# GUIDELINE
# WORKING IN CELL CULTURE LABORATORIES

## CONTENTS



## REVISIONS

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| *Ed.* | *Date* | *Description of change(s)* | *Author of change(s)* | *Approval* |

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| 0 | 10.05.2013 | EDITION FOR PUBLICATION | Lacerra G.[(1)], Digilio A.[(1)], Barra A.[(1)], Lanati A.[(2)], Liguori G.L.[(1)]<br><br>*(1) qPMO-WP1, IGB-CNR*<br>*(2) VQ consultant* | **qPMO-WP1, IGB-CNR** |
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## 1. PURPOSE

The following are guidelines for operating cell culture laboratories dealing with biological agents which are nonpathogenic to humans (groups 1 and 2), drawn up with the objective of minimizing contaminations and facilitating experimental activity in a shared work environment.

## 2. REFERENCE SOURCES AND STANDARDS

- "Introduzione alle colture cellulari" [Introduction to cell cultures], by Gian Luigi Mariottini et al., Editore Tecniche Nuove, 2010
- GL: "**The Drafting of Guidelines for Scientific Research Laboratories According to Quality Principles**" Lacerra G, Digilio FA, Lanati A, Liguori GL. Edition 0, date 11.03.2013
- Legislative Decree n. 206 of 12 April 2001

For the section regarding mycoplasma contaminations:

- European Pharmacopoeia Suppl. 2001, 2.6.7
- FDA Points to Consider (May 1993)
- *Principi di Microbiologia Medica* [Principles of Medical Microbiology], by Michele La Placa, Bologna, Società Editrice Esculapio, 2006

## 3. ACRONYMS AND SPECIAL TERMS

| Acronym | Meaning |
|---|---|
| ad$H_2O$ | Autoclaved distilled water |
| WP1 | Workpackage 1 |
| CC Lab | Cell culture laboratory |
| GL | Guideline |
| qPMO | Quality Project Management Open-Lab |
| VQ | Valore Qualità |

## 4. GUIDELINE AREAS

### 4.1. MILIEU

### Organization

It is important to take actual research procedures and space availability into account when organizing the workspaces for cell cultures. To organize shared workspaces, it is necessary to make agreements and obtain authorizations from the Director of the Institute. The following are optimal arrangements:

- Spaces for mycoplasma-free cell cultures into which only certified or tested mycoplasma-free cell lines and derivatives can be introduced.
- A small room with a hood and incubator (possibly self-sterilizing) for quarantining cell lines which are to be tested and for treating infected lines. If it is not possible to have a separate room, at least one incubator and hood should be devoted to quarantining and initial testing. To avoid cross infections, initial test and mycoplasma treatment sheduling need to be staggered so that they occur on different days. Periodically, as well as after each introduction of mycoplasma-infected lines, the incubator must run a self-sterilization cycle. Once they have tested negative for mycoplasma, cell lines can be moved into the mycoplasma-free rooms.
- Spaces for mycoplasma-infected cell cultures which can not be substituted or upon which experiments are still being performed, into which only mycoplasma-infected cell lines can be introduced.

- Spaces for primary cultures. Primary cultures may be contaminated with mycoplasmas. Thus, it is useful, if possible, to dedicate a space to these cultures. Different hoods and incubators are to be used for animal primary cultures and human primary cultures.
- If having separate spaces is impossible, it is useful to specially allocate a hood and an incubator each to mycoplasma-free cultures and mycoplasma-contaminated cultures.

### Cleaning

To reduce contamination by staff, putting adhesive dust-catching mats at the entrance to all CC Labs is recommended.

Regular daily cleaning of the cell culture rooms is performed by a designated company which cleans floors and windows according to procedures that ensure the highest possible level of hygiene. It is helpful to work with the company to define the specific cleaning procedures required if they are different from those performed in other laboratories.

The cleaning of CC Lab incubators, hoods, work surfaces and equipment is the responsibility of all of its users. Coordinators need to organize, using the attached forms, shifts for who is to be in charge of the rooms and their cleaning, both regular (approximately every six months) and as needed. All CC Lab users are required to take part in these shifts, with appropriate data recorded, and any nonroutine cleaning should also be recorded (Form 1).

The use of bleach should be avoided when cleaning steel items because it can corrode them. Instead, use denatured alcohol, 70% alcohol, or benzyl quaternary ammonium compounds (e.g., Incubator Clean).

In weekly turns, all surfaces are to be cleaned. For the cleaning of instruments, see the appropriate section below.

When necessary, UV lights may be turned on in the room at night.

### Monitoring

The CC Lab Coordinator performs monthy checks to verify that cleaning activities are regularly and properly carried out by the designated users and that details, dates and signatures are recorded (Form 2).

## 4.2. MANPOWER

For the optimal management of CC Labs, it is helpful to assign specific tasks to specific individuals.

**General Manager:** He/she is the Director of the Institute and is responsible for defining the objectives of the CC Labs, ensuring the availability of the necessary resources for achieving and maintaining the established level of quality, selecting the Coordinators and their staff.

**CC Lab Coordinators:** These are selected by the Director. Each CC Lab has a Coordinator whose name and contact information are posted at the entrance of their CC Lab. The role of the Coordinator is to ensure that all users can work in optimal conditions, in accordance with the rules and respecting shared workspaces. He/she should be contacted whenever there are problems regarding the laboratories. The Coordinator authorizes users who have applied for access and records them in a list and has the right to deny access to users who lack the required training.

**Tutors:** These are researchers who are entrusted with responsibility for the research of internal staff and external personnel (undergraduates, fellows, graduate students, post-docs, etc.). Tutors are required to enforce the guidelines below and are directly responsible for any breaches of protocol by CC Lab users. Each Tutor is responsible for training the users they are responsible for in the use of CC Lab equipment and

specific experimental procedures, in accordance with the relevant guidelines. Tutors are required to send to the Coordinators lists of the internal and external personnel who are permitted access to the CC Labs.

**Technical Support Services:** This service is responsible for the care and maintenance of the equipment. Technical Support Services should be contacted in cases of equipment malfunction in the CC Labs and can respond directly or make use of outside services to resolve the problem. A list of support services for the CC Labs, along with contact information, can be found in section 6.

**Contamination Monitoring Service:** This service monitors mycoplasma contaminations and provides guidance on how to treat and eradicate it. It is good practice to have a dedicated monitoring service. The service organizes periodic testing sessions for the presence of mycoplasma in cell cultures. The testing date can be communicated through e-mail to the whole Institute or solely to users who work with cell cultures. To request a test, find out how to provide the material to be tested, and other issues, users need to follow the instructions provided by the service.

**Users:** These are internal staff and external personnel affiliated with the Institute (undergraduates, fellows, graduate students, post-docs, etc.) who are authorized to work in CC Labs. Users are obliged to follow the guidelines, and if someone is found to be violating them, the CC Lab Coordinator is authorized to contact their Tutor. Access to CC Labs is restricted to authorized users only. To work in a CC Lab, it is necessary to wear a (dedicated) lab coat, wash one's hands, and wear gloves.

### 4.3. MACHINES

#### Space Assignment

For the allocation of space (shelves in the incubators, refrigerators, and freezers), Tutors must contact the CC Lab Coordinator. Decisions regarding the allocation of space must be respected.

#### Refrigerators

Stored materials should be marked with the date and the laboratory they come from. Cardboard containers are not to be used in refrigerators. Users should periodically empty regrigerators of their unused materials, in conjunction with the cleaning of the room or as needed.
For the maintenance of refrigerators, it is advisable to periodically (every six months) check and record the internal temperature on the appropriate Form 3.

#### Water baths

Water baths are filled with distilled water treated with antifungal and antibacterial agents, according to the proportions recommeded by the service in charge. Water levels should be between the maximum and minimum limits specified on the tubs.
Water baths must be emptied and cleaned whenever contaminations are detected in the room, as well as during periodic cleanings (approximately every six months). Washing is done with water and detergent, using a cloth or paper towel to remove deposits, followed by a plain-water rinse and then a distilled-water rinse. The bath should be dried with paper and then filled as described above.
It is good to periodically check and calibrate the internal temperature, recording it on the appropriate Form 3.

#### Incubators

Culture plates and flasks to be stored in the incubators should be marked with the user's or group's code and placed on the shelves assigned to each group. Users should clean the incubator shelves frequently and as needed with 70% alcohol.
Cleaning and maintenance done by the users should be recorded on the Form 4.
The incubator shelves and chamber should be cleaned with appropriate detergents at least once every six months as reported. Removable parts should be washed under running water with detergent, then rinsed with ad$H_2O$. After being dried and wrapped in aluminum foil, they are oven sterilized. Nonremovable parts are washed in ad$H_2O$ and detergent, rinsed with ad$H_2O$, and then cleaned with 70% ethanol and disinfectants which are not toxic to cell cultures, such as Incubator-Clean. For incubators that have a sanitizing cycle, it should be activated after the shelves and chamber have been cleaned with ad$H_2O$ and detergent, as specified above.
The water in the pan which is fitted in every incubator should be changed monthly using ad$H_2O$ to which a suitable disinfectant has been added.
Below the maintenance's responsibility of Technical Support Services, which must record the details, date, and signature: temperature and $CO_2$ levels of each incubator should be calibrated monthly (Form 5); incubator filters should be replaced every six months or once a year (in conjunction with general cleaning) or when necessary (Form 5).

### Laminar flow hoods

Hoods may be reserved using the attached Form 6. Reservations must be respected; if it is not possible, they must be cancelled as early as possible. The surfaces of all materials to be placed under the hood must be cleaned with denatured alcohol. There should be as little equipment under the hood as possible so as not to block the laminar airflow. Hoods are shared, so no materials should be left under them at the end of the activity.
At the end of their work, each user must clean the work surface and glass shield with 70% alcohol.
Users must clean the work surface, the space underneath, and the walls whenever materials are spilled and periodically, as indicated, with the date and signature recorded on the appropriate Form 1. For cleaning the hood, all parts must first be washed with ad$H_2O$ and detergent, followed by a rinse with ad$H_2O$ and cleaning with 70% ethanol. UV lights may be turned on after each use and must be turned on at the end of the day and after each cleaning of the hood.
Maintenance is provided by an external company which must record the details, date, and signature on an appropiate form (provided by the company); a semi-annual check of the intake flow of the hoods is recommended; filter replacement is arranged with the Coordinators of the CC Labs, with notice posted on the hoods at least one week in advance.

## 4.4. MATERIALS

### Shared materials and detergents

Each CC Lab must be supplied with the following materials for cleaning hands and surfaces: soap, paper towels, 70% ethanol, bleach, and disinfectant for incubators and hoods. These materials can be supplied in turns by the various groups affiliated with a CC Lab or with the Institute. In the latter case, it is recommended that materials be stored in a fixed location outside of the CC Lab from where it can be picked up by personnel assigned by the CC Lab Coordinator.

### Group materials

Each group must set up a discard rack/cart in the CC Lab for the storage of all disposable material. All material brought into the CC Lab must be disposable, sterile, and sealed or thoroughly sterilized and marked with the date and the group it belongs to.
Before starting work, personnel should equip themselves with all necessary material. Avoid transferring media already in use, pipettes and disposable materials from one CC Lab to another. Keep cardboard packaging to a minumum.
At the end of their activities, each user must leave the CC Lab in order and put pipettes and plasticware in the discard rack/cart, leaving all surfaces free and clean.

## 4.5. METHODS

### 4.5.1. REMOVING CELLS FROM LABORATORY ANIMALS FOR PRIMARY CULTURES

When removing cells directly from laboratory animals for primary cultures, it is important to remember that these animals may be infected with mycoplasma and that the infection can be transmitted during the removal process. Each user should follow the specific protocol agreed with the Tutor. To limit infections, it is recommended to:

- ask the animal housing unit if it conducts periodic testing for mycoplasma infections. If it does not, provide for appropriate safeguards;
- conduct dissection in a sterile environment;
- wash the removed materials several times with sterile solutions and transfer them in sterile containers before moving them under the laminar flow hood in the appropriate CC rooms reserved for their use.

### 4.5.2. ASPIRATION OF LIQUIDS

It is necessary to establish a system for the aspiration of liquids under the hood, for example by using a Büchner flask connected to a vacuum system or vacuum pumps.
To avoid wasting water if using water pumps or having to drain the vacuum system, it is best to activate the aspiration system only when it is to used, and to turn it off after use.
Prior to starting aspiration, each user should attach a sterile pipette tip or pasteur pipette to the end of the aspiration hose. At the end of the work, all users are required to sanitize the aspiration junction by aspirating disinfectant solution (quaternary salt, hypochlorite, etc.), followed by ad$H_2O$.
According to the schedule on the attached Form 7, flasks are emptied into liquid waste collection tanks; they are changed weekly or whenever the liquid approaches the fill limit indicated.

### 4.5.3. WASTE

Glass and sharp waste are collected in the appropriate yellow containers. The glass must not overflow the container, and once it is full, it is closed and put inside the solid waste container. All CC Lab users are required to properly close the solid waste container when it is full. In turns, it is taken out of the CC Lab on the day of waste collection according to the schedule on the attached Form 7. All waste containers are provided by the Health and Safety Office. Liquid wastes are decontaminated before disposal (according to the law as specified by directives of the responsible office of the Institute).

### 4.5.4. MANAGEMENT OF CONTAMINATIONS AND DECONTAMINATION

In case of fungal or bacterial contaminations, it is necessary to:

- inform the CC Lab Coordinator;

- decontaminate the contaminated plates, flasks, and equipment used. Decontamination is done outside of the CC Lab, in the users' laboratory, according to the following procedures: add hypochlorite to the plates and flasks, agitate, wait approximately 10 minutes, then pour it into the liquid waste tank; wash the equipment well with soap followed by 10% hypochlorite, then autoclave them;
- dispose of or inspect the materials used (media, plastic, etc.);
- clean the incubator and hood used;
- immediately remove solid and liquid wastes from the CC Lab.

### 4.5.5. MANAGEMENT OF MYCOPLASMA CONTAMINATIONS

Mycoplasmas are parasites of various animal and plant species that are widely distributed throughout the environment. In humans, they cause pathologies of the respiratory system and genitals, multiplying on the surface of mucosal epithelia and showing little tendency to go beyond them. Another characteristic of mycoplasma infections is that they can be transmitted by aerosols. Nine types of mycoplasmas are responsible for 98% of contaminations.

Mycoplasma contaminations are quite common in cultured cell systems and, being small bacteria (<1um) lacking a cell wall, they are immune to most common antibiotics which target cell wall synthesis. Mycoplasma infection causes changes in cellular functions and can thus distort experimental results. Generally, these infections originate from users, other contaminated cultures, primary cultures of infected animals, or infected bovine serum.

To reduce the risk of cell culture mycoplasma contaminations, it is necessary to act on the following three levels:

#### Prevention

To prevent mycoplasma contaminations, it is imperative to strictly follow the guidelines for working in a CC Lab. In addition, it is important to conduct periodic controls as specified below.

#### Detecting mycoplasma using test kits

For the determination of the presence of mycoplasma infections in cell cultures, it is necessary to begin with an analysis of the available systems, favoring those that ensure high sensitivity, are representative of multiple strains, have shorter execution times, and finally, are recognized by the European Union Pharmacopoeia. Using complementary approaches is recommended for the early identification of infections: for example, if the enzyme immunoassay method is negative, it is useful to try identification by PCR which is more sensitive and thus allows the detection of low levels of infection.

Requests are made to the service responsible for testing, using the attached Form 8 in which the applicant, group leader, and room manager's names, along with the number of the incubator housing the line, are entered. The test results are sent to the three people listed above. The service assigns an identification number to each cell line of each user, which must be referred to by the users in subsequent controls.

It is appropriate to conduct tests in the following circumstances:

- before storage in liquid nitrogen;
- on all previously untested cell lines stored in liquid nitrogen;
- following changes in cell characteristics;
- in case of problems with result reproducibility;
- every week for 2 to 3 weeks following contamination of an incubator. To be definitively negative, a

cell line must not show infection for at least two successive tests;

- on new cell lines. It is good practice to assume that any cell line can be a possible source of mycoplasma contamination. For this reason, new cell lines lacking adequate certification must be grown in isolation and tested for mycoplasma. Only after the culture shows negative results can it be transferred to mycoplasma-free environments;
- on cell derivatives. It is good practice to buy cell derivatives (hormones, immune stimulators, sera, virus stocks) which are certified mycoplasma-free. It is also necessary to verify the absence of mycoplasma in laboratory-produced materials (master or control cell cultures, vaccines for human and veterinary use, etc.).

### Care

Because caring for cells infected by mycoplasma takes time, is expensive, and has no guarantee of success, it is preferable, when possible, to eliminate the infected line and acquire fresh cultures of the same cell line. In case of contamination:

- dispose of replaceable cell cultures, checking cryopreserved cultures and cell derivatives;
- quarantine and begin treatment of irreplaceable primary cell cultures. For each cell line, decide with the responsible service whether to initiate treatment with antibiotics and specific treatment details;
- check for infection when the treatment is completed. If the results are negative for two successive tests, the culture can be expanded, frozen, and tranferred to mycoplasma-free CC Lab rooms. If the treatment has not been effective, the Tutor decides whether or not to continue the experimental activity, with the restriction of working only in the nonmycoplasma-free rooms of the CC Lab.

## 5. FORMS

Form 1 Cleaning Shifts
Form 2 Cleaning Supervision
Form 3 Temperature Control
Form 4 Incubator Maintainence
Form 5 Incubator Calibration and Filter Changes
Form 6 Hood Reservation
Form 7 Waste Removal
Form 8 Mycoplasma Test Requests

## 6. SUPPORT SERVICES

**Health and Safety Office** **Person in charge …….. Telephone ……**
For solid waste containers, sharp waste containers, and liquid waste tanks.

**Technical Support Services** **Person in charge …….. Telephone ……**
For incubator $CO_2$ and temperature calibration, incubator filter changes. The hood intake flow checks and filter replacement are supported by an outside company. They should be contacted in case of instrument malfunctions, and they can intervene directly or make use of external services to solve the problem.

**Materials Management Service** **Person in charge …….. Telephone ……**
For materials furnished by the Institute.
**Contamination Monitoring Service** **Person in charge …….. Telephone ……**

For test requests and treatment instructions.

## 7. AUTHORS AND REVIEWERS

The following groups have contributed to the edition for publication (edition 0):

### 7.1. DRAFTING GROUP

**qPMO-WP1, IGB-CNR Naples**

Giuseppina Lacerra
F. Anna Digilio
Adriano Barra
Giovanna L. Liguori

**VQ consultant**

Antonella Lanati

### 7.2. COLLABORATORS

**IGB-CNR, Naples**

Daniela Alfano, Antonio Baldini, Pasquale Barba, Giancarlo Bellenchi, Annalisa Fico, Stefania Filosa, Paola Franco, Francesca Fusco, Sergio Rossi.

### 7.3. REVIEWERS

**qPMO**

Marta Di Carlo, Antonella Bongiovanni (IBIM, CNR)
Gianni Colotti (IBPM, CNR)
Annamaria Cirafici, Anna Mascia, Annamaria Kisslinger (IEOS, CNR)

**External Reviewers**

Federica Gemignani (Department of Biology, University of Pisa)
Maria De Angioletti (ICCOM-CNR and Tuscany Tumor Institute, Florence)