebrates in culture areas.

5.3.1.6 Contamination by Transmissible Spongiform Encephalopathy (TSE)

TSE (including what is known as bovine spongiform encephalopathy, BSE or mad cow disease) is unlikely to be present in cancer cells or tissue culture products. However, a risk of exposure must be regarded as potentially present, particularly since all the routes of transmission have not been identified. Some regulatory authorities now demand that serum used in the production of pharmaceutical, veterinary, and sometimes diagnostic products can only be obtained from specified countries of origin where BSE has not been diagnosed. Some countries, including the USA, will only allow import of cells that have been cultured in media containing serum from BSE-free areas.

5.4. Remediation for Contaminated Cultures:

(i) 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 recognized source. Autoclaving is the preferred method for dealing with contaminated cultures — it always works and is guaranteed to keep the infection from spreading to other cultures.

(ii) However, occasionally contamination will be found in a valuable culture that cannot be replaced and attempts will be made to save it. This is a task that should not be undertaken lightly as it usually entails considerable effort and frequently turns out to be unsuccessful. In addition, cultures can lose important characteristics as a result of the clean up procedure.

- If the contaminant is a fungus or yeast, success is unlikely since antifungal agents, such as amphotericin B (Fungizone) and Nystatin, will not kill these organisms, but only prevent their growth.
- Many bacterial culture contaminants come from human or animal sources and are likely to have developed resistance to most commonly used cell culture antibiotics.
- Most clean up attempts, however, are usually made against mycoplasma infected cultures (see below).

(iii) If a culture is inadvertently contaminated with mycoplasma, two solution options are available.

1. Simply to autoclave the culture, dispose of it, and start with a new culture.

2. If necessary, and only if the contamination is not extensive, then it is often possible to rescue the cells by treatment with one of the commercially available antibiotics or other chemicals that are toxic to mycoplasma but safe for cells. This must only be considered for a remedial action, not as a routine supplement to growth media (and thereby a substitute for good cell culture practice)

    - Proper antibiotic treatment should kill mycoplasma rather than inhibit growth. Unfortunately, complications can arise due to antibiotic resistance, cellular toxicity, or cytotoxic side effects to the cell culture itself.
    - Most typical cell culture antibiotics are not effective against mycoplasma contamination but other types have shown success in eliminating mycoplasma from cell cultures. Ciprofloxacin, BM-Cyclin and quinolone derivatives provide reasonable mycoplasma elimination rates but none are proven to be 100% effective.
    - Non-antibiotic treatments can target mycoplasma by damaging the plasma membrane. However, they may also have cytotoxic effects to the cell culture, and to be effective must come in direct contact with the mycoplasma organism; reduced effects may be seen in adherent and clustered cells.

NOTE: None of these methods are 100% successful and clean up should only be tried as a last resort. Often these treatments reduce the level of contamination below that which can be detected by indirect methods such as DNA staining or PCR. As a result, clean up attempts often appear successful for the first month or more following treatment because the low level of surviving mycoplasmas can escape detection, but eventually the few remaining undetected mycoplasmas recover leading to more serious problems.

(iv) Culture collections such as ECACC will attempt to eradicate any contaminants if required. Contact ECACC for further details.

(v) 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.

5.5. Environmental Monitoring

At the time of writing a formal program of environmental monitoring is not required in the CBE Laboratories. However, there may be a requirement following a contamination event. Testing inside the Class II BSC for example should involve:

- Incubation of settle plates (both tryptone soya agar and sabouraud dextrose agar) in the Class II BSC for 4 hours during a routine working day. These plates should then be transported to a recognised testing service for analysis, e.g. Swann-Morton http://www.swann-morton.com/settle_plates.php

  NOTE: Sterility test media is very useful for evaluating the amount or source of particulate contamination in an area, near a piece of equipment or by a technique. BSCs, and especially incubators, are frequently blamed by laboratory personnel as the source of their contamination problems. Until these areas are screened and eliminated as the source of the problem, the real problem, often simply aseptic technique, can not be dealt with effectively. These suspected problem areas can be screened by dispensing the test medium into 96 well culture plates or 100 mm culture dishes (use agar-gelled media for the dishes). The vessels are then opened (with unopened vessels as controls) for 30 to 60 minutes at several locations within the test site prior to being sealed and incubated. Cultures can be initially checked for contamination after two to three days although slow growing contaminants may take two weeks or longer to appear. The rate of contamination (number of colonies or contaminated wells/vessel or unit area/unit time) can then be calculated and analyzed. Besides giving an accurate level of the bioburden in that area, microscopic observation of the contaminants in the liquid test media also allows their morphological comparison with the microorganisms found causing problems in the cell cultures. Past experience with this approach has shown it is a very useful tool when teaching aseptic technique as it clearly demonstrates that the air in a room or even inside a humidified incubator is usually not a major source of contamination in a well maintained laboratory. It is also a useful tool in tracking down mysterious contamination outbreaks.

6. DOCUMENTATION

Standard documentation as used for SOP016, SOP017, SOP028 and SOP035 should be used for record keeping. Biological testing records from the appropriate facilities should also be kept in one folder with the Laboratory Manager to ensure traceability. In addition, all product information sheets received following product orders should be stored by the Laboratory Manager.

ANNEX I: STEPS FOR PREVENTING OR REDUCING CHEMICAL CONTAMINATION

Chemical contamination is best described as the presence of any nonliving substance that results in undesirable effects on the culture system. To define it further is difficult; even essential nutrients become toxic at high enough concentrations. Toxicity is not the only concern since hormones and other growth factors found in serum can cause changes that, while not necessarily harmful to cultures, may be unwanted by researchers using the system.

1. Types and Sources of Potential Chemical Contaminants

- Metal ions, endotoxins, and other impurities in media, sera, and water
- Plasticizers in plastic tubing and storage bottles
- Free radicals generated in media by the photoactivation of tryptophan, riboflavin or HEPES exposed to fluorescent light
- Deposits on glassware, pipettes, instruments etc., left by disinfectants or detergents, anti-scaling compounds in autoclave water, residues from aluminium foil or paper
- Residues from germicides or pesticides used to disinfect incubators, equipment, and labs
- Impurities in gases used in CO2 incubators

NOTE: Serum proteins have the ability to bind substantial quantities of chemical contaminants, especially heavy metals that may have entered the culture system from other sources, rendering them less toxic. Switching from serum-containing medium to a serum-free system can unmask these toxic chemical contaminants, exposing the cells to their adverse effects.

2. Steps for Reducing Chemical Contamination Problems:

The majority of chemical contaminants are found in cell culture media and come either from the reagents and water used to make them, or the additives, such as sera, used to supplement them. Mistakes in media preparation protocols, reading reagent bottle labels, or weighing reagents are other common sources of chemical contamination:

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- Reagents should always of the highest quality and purity.
- Reagents should be properly stored in order to prevent deterioration.
- Reagents should be either certified for cell culture use by their manufacturer OR evaluated by the researcher before use.

(i) Media and Sera

Sera used in media are a source of both biological and chemical contaminants. Due to cell culture-based screening programs currently used by good sera manufacturers, it is unusual to find a lot of foetal bovine sera for example that is toxic to a majority of cell cultures. However, it is common to find substantial variations in the growth promoting abilities of different lots of sera for particular cell culture systems, especially for cultures that have specialized or differentiated characteristics, e.g. uncontrollable lot-to-lot variation in hormone and growth factor concentrations.

Sera should be carefully tested before purchase, OR serum free media should be used where possible.

(ii) Water

The water used for making media and washing glassware is a frequent source of chemical contamination and requires special care to ensure its quality.

- Double or triple glass distillation systems should be used to ensure high quality water suitable for cell culture media and solutions, OR
- Purification systems that combine reverse osmosis, ion exchange and ultrafiltration can be used.
- Water systems must be properly maintained and serviced to ensure continued water quality.

- Highly purified water can leach potentially toxic metal ions from glassware or metal pipes, and plasticisers from plastic storage vessels or tubing. These contaminants can then end up in media or deposited on storage vessels and pipettes during washing and rinsing.

- Water used to generate steam in autoclaves may contain additives to reduce scale build-up in pipes; these potentially toxic additives can also end up on glassware.

- Media stored in glass or plastic bottles that have previously contained solutions of heavy metals or organic compounds, such as electron microscopy stains, solvents and pesticides, can be a source of contamination. The contaminants can be adsorbed onto the surface of the bottle or its cap (or absorbed into the bottle if plastic) during storage of the original solution. If during the washing process they are only partially removed, then once in contact with culture media they may slowly leach back into solution.

- Residues from chemicals used to disinfect glassware, detergents used in washing, or some aluminium foils and wrapping papers for autoclaving or dry heat sterilization can also leave potentially toxic deposits on pipettes, storage bottles and instruments.

- Exposure of media containing HEPES (N-[2-hydroxylethyl] piperazine-N'-[2-ethanesulfonic acid]) — an organic buffer commonly used to supplement bicarbonate-based buffers), riboflavin or tryptophan to normal fluorescent lighting can cause chemical contamination. These media components can be photoactivated producing hydrogen peroxide and free radicals that are toxic to cells; the longer the exposure the greater the toxicity.

- Short term exposure of media to room or BSC lighting when feeding cultures is usually not a significant problem; but leaving media on lab benches for extended periods, storing media in walk-in cold rooms with the lights on, or using refrigerators with glass doors where fluorescent light exposure is more extensive, will lead to a gradual deterioration in the quality of the media.

- The incubator can also be a source of chemical contamination. The gas mixtures (usually containing carbon dioxide to help regulate media pH) perfused through some incubators may contain toxic impurities, especially oils or other gases such as carbon monoxide that may have been previously used in the same storage cylinder or tank. This problem is very rare in medical grade gases, but more common in the less expensive industrial grade gas mixtures.

    - Care must be taken when installing new cylinders to make sure the correct gas cylinder is used.
    - Other potential chemical contaminants are the toxic, volatile residues left behind after cleaning and disinfecting incubators. Disinfectant odours should not be detectable in a freshly cleaned incubator when it is placed back into use.

NOTE: Chemical contaminants tend to be additive in cell culture; small amounts contributed from several different sources that are individually non-toxic, when combined together in medium, may end up overloading the detoxification capabilities of the cell culture resulting in toxicity-induced stress effects or even culture loss.

(iii) Endotoxins

Endotoxins, the lipopolysaccaride containing by-products of gram negative bacteria, are another source of chemical contaminants in cell culture systems. Endotoxins are commonly found in water, sera and some culture additives (especially those manufactured using microbial fermentation) and can be readily quantified using the Limulus Amebocyte Lysate assay (LAL).

These highly biologically reactive molecules may affect the growth or performance of cultures and are a significant source of experimental variability (Reviewed in references 6 and 39). Cell culture produced therapeutics is compromised by high endotoxin levels, efforts must be made to keep endotoxin levels in culture systems as low as possible.

- Sera are a major source of endotoxins in cell cultures but most manufacturers have significantly reduced levels in sera by handling the raw products under aseptic conditions.
- Poorly maintained water systems, especially systems using ion exchange resins, can harbour significant levels of endotoxin-producing bacteria and may need to be tested if endotoxin problems are suspected or discovered in the cultures.

3. Detecting Chemical Contaminants

Determining that a chemical contaminant is the cause of a cell culture problem is usually much more difficult than with biological contaminants because it is so hard to detect. Often the first signs that something is wrong are widespread alterations in the growth, behaviour or morphology of the cultures in the laboratory; however, it may take weeks before these changes are noticed. Once noticed, the cause is frequently misconstrued to be of biological origin; only after extensive and unsuccessful testing for the usual microbial suspects does attention focus on the possibility it might be a chemical contaminant.

Begin the problem solving process by:

- Identifying all changes that have occurred in the lab in the weeks prior to the problem being noticed, especially in equipment, solutions, media and supplies that may be related to the problem. Good record keeping is essential for this process to be successful.
- Bring together laboratory personnel to brainstorm for all of the possible causes and then select the best possibilities for evaluation.
- Simple comparison experiments can be done to eliminate each possibility as the source of the problem; media, solutions, sera and other products to use as controls in the testing can be obtained from other labs or sources.
- The best way to avoid chemical contamination is to test all new lots of reagents, media and especially sera, and test the water purity at least yearly using the most sensitive culture assay available.

ANNEX II: STEPS FOR PREVENTING OR REDUCING BIOLOGICAL CONTAMINATION

1. Steps for Reducing Cross-Contamination by Other Cell Cultures

Whenever a rapidly-growing, continuous cell line is maintained in a laboratory there is a risk that it may cross-contaminate other, more slowly-growing lines. This may have limited importance if the property under examination is cell line specific and intrinsically important, but if comparisons are to be made between cell lines or if extrapolations are made to the tissue of origin or a particular class of cell lines, then interpretation of the data can only be made if the target cell lines are correctly identified.

The seriousness of cross-contamination, while not as common as microbial contamination, cannot be overstated. The validity of experimental results from cultures having inter- or intraspecies contamination is, at the very least, questionable. Furthermore, their use can lead to the embarrassment of having to retract published results.

(i) Identification of Cell Line Cross-Contamination

Whenever the invading cell is better adapted to the culture conditions and thus faster growing than the original cells, it will almost always completely replace them. Because of the outward physical similarities of different cell lines and the wide morphological variations that can be caused by the culture environment, it is impossible to rely only on microscopic observation to screen for cross-contamination of cultures.

Characterisation of a cell line, using the correct parameters, will confirm its identity and may enable confirmation of its origin. It will also reveal any signs of instability and variation occurring in early passage finite cell lines due to selection and overgrowth, and in continuous cell lines due to genetic instability. The procedures used will depend on the type of technology accessible to the user, but certain general recommendations can be made.

- DNA fingerprinting or profiling are the recommended methods for confirming the origin of a cell line and to check for cross-contamination between cells. Ideally it will be possible to compare the DNA with that of the tissue of origin. Unfortunately this is only possible in a minority of the cell lines already available. Nevertheless, it is desirable that a DNA fingerprint or profile is defined before the cell line is used, so that at least it can be distinguished from other cell lines in the same laboratory and other common cross-contaminants and can, therefore, be tracked through subsequent transfers.

- Where possible, new cell stocks should be characterised for features which will enable monitoring of genotype and phenotype variability. These include the karyotype (by G-banding or fluorescence in situ hybridisation) and production of cell or tissue specific markers (e.g. cell surface markers etc).

(ii) Prevention of Cell Line Cross-Contamination

- Simple accidents are one of the most common means by which other cell lines gain entry into cultures. A problem arises when a small number of cells from a rapidly growing cell line are inadvertently transferred into a culture of more slowly growing cells. Such transfer can occur by a variety of routes such as the accidental touching of a pipette on the neck of a bottle of medium or some other common reagent, or by the presence of an aerosol in the BSC at a time when flasks are uncapped.

> EXAMPLE: *A single cell from a line with a population doubling time of 12 hours is transferred into a culture of 105 cells of a cell line with a population doubling time of 48 hours. By the time the slow-growing cell line has expanded from 105 to 3.2 x 106 cells (i.e. 5 doublings or 10 days), the rapidly growing cells will have doubled 20 times (i.e. from a single cell to 106 cells), and hence comprise nearly 25% of the total cell population. After one further passage, the rapidly growing cells will predominate.*

- Whenever possible, cells should be obtained from certified sources, and appropriate procedures should be applied to minimise the risk of cross-contamination during their storage and use in the laboratory.

- Derivation of new cell lines should allow for future authentication by storing samples of tissue or DNA from the source individual for subsequent DNA fingerprinting. Cell lines new to a laboratory should be developed into frozen stocks and typed. Change in cell behaviour or morphology may indicate a cross-contamination and constant vigilance and attention to good tissue culture practice are essential.

- Simple precautions must be taken to minimise the possibility of cross-contamination, including:

    - Only one cell line should be used in a BSC at any one time. After removal of the cells from the BSC, it should be swabbed down with a suitable liquid disinfectant and the cabinet run for 5 minutes before the introduction of another cell line.
    - Bottles or aliquots of medium should be dedicated for use with only one cell line.
    - The formation of aerosols must be kept to a minimum.
    - Regularly return to frozen stocks (except where essential, never grow a cell line for more than 3 months or 10 passages, whichever is the shorter period).
    - All culture vessels must be carefully and correctly labelled (including full name of cell line, passage number and date of transfer), as must storage containers.

2. Mycoplasma Contamination

The small size (0.15–0.3 μm) and lack of a cell wall allow mycoplasmas to grow to very high densities in cell culture, often without any visible signs of contamination — no turbidity, pH changes or even cytopathic effects. Even careful microscopic observation of live cell cultures cannot detect their

presence. These same two characteristics also make mycoplasmas, like viruses, very difficult to completely remove from sera by membrane filtration.

Aggressive management against mycoplasma contamination must be the central focus for any cell culture laboratory contamination or quality control program.

(i) Sources of Mycoplasma Contamination

- The **primary source** of mycoplasma contamination is cross-contamination of healthy cells with other infected cell lines:
    - Cross-contamination can occur when untested, infected cells or media materials come into contact with a clean cell culture. Previously infected cells can come from other research laboratories as shared research, donations or gifts, or from commercial suppliers.
    - Between 15 and 50% of cell lines submitted to cell banks are contaminated with mycoplasma.
- The second common source is poor aseptic technique:
    - Cross-contamination can also occur in conjunction with poor aseptic technique via aerosolization during routine handling of cells. Even in the protective environment of a BSC, airborne particles and aerosols can result from pipetting, dust in protective garments, dry, flaking skin, or even talking or sneezing while working around cell cultures.
    - Improperly sterilized or stored consumable supplies, media, or other materials may become contaminated before or during use.
    - Materials and equipment stored in BSCs can disrupt air flow patterns, and increase surface area for potential mycoplasma contamination. Mycoplasma contamination can often be recovered from equipment and media bottles used in a hood and even the internal surface of the hood several weeks after exposure to the contaminants.
    - Incubators with integrated fans and air currents created during opening and closing of the internal incubator door may spread mycoplasma-containing particles.
    - Water baths, waste containers, and even cooling coils on refrigerators and freezers are major and often overlooked sources of mycoplasma contamination.
- The last important source of mycoplasma is sera and other biologicals that are sterilized by filtration.
    - While sera and other animal-derived products, historically a common source of mycoplasma contamination, have improved dramatically in quality, they cannot be automatically eliminated as a source of contamination, especially if they are from an unknown or questionable manufacturing source.

NOTE: Laboratories which do not test for mycoplasma probably harbour contaminated cell lines and may even have their entire stocks contaminated, as mycoplasma spreads readily among cell lines via reagents and media, the operator and the work surface.

The presence of mycoplasma may invalidate the results obtained with that culture. The presence of mycoplasma-infected cultures can result in the shut-down of the entire laboratory until the infection can be eliminated, whereupon complete restocking is required.

NOTE: Regulatory bodies now insist that cell cultures used for the production of reagents for diagnostic kits or therapeutic agents are free from mycoplasma infection. Also, some scientific journals have the policy of requiring statements from authors that the culture work reported in those journals is carried out with mycoplasma-free cells.

(ii) Identification of Mycoplasma Contamination

There are two basic testing methods for mycoplasma: direct culture in media, or indirect tests that measure specific characteristics of mycoplasma. Direct culture is the most effective and sensitive method for detecting mycoplasma, but it is also the most difficult and time consuming. It is best contracted to an outside testing facility.

- Direct culture testing is the most sensitive method of cultivatable mycoplasma detection but also the most cumbersome and time-consuming; results can take up to four weeks. As it requires live mycoplasma controls, it is recommended that the laboratory reduce risk by using an outside testing facility.

- There are a wide variety of indirect test methods available for mycoplasma detection, including PCR-based kits, DNA fluorochrome staining, autoradiography, ELISA, immunofluorescence and specific biochemical assays. These tests are faster than direct culture and they can detect the fastidious, difficult to cultivate strains that are occasionally missed by direct culture. However they lack the sensitivity of direct culture, requiring much higher levels of contamination for detection. As a result, they have more frequent false negatives than direct culture methods, potentially leaving researchers who rely solely on a single indirect test with a false sense of security.

It is recommended to use a combination of both the direct and indirect methods to ensure accurate results. Direct culture can provide very high sensitivity while DNA fluorochrome staining for example can detect any fastidious mycoplasma that the direct culture misses.

NOTE: Both the FDA and USDA requires this approach for cell culture derived products. If resources do not permit the combined approach, then the DNA fluorochrome staining procedure using an indicator cell line, combined with one other indirect test method should provide a minimum level of security.

(iii) Prevention of Mycoplasma Contamination

Proper aseptic cell culture technique combined with the strategic use of a tested cell repository and limited use of antibiotics will greatly reduce the opportunities for contamination via this route. This reduces the risk not only of mycoplasma infection, but bacterial, viral, chemical, and other contaminants as well. The following guidelines can help to reduce the risk of contamination.

(a) Reduce aerosol generation and airflow patterns:

- Wear dedicated personal protective equipment to shield cells from aerosol and debris contamination caused by street clothes, skin, hair, and even breathing.
- Clear work surfaces and BSC hoods of all clutter and storage boxes, and thoroughly clean with a suitable disinfectant between uses, and only use these surfaces and hoods for one cell line at a time.
- BSC hoods should continually run unless they won't be used for extended periods of time. To reduce turbulent airflow in the laboratory, the number of people in the laboratory should be as few as possible.

(b) Increase attention to detail:

- Maintain and separately store media and reagents for each cell line and clearly label to eliminate any doubt or confusion.
- Clearly label the cell lines as well and test prior to and after freezing in a cryogenic cell repository to ensure that they are free from mycoplasma contamination. It is important that frozen stocks are created immediately after testing.
- If cells are cultured for more than 3 months after testing, they should be re-tested.
- Additionally, rotate the frozen stock periodically to reduce dependence on the active cell culture.
- Clean, service, and calibrate incubators, water baths, environmental monitoring tools, and other equipment at regular intervals.
- Similar attention to care and cleaning should apply to the surrounding environment, including areas behind and underneath equipment, storage shelves and cabinets, and even low-traffic sections of the laboratory.

(c) Perform routine testing:

- Cell cultures should be tested for mycoplasma contamination on a regular basis, depending on the needs of the laboratory.
- Media and reagents should also be subject to rigorous testing prior to use.
- It is essential to quarantine all cultures brought into the laboratory until they have tested free from mycoplasma contamination because high levels of contamination are common and because of the proven ease with which mycoplasmas can be spread from contaminated

cultures. As a minimum safeguard against microbial contamination, screening for mycoplasma is essential before the cell line leaves quarantine.
- Even equipment and work surfaces should be monitored and tested for exposure to mycoplasma.
- Sera and other biologicals should only be purchased from sources that have a good reputation and that use currently acceptable filtration (0.1 µm or smaller) and testing procedures.

(d) Consider the human factor:

- Accidents due to human nature are often unavoidable. Mistakes are much more likely to happen when operators are inexperienced, feel stressed, overworked, distracted, or rushed. Proper and repeated training sessions educate personnel on correct aseptic technique in cell culture, and also keep the lessons and skills learned top of mind. Additionally, being mindful of and focusing on the tasks at hand can prevent countless errors.

(e) Use of antibiotics:

- When overused, antibiotics can provide a false sense of security and mask mycoplasma contamination. It can also promote resistance, even to antibiotics specifically targeted for mycoplasma infections. Antibiotics should be used with great care and only when absolutely necessary.

3. Contamination by Bacteria, Moulds, and Yeasts

Bacteria, Moulds, and Yeasts are found virtually everywhere and are able to quickly colonize and flourish in the rich and relatively undefended environment provided by cell cultures. Because of their size and fast growth rates, these microbes are the most commonly encountered cell culture contaminants:

- The common contaminants, fungi, yeasts, and bacteria and will usually become apparent by naked eye or microscopic examination (increase in turbidity of the medium and/or a decrease in pH (yellow in media containing phenol red as a pH indicator)), if the cells are cultured in the absence of antibiotics. It is recommended that cells are inspected daily, and must always be examined under an inverted phase microscope before use in an experiment.

- When antibiotics are routinely used in culture, resistant organisms may develop into slow growing, low level infections that are very difficult to detect by direct visual observation.

- For most purposes, it is sufficient to grow the cells antibiotic-free to demonstrate that they are free from microbial contamination. However, if a microbial contamination is suspected but not evident on visual examination or more stringent quality control is required, it may be necessary to culture a sample of the cells and/or medium in nutrient broth or on nutrient agar.

- However, the direct culture tests and the indirect DNA fluorochrome test for mycoplasma, although not designed for this purpose, will also detect most bacteria, yeasts and fungi, including intracellular forms, reducing the need for the traditional tests. Special culture procedures are also available for detecting suspected protozoan contaminants in culture.

- With correct working practice it is not necessary to use antibiotics when working with established cell lines. Antibiotics should not be used except in high risk situations, e.g., when initiating a primary culture. Microbial contamination may be overt, and hence act as a signal to discard the culture, but if antibiotics are used, contamination may be repressed but not eliminated. Such cryptic contamination may co-exist with the cell culture and only appear when the culture conditions change or a truly resistant organism appears.

- If a cell culture is contaminated with bacteria or fungi, then the best method of elimination is to discard the culture and obtain fresh stock cultures or new supplies. In the case of irreplaceable stocks, it will be necessary to use antibiotics. The more antibiotics that are tested, the more chance there is of finding one that eliminates the infection. However, if the cells have been routinely grown in media supplemented with antibiotics (which is not recommended), it is almost certain that the contamination will be with organisms that are already resistant to this and some other antibiotics.

- To eliminate infection, the cells should be cultured in the presence of the antibiotic for at least three passages. If the contamination appears to be eradicated, then the cells should be cultured in antibiotic free medium for one month before re-testing.

4. Contamination by Viruses

Due to their extremely small size, are the most difficult cell culture contaminants to detect in culture, requiring methods that are impractical for most research laboratories. Their small size also makes them very difficult to remove from media, sera, and other solutions of biological origin.

- Most viruses have stringent requirements for their original host species' cellular machinery (may also be tissue specific) which greatly limits their ability to infect cell cultures from other species. Although viruses may be more common in cell cultures than many researchers realize, they are usually not a serious problem unless they have cytopathic or other adverse effects on the cultures.

- Since cytopathic viruses usually destroy the cultures they infect, they tend to be self-limiting. Thus, when cultures self-destruct for no apparent reason and no evidence of common biological contaminants can be found, cryptic viruses are often blamed. However, the he real cause of this culture destruction may be something else, possibly mycoplasma or a chemical contaminant, and as a result will go undetected to become a more serious problem.

- A major concern of using virally infected cell cultures is not their effects on the cultures but rather the potential health hazards they pose for laboratory personnel. Special safety precautions should

always be used when working with tissues or cells from humans or other primates to avoid possible transmission of viral infection (HIV, hepatitis B, Epstein- Barr, simian herpes B virus, among others) from the cell cultures to laboratory personnel.

- Viruses can cause lytic infections, thus destroying the host cells, but may also become established as persistent, sub-lethal infections, which are maintained with passage of the host cell line. Many cell lines both carry and express virus sequences without producing infectious virus particles. In a small number of cases, infectious human pathogens are released into the culture medium from lymphoblastoid cell lines (for example, Epstein-Barr virus from the B95-8 cell line, and human T-lymphotrophic virus II from MT4 cells).

- Mammalian genomes contain many retrovirus-like sequences, which, whilst not overtly infectious, may be released in large quantities as retrovirus-like particles in murine myeloma cells, hybridomas and other cell lines (for example, CHO cells and BHK cells). The expression of such virus-like sequences is also observed at the RNA level in many human cancer cell lines and also in primate cell lines.

- As long as serum is used to supplement media and natural trypsin is used in subculture, there will always be a risk that endogenous infections in the source of the reagent will infect the culture. The source of viral contamination can be from the tissue from which the cells are derived (e.g. HIV from Kaposi's sarcoma cells, EBV from lymphoma cells). Alternatively, contamination can be derived from growth media from other infected cultures or, as a more remote possibility, from laboratory personnel.

- Viral contamination presents an even greater detection problem than mycoplasma because of the diversity of potential viral contaminants. PCR and immunostaining are probably the most useful methods. Proper validation should ensure that the culture is virus-free, but that has to be qualified by the availability of the correct primers or antibodies, and presumes that the likely identity of contaminants is predictable.

- There is as yet no known way of eliminating viral contaminants, other than by discarding stocks. As for other forms of contamination, prevention is better than cure, and screening incoming biologicals is essential. Most reliable suppliers will screen serum and other biologically derived reagents but the individual laboratory should carry the responsibility for screening incoming cell lines.

5. Good Cell Culture Practice: Operational Steps for Reducing Contamination Problems:

    - Use good aseptic techniques
    - Reduce accidents
    - Keep the laboratory clean
    - Routinely monitor for contamination
    - Use frozen cell repository strategically
    - Use antibiotics sparingly if at all

(i) Use Good Aseptic Techniques (refer to CBE Code of Practice for further guidelines)

Good aseptic technique is essential (Freshney, 2000). It will protect the cell line against microbial and cross contamination, provide consistency in handling, and minimize variations in culture behaviour. This should be confirmed by regular observation, by eye and on the microscope, and any variations noted and accounted for. Records should be kept.

The following steps are recommended for developing sound, rational aseptic techniques:

1. Understand the nature and potential sources of biological contamination. This is reviewed in the beginning of this document.

2. Based on the nature of your work, determine the level of risk or danger to yourself and other laboratory personnel and then design your culture techniques accordingly. This is especially true when working with cultures that are virally contaminated or derived from human and other primate sources. Ensure that all laboratory personnel have been trained in the safe handling and disposal of any potentially hazardous cultures and materials; refer to the Training Manual and COP for guidance.

3. Based on the potential costs and consequences if the cultures are lost, determine how rigorous your technique must be and what degree of redundancy if any, is required.

4. Very valuable or irreplaceable cultures can be carried by two or more workers using media from different sources and separate incubators to reduce the chance of their simultaneous loss.

5. Evaluate whether workers need to be gloved, gowned and masked to reduce the potential for contamination.

6. The nature of your working environment and any problems it may present must also be considered in choosing appropriate aseptic techniques.

7. Avoid unintentional use of non-sterile supplies, media or solutions during routine cell culture procedures. This is a major source of biological contaminants. These products may be contaminated as a result of improper sterilization or storage, or may become contaminated during use.

(ii) Actions to reduce the probability of contamination:

1. Unsealed culture plates and dishes, as well as flasks with loose caps to allow gas exchange, provide another common way for contaminants to enter cultures. It is very easy for the space between the top and bottom sidewalls of a dish, or a flask and its cap to become wet by capillary action with medium or condensation. This thin film of liquid then provides a liquid bridge or highway for microorganisms to either swim or grow into the culture vessel. Even without any detectable film, fungi, as well as other microorganisms, can grow on the outside of culture

vessels; eventually their hyphae grow right up the side wall of the dish or past the cap into the neck of the flask. This is more often observed in long term cultures (a month or more) maintained in the same unsealed culture vessel. Small insects and other invertebrates can also make temporary visits into unsealed cultures, especially dishes and plates, leaving behind (unless they fall in and drown) only the contaminants carried on their feet.

2. Use sealed culture vessels whenever possible, especially for long term cultures. Multiple well plates for example should be sealed with labelling tape or placed in sealable bags, 35 and 60 mm dishes can be placed inside 150 or 245 mm dishes. Use vented cap flasks whenever possible. These have hydrophobic filter membranes that allow sterile gas exchange but prevent the passage of microorganisms or liquids.

3. Avoid pouring media from cell culture flasks or sterile bottles by using 50 or 100 mL pipettes to transfer larger volumes. Use a disposable aspirator tube and vacuum pump to quickly and safely remove medium from cultures. A drop of medium remaining on the vessel's threads after pouring can form a liquid bridge when the cap is replaced providing a means of entry for bacteria, yeasts and molds.

4. If pouring cannot be avoided, carefully remove any traces of media from the neck of the vessel with a sterile gauze or alcohol pad.

5. Always carry unsealed cultures in trays or boxes to minimize contact with airborne contaminants.

6. Do not use the BSC as a storage area. Storing unnecessary boxes, bottles, cans etc. in the BSC, besides adding to the bioburden, disrupts the airflow patterns.

7. Never mouth pipette. Besides the risk of injury to laboratory personnel, mouth pipetting has been implicated as the likely source of human mycoplasma species (*M. orale* and *M. salivarium*) often found in cell cultures.

8. Use clean lab coats or other protective clothing to protect against shedding contaminants from skin or clothes. Their use should be restricted to the cell culture area to avoid exposure to dirt and dust from other areas.

9. Work with only one cell line at a time in the BSC and always use separate bottles of media, solutions, etc. for each cell line to avoid possible cross contamination. Use disinfectant to wipe down the BSCs work surfaces between cell lines.

10. Use antibiotic-free media for all routine culture work.

11. Whenever possible, package sterile solutions, such as trypsin, L-glutamine and antibiotics, in small volumes (i.e., stored in 15 mL tubes) to reduce the number of times each tube must be entered and thus reduce the probability of contamination.

12. Leave BSCs running 24 hours a day. Only turn them off when they will not be used for extended periods.

13. Glassware, including storage bottles and pipettes, is usually sterilized by autoclaving or dry heat sterilization. Ensure proper maintenance and operation of sterilization autoclaves and ovens. Packing too much into an autoclave or dry heat oven will cause uneven heating, resulting in pockets of non-sterile supplies. Using too short a sterilization cycle, especially for autoclaving volumes of liquids greater than 500 mL per vessel or solutions containing solids or viscous materials, such as agar or starches, is a common mistake. The size, mass, nature and volume of the materials to be sterilized must always be considered and the cycle time appropriately adjusted to achieve sterility.

14. Ensure that sterility is maintained by properly storing the supplies and solutions in a dust- and insect-free area to prevent recontamination.

15. Care must also be taken to avoid condensation on bottles of solutions stored in refrigerators and cold rooms.

16. Plastic disposable cell culture vessels, pipettes, centrifuge tubes, etc. are usually sterilized by their manufacturer after they are sealed in their packaging. Care must be taken when opening and resealing the packaging to avoid contaminating the products within.

17. Most media, sera and other animal derived biologicals are not heat sterilizable and require membrane filtration (Sometimes radiation is also used.) to remove biological contaminants. Products filter sterilized in the laboratory should always be tested for sterility before use; commercially produced sterile products are tested by the manufacturer before being sold. While filtration through 0.2 µm membranes is very effective in removing most biological contaminants, it cannot guarantee the complete removal of viruses and mycoplasmas, especially in sera. While these products are not considered a major source of mycoplasma contamination, they must still be considered as potential sources to be evaluated whenever mycoplasmas are detected in cultures.

(iii) Reduce Opportunities for Accidents

Cell culture-related accidents are one of the leading causes of cross-contamination by other cell cultures. Accidents usually involve people, and reducing them must take into consideration both human nature and stress. The following recommendations will both reduce the potential for errors as well as provide a valuable aid in tracking down the cause of problems.

1. Be very careful when labelling solutions, cultures, etc. Always clearly indicate if solutions or other supplies have been sterilized. Reduce misunderstandings in crowded or busy labs by using a colour coding system: assign each worker their own colour for labelling tape and marking pen inks.

2. Be very careful with the use and choice of acronyms. Everyone in the laboratory should understand and agree to their meaning.

3. Whenever possible use standardized record keeping forms; this simplifies their use and makes it more likely that good records will be kept.

4. Use written protocols and formulation sheets when preparing media and solutions, listing the reagents used, lot numbers, weights, volumes, pH and any special treatments that were done.

(iv) Clean Up the Work Area and Surrounding Environment

- In most laboratories, the greatest sources of microbial contamination are airborne particles and aerosols generated during culture manipulations. The microbial laden particles are relatively large (generally 4 to 28 µm in diameter) and settle at a rate of approximately one foot per minute in still air. As a result, the air in a sealed, draft-free room or laboratory (no people, open windows or doors, air handling units, air conditioners, etc.) is virtually free of biological contaminants. However as soon as people enter the room, particles that have settled out will be easily resuspended. In addition certain equipment and activities can generate large amounts of microbial laden particulates and aerosols: pipetting devices, vacuum pumps and aspirators, centrifuges, blenders, sonicators, and heat sources such as radiators, ovens, refrigerators and freezers.

- The major source of mycoplasma contamination is infected cultures acquired from other research laboratories or commercial suppliers. Entry of a single mycoplasma infected culture into a laboratory can quickly lead to the infection of all the other cultures in the laboratory. This explains the frequent finding that if one culture in a laboratory is mycoplasma contaminated then usually most if not all of the other cultures will be as well.

- Another major source of particulates and aerosols are laboratory personnel. Street clothes and dirty lab coats are dust magnets. Talking and sneezing can generate significant amounts of aerosols that have been shown to contain mycoplasma. Dry, flaky skin is another source of contamination laden particles; this common condition is aggravated by the frequent hand washing required in the laboratory; even the lotions designed to moisten dry skin have occasionally been found to be contaminated.

- Incubators, especially those maintained at high humidity levels, can be a significant source of biological contamination in the laboratory. Dirty water reservoirs, and shelves or culture vessels soiled by spilled media, allow the growth of spore-generating fungi. The fans used in many incubators to circulate the air and prevent temperature stratification can then spread these spores and other particulates. Some incubators humidify incoming gases by bubbling them through the water reservoirs at the bottom of the incubator; the aerosols generated by this will quickly spread any contaminants in the water.

- While laminar hoods and incubators are the major sites where biological contamination occurs, transporting cultures between these two sites also provides opportunities for contamination. Most cell culture laboratories try very hard to keep their incubators and laminar flow areas clean, but sometimes they overlook the potential sources of contamination found in less clean laboratory areas transversed going from one location to the other. Rooms containing open windows, air conditioners, microbiology and molecular biology work areas, and the other major particle generators discussed above, add to the potential hazards of moving cultures around the laboratory. This problem increases both with the distance travelled and when the culture vessels are unsealed.

1. Reducing the amount of airborne particulates and aerosols in the laboratory, especially around the incubator and the BSC, will reduce the amount of contamination.

2. Routinely wipe floors and work surfaces to keep down dust.

3. Incubators, especially those that maintain high humidity levels, require periodic cleaning and disinfecting.

4. Often overlooked but important sources of contaminants are the water baths used to thaw sera and warm media. Dirty water baths not only coat bottles with a layer of heavily contaminated water immediately before they are placed under the hood, but the water dripping from bottles generates heavily contaminated aerosols which can end up on lab coats and hands. Water baths should be emptied and cleaned on a regular basis, well before odour or visible turbidity develops.

5. Pipette disposal trays and buckets, and other waste containers provide a source of potentially heavily contaminated materials in close proximity to the laminar flow hood and are a potential mycoplasma source. Waste containers should be emptied daily and the wastes disposed of safely. Autoclaving of any wastes that have been in contact with cells is recommended.

6. The cooling coils on refrigerators and freezers are a major source of microbial laden airborne particulates that are often overlooked in otherwise very clean laboratories. These should be vacuumed at least yearly; besides removing a significant source of contamination; regular vacuuming will extend the life of the cooling units and allow them to run more efficiently.

7. A pest management program is recommended to reduce the presence of insects and other multi-legged creatures that can be sources of contamination.

(v) Strategic Use of Antibiotics

- Experienced cell culture users have recommended for many years that antibiotics never be used routinely in culture media. Over reliance on antibiotics leads to poor aseptic technique. It also leads to increased antibiotic resistance among common culture contaminants. Routine use can also lead higher rates of mycoplasma contamination because everyone generates

and sheds a relatively constant flow of particles, consisting of fibres, aerosols and droplets, as they work in the laboratory.

- These particles can have a mixture of bacteria, yeast, fungi, and even mycoplasmas bound to them. If one of these contamination-laden particles enters an antibiotic-free culture, the chances are that at least one of the contaminants will produce a highly visible infection within 24 to 48 hours. As a result the contaminant is quickly detected and the culture discarded. It is very unlikely that particles shed by laboratory personnel would ever consist of just difficult to detect contaminants, such as mycoplasmas, that could enter cultures and not cause visible signs of contamination. However, if the culture contains antibiotics, there is a chance that the antibiotics will prevent the growth of the usually more easily detected contaminants but allow mycoplasma or other cryptic contaminants to grow undetected. As a result, instead of being discarded, the cryptically infected culture remains in use, is utilized in experiments, and becomes a potential source of serious contamination for the other cultures in the laboratory.

- Antibiotics should never be used as a substitute for good aseptic technique; however they can be used strategically to reduce the loss of critical experiments and cultures. The key is to use them only for short term applications:

  (i) for the first week or two of primary cultures,
  (ii) for experiments in general where the cultures will be terminated in the end.
  (iii) Whatever their use, the antibiotics ultimately chosen should be proven effective, noncytotoxic and stable.

- 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.

- It is important to remember that antibiotics are agents that arrest or disrupt fundamental aspects of cell biology. It is important to obtain antibiotics from companies that are willing to provide certification for the concentration and purity of the antibiotics they supply. Where possible, the use of antibiotics should be avoided. It should not become routine in the cell and tissue culture laboratory, and can never be relied on as a substitute for effective aseptic techniques.

ANNEX III METHODOLOGY FOR TESTING FOR A BACTERIA CONTAMINATION USING AGAR

This protocol is useful if you are unsure whether you have a contamination in mammalian cell culture or not. If you have an obvious contamination DO NOT risk performing this protocol. Instead dispose of your cultures according to SOP 003 section 7.1.3.5.

If a contamination is confirmed, you must:

- Inform your lab leader
- Dispose of contaminated cultures and agar using the autoclave and following SOP 003
- Inform lab management using the contamination sheet

Note: before carrying out this protocol you must:

- Check with another experienced cell culture lab user or lab leader to get a second opinion on contamination status
- Inform lab management (Carolyn/Kul/Jen)
- Discuss with the lab leader of H29 (currently Jenna Davis) on using that lab and required contamination control protocols. These include:
    - Arrange for H29 lab induction to be able to carry out the work yourself or arrange for the lab leader to prepare your plates.
    - Changing lab coat and gloves upon entry

Consumables:

- Sterile petri dishes: Fisher Scientific Product number 12654785 https://www.fishersci.co.uk/shop/products/sterilin-polystyrene-petri-dishes/12654785
- L shaped spreaders: Fisher Scientific Product number: 15625467 https://www.fishersci.co.uk/shop/products/fisherbrand-l-shaped-cell-spreaders-2/p-4249846#?keyword=L-Shaped+Spreader
- Agar powder: Sigma Aldrich L3147-250G https://www.sigmaaldrich.com/catalog/product/sigma/l3147?lang=en&region=GB
- Glass bottle for Agar prep (you can find these in the sink cupboards in individual labs)
- Sterilely collected suspected contaminated medium sample (1 mL)

Method

- Add 10g of agar powder to 250mL of DI water in an autoclavable glass bottle.
- Shake vigorously until as much of the powder has dissolved as possible
- Autoclave the agar on cycle 5. Be sure to leave the lid of the bottle slightly open to allow steam to penetrate the bottle.
- Remove the autoclaved agar from the autoclave and transfer to H29
- Wait until the bottle is cool enough to handle
- In a BSC Pour the agar into sterile petri dishes from the glass bottle, filling them half way up
- Leave to solidify with lids off

- Use a sterile pipette to transfer 100µL of culture medium to agar plates
- Use an L shaped spreader to spread the culture over the surface of the agar
- Ensure you use a control agar plate
- Place in incubator in H29 – **Note**: you must liaise with the lab leader of H29 for this step.

Standard Operating Procedure SOP036

Title: MAINTENANCE OF A QUALITY LABORATORY ENVIRONMENT

Location: CBE Laboratories

SOP Version History

Version Reviewed	Date Revised/ Reviewed	Revision Summary	New Version Number
1.0	01.04.11 P.Hourd	Minor revision to Section 7.13 to add note on checking for cross contaminated or misidentified cell lines; list available from HPA website. Added Section 7.1.2 (v) cross contaminated or misidentified cell lines	2.0
2.0	01.10.12	Annual Review – minor format change	Not issued
2.0	01.04.2019 E Cheeseman	Minor text revisions Annex III added for testing for bacteria contamination	3.0