Chemistry
UNIVERSITY OF TORONTO

Lash Miller Laboratories St. George Campus

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BEST PRACTICES TO AVOID CONTAMINATION IN SHARED CL2 LAB (DB440) GUIDELINES

1) **Types and sources of potential chemical contaminants:**
    a. Metal ions, endotoxins, and other impurities in media, sera, and water
    b. Plasticizers in plastic tubing and storage bottles
    c. Free radicals generated in media by the photoactivation of tryptophan, riboflavin or HEPES exposed to fluorescent light
    d. Deposits on glassware, pipettes, instruments etc., left by disinfectants or detergents, antiscaling compounds in autoclave water, residues from aluminum foil or paper
    e. Residues from germicides or pesticides used to disinfect incubators, equipment, and labs
    f. Impurities in gases used in CO2 incubators

2) **How do biological contaminants enter cultures?**
    a. *Contact with nonsterile supplies, media, or solutions*
        - Unintentional use of nonsterile supplies, media or solutions
        - Improper maintenance and operation of sterilization autoclaves (uneven heating from packing too much into autoclave, too short a sterilization cycle)
        - Improper storage of supplies and solutions (must be dust and insect free, avoid condensation on bottles of solutions in fridges)
        - Good aseptic technique maintains sterility of supplies and solutions
    b. *Particulate or aerosol fallout during culture manipulation, transportation, or incubation*
        - Pipetting devices, vacuum pumps/aspirators, centrifuges, blenders, sonicators, and heat sources can generate large amounts of microbial laden particulates and aerosols
    c. *Microorganisms swimming, crawling, or growing into culture vessels*
        - Unsealed culture plates and dishes, as well as flasks with loose caps to allow gas exchange, provide a common way for microorganisms 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 for microorganisms to 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.

d. *Accidents and mistakes*

Other Sources of Contamination:

a. Poor housekeeping could be a source of contamination: Mopping/disinfecting of the floors is recommended to take place once a month (with Lysol for example), inspecting the water baths for growths, and changing its contents, disinfecting surfaces before and after each use, removing waste once buckets are full
b. Cardboard boxes are a source of contamination as they collect spores, which can travel into the incubators and infect the cell cultures. Therefore, cardboard boxes cannot be stored in a cell culture lab, and any shipments that come in cardboard boxes must be transferred to plastic containers, and boxes should be disposed of immediately.
c. Do not use Styrofoam boxes for ice due to their ability to absorb contaminants. Proper ice buckets should be used, because they are made of materials that can be easily disinfected.

<u>Controlling cell culture contamination</u> **1) Use good aseptic techniques:**

a. Understand both the nature and potential sources of biological contamination and design your culture techniques accordingly
b. Based on the potential costs and consequences if the cultures are lost, determine what degree of redundancy, if any is required. Ex. Replenishing your stocks after your passage 1 (P1) every time you thaw a new vial.
c. Make it more difficult for microorganisms to gain entry by using sealed culture vessels whenever possible, especially for long term cultures. Use vented cap flasks whenever possible. These have hydrophobic filter membranes that allow sterile gas exchange but prevent the passage of microorganisms or liquids.

d. Avoid pouring media from cell culture flasks or sterile bottles by using 50 or 100 mL pipettes to transfer larger volumes. Using a disposable aspirator tube and vacuum pump is an economical way 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. If pouring cannot be avoided, carefully remove any traces of media from the neck of the vessel with a sterile gauze or alcohol pad.

e. Always carry unsealed cultures in trays or boxes to minimize contact with airborne contaminants. Square 245 mm dishes are excellent carriers for 384 and 96 well plates as well as for 35mm and 60 mm dishes.

f. Do not use the biosafety cabinet (BSC) as a storage area. Storing unnecessary boxes, bottles, cans etc. in the BSC disrupts the airflow patterns, facilitating contamination.

g. 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 crosscontamination.

h. Use 70% ethanol to wipe down the BSC's work surfaces before and after each use, and between cell lines.

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

j. 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.

k. Sterile filter pipettes and pipette tips are highly recommended as they prevent aerosolized contamination from reaching the cell culture. They should be certified DNase free, RNase free and non-pyrogenic. See photos below.

l. 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.

2) Reduce accidents:

a. Be very careful when labeling solutions, cultures, etc. Only use your solutions, do not open other people's reagents.

b. Always clearly indicate if solutions or other supplies have been sterilized, the date of preparation, date of opening, expiration date (if applicable)
c. 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. These will both reduce the potential for errors as well as provide a valuable aid in tracking down the cause of problems.

3) **Keep the laboratory clean. The items below are included in the Shared Lab Duties for the use of DB440:**
   a. Routinely wipe floors and work surfaces to keep down dust.
   b. Incubators, especially those that maintain high humidity levels, require periodic cleaning and disinfecting.
   c. Water baths should be emptied and cleaned on a regular basis, well before odor or visible turbidity develops.
4) **Routinely monitor for contamination through (1) sterility tests, (2) detecting mycoplasma in cultures and (3) other biological contaminants, and (4) detecting chemical contaminants:**

Recommended Sterility Tests (as applicable):

a. **All autoclaves used to sterilize glassware**, solutions and other supplies must be regularly maintained, and personnel properly trained in their loading and operation. Thermometers and chart recorders should be tested and calibrated periodically to ensure their accuracy. Autoclave thermometers, spore test strips and capsules, or other testing devices can be placed inside autoclaves or into bottles of solutions or other packaged supplies during every run, or as necessary, to ensure proper loading and operation.

b. **Samples of all in-house filter-sterilized solutions** should be tested for sterility each time they are prepared and the solutions not used until testing is complete. Standard microbiological testing methods for bacteria, yeasts and fungi usually require placing samples for testing into several different broths (trypticase soy, thioglycolate and Sabouraud broths, for example) and semisolid media (brainheart infusion, blood agar), and then incubating them at both 30° and 37°C for at least two weeks.

c. **Cell culture media**, especially unopened bottles of media that are outdated or no longer used in the lab (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° and the other at 37°C for at least two weeks.

- This sterility test media substitute is also very useful for evaluating the amount or source of particulate contamination in an area, near a piece of equipment or by a technique. 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.

Testing for Mycoplasma:

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 requires several carefully tested liquid and semisolid media and controlled environmental conditions, and must be run with live mycoplasma controls. Additionally, although direct culture is the most sensitive method, it is the slowest (requiring up to 28 days) and it may not reliably detect some fastidious strains of mycoplasma, making it less than 100% effective. Budget permitting, direct culture testing is best contracted to an outside testing facility due to the ease with which mycoplasma can spread in the laboratory.

- 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, all are commercially available in kit form, and they can detect the 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.

- **The most widely used and recommended indirect test** is DNA fluorochrome staining. This easy and relatively fast procedure stains DNA using a fluorescent dye. When stained and fixed cells are examined under a UV microscope equipped with the proper filter package, DNA fluoresces brightly. This staining method can be combined with an indicator cell line to increase its sensitivity. Interpreting results is not always easy, especially with hybridoma cultures; suitable positive and negative control slides should always be used to help interpret staining results. The best overall testing approach is a combination of both methods: direct culture can provide very high sensitivity while DNA fluorochrome staining can detect any mycoplasma that the direct culture misses. 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.

<u>Testing for Other Biological Contaminants:</u>

- The traditional microbiological media described earlier for testing the sterility of solutions can be adapted for testing cultures for bacteria, yeasts and fungi. 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.

<u>Avoiding Chemical Contamination</u>

- 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.

<u>Characterization methods for monitoring cell cultures:</u>

- 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.

General steps for setting up testing/monitoring program:

- Test all current in-house cell lines using the methods described above to ensure they are free from mycoplasma and other microbial contaminants, and to check their identity. Then incorporate these tested cultures into your cell repository and rely only on them for all future experiments.
- Quarantine and then test all incoming cell lines and any cultures currently stored in your cell repository that were not tested when they were frozen.
- Test all cell lines that are in continuous use at least every three to four months and any time they behave suspiciously. Better yet, periodically discard these cultures and replace them with cultures from your tested cell repository. (See cell repository section below).
- New lots of sera should be evaluated for any critical applications before widespread use. The simplest test method is to use the new serum in an indicator cell culture for several weeks and then test the culture for mycoplasma contamination using DNA staining or other suitable test.

5) Use frozen cell repository strategically:

a. 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. Freezing cultures also stops biological time for them, preventing them from acquiring the altered characteristics that can normally occur in actively growing cells as a result of environmental or age related changes. However, a cell repository is only a reliable resource if the cultures it contains have been properly tested, labeled and stored.

b. A cell repository can also be used strategically to convert continuously carried cultures into a series of short-term cultures, which reduces both the amount of quality control testing required and potential problems from cryptic contaminants. When cultures are continuously carried for long periods in the laboratory, they should be tested for contaminants at least every three to four months (more often for critical applications). 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. However, regular testing, although very important to ensure the integrity of your cultures, can require considerable effort, especially in laboratories using multiple cell lines. Rather than test cultures several times a year, it is easier to simply discard them 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. Tested stocks should be set up in the cell repository for each culture that is routinely used in your laboratory.

c. **A better alternative** may be to first develop a seed or master stock (10 to 20 vials is usually sufficient, depending on your envisioned needs), and then from that, develop a working stock (approximately 20 vials). When the original working stock is depleted, it is replaced by using a vial from the seed stock to develop a new working stock. 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. 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.

Endotoxin 1) Endotoxin can be a problem for some cell culture users; to reduce the possibility of endotoxin related problems, use cell culture media, sera, and plasticware that are certified either by their manufacturers

or through in-house testing to be nonpyrogenic.

2) Test the source of the water used when making solutions and for washing glassware and plasticware to ensure the water is not a source of endotoxin.
    a. High purity water is essential in any cell culture laboratory, not only for preparing media and solutions, but also for glassware washing. Traditional glass distillation is very effective in removing endotoxin if the equipment is maintained and used properly. Reverse osmosis is also very effective.
    b. Poorly maintained water systems, especially systems using ion exchange resins, can harbor significant levels of endotoxin-producing bacteria and should be tested if endotoxin problems are suspected or discovered in cultures.
    c. Equally important are the storage conditions used for the water after it has been purified. Bacteria are often found growing in glass or plasticware storage containers and associated tubing and can quickly raise endotoxin levels in the stored water. A LAL (Limulus Amebocyte Lysate) assay can be done to check the endotoxin levels in a water purification system. As an alternative, nonpyrogenic water for injection can also be used for preparing media and other critical solutions.
3) As an extra precaution, the culture medium can be tested for endotoxin after any additional components have been added.

References

- Understanding and Managing Cell Culture Contamination
- Endotoxins and Cell Culture
    - An updated version can be found here