POLITECNICO
MILANO 1863

BIOLOGY LABS MANUALS

Dept. Chemistry, Materials and Chemical Engineering “G. Natta”
Building 6, Politecnico di Milano

Version: October 2019

GENERAL INDEX

PREFACE

The present "BIOLOGY Labs- Manuals" describes the risks, the directives for the safety, the instruments and the experimental protocols of the BIOLOGY LABS facility.

- *Working facilities:* The working area named "Biology labs" is composed by two adjacent units: the "TechnoBiology" labs and the "Mechanobiology" labs. The TechnoBiology labs are made of two laboratories: "ATHENA", the microbiology laboratory for microbiota and bacteria culturing; "MINERVA", the cell biology laboratory for cell culturing and manipulation. The MechanoBiology labs are made of three laboratories: Mechanobiology lab, LuCid lab and Nichoid lab.

- *Biosafety Level:* BIOLOGY LABS are classified as basic laboratories (level of biosafety 2). The actions to fulfill the legislation guidelines/requirements for Biosafety Level 2 (BL2) laboratory setting/classification are described in the Legislative Decree number 81/2008 Title X (integrated with Legislative Decree 106/2009 and ensuing amendments) "Testo unico per la sicurezza sul lavoro" (http://lavoro.gov.it/documenti---e---norme/studi---e--statistiche/Documents/Testo%20Unico%20sulla%20Salute%20e%20Sicurezza%20sul%20Lavoro/Testo---Unico---81---08---Edizione---Giugno%202016.pdf), where control measures for biological risk are defined in the Annex XLVII.

- *Safety procedure-biological agents:* The access and manipulation of biological samples in the above mentioned working area is strictly controlled and allowed only to biological samples that require a BL2 (or lower) laboratory setting. Any activity not explicitly referred to laboratories with a higher level of biosafety (3 or 4) must be considered as referred to the activities normally performed in BIOLOGY Labs.

- *Safety procedures-information:* Two Laboratory Manuals, named Biology labs Manual: part I and II, where BL2 safety and experimental procedures are described in detail, have been finalized and will be regularly revised/implemented in accordance with the normative. The Biosafety procedure Manual (Manual Part I) describes the main guidelines to manage the biohazard related to the cell/microbiological practices, in particular: (a) classification criteria of biological agents and laboratories; (b) protective equipment to be used by the personnel in the labs; (c) good laboratory practices; (d) procedures for biohazard waste treatment. The Assays and experimental procedure Manual (Manual Part II) describes the experimental methodologies to be followed and the description of all the equipment included in the laboratories. The two Manuals complements, and does not replace, each other.

- *Safety procedures- formation:* Both above-mentioned manuals are provided and discussed with all the lab personnel prior they start the lab activities. The access to the laboratory is restricted and allowed only to personnel previously trained on the biohazard associated with BL2 materials handling. Before accessing the laboratories, it is mandatory to participate to the appropriate Safety Courses. Before starting every activity, it is mandatory to read all the safety datasheets of the chemical/biological reagents required for the experiments.
  The Emergency Procedures and Rules described in this manual are presented in the aforementioned Safety Courses. They must be considered as an integral part to complete the provisions of the Dept. Chemistry, Materials and Chemical Engineering "G. Natta", Politecnico di Milano, where BIOLOGY Labs are located. Operators must discuss, plan and approve each activity with the Safety officer and the Lab Manager in duty. Working activities in BIOLOGY laboratories must be indicated on

TechnoBiology/MechanoBiology reserved areas. On the reserved areas, it is also possible to reserve equipment and space for such activities.

- *Safety procedures-vigilance:* Politecnico di Milano, in accordance with the above mentioned decree and with the Legislative Decree 363/1998 "Regolamento recante norme per l'individuazione di particolari esigenze delle università e degli istituti di istruzione universitaria ai fini delle norme contenute nel d.l. 19.09.94, n.626, e successive modificazioni ed integrazioni" (http://www.gazzettaufficiale.it/eli/id/1998/10/21/098G0414/sg;jsessionid=Ou4ATnv6cCX0LZdYrEQZuw__.ntc-as3-guri2b) has appointed a person in charge of monitoring the compliance of the lab activities and personnel working practices with the BL2 requirements.

  In case of emergency, contact the Lab Managers on duty, the Safety officers (RADRL *: Responsible for teaching activities and research in the laboratory*) and the switchboard immediately. The mobile numbers of the Lab Managers and the Safety Officers (RADRL) are specified at the entrance of each unit of the BIOLOGY LABS. In the event of a failure to respond, call the emergency services independently and then inform the switchboard.

Emergency contact information:

Politecnico di Milano switchboard - Leonardo Campus (inside): +39 02 2399 3000

Department CMIC switchboard - Leonardo Campus (inside): +39 02 2399 3200

Safety officers (RADRL)

Prof. Manuela Raimondi (MechanoBiology): +39 02 2399 4306

Prof. Carmen Giordano (Minerva, Athena): +39 02 2399 3122

Department/Institution Safety personnel

Lucio Ogliani: +39 02 2399 3233

Mirvana Lauria: +39 02 2399 3066

Oscar Bressan: +39 02 2399 3210/3262/3246

Claudio Brambilla: +39 02 2399 3202

Poison Control Center, Niguarda Hospital: +39 02 6610 1029

European Emergency Unique Number (NUE: Numero Unico di Emergenza europeo): 112

or, alternatively, the following numbers:

Ambulance: 118

Carabinieri: 112

Police: 113

Firefighters: 115

GENERAL RULES

Before entering the laboratory

Learn the name of the Safety Officer on duty and create **a new contact "Safety Officer" with his/her name and telephone number on your cellular phone before entering the laboratory**. Always keep you cellular phone in your pocket in the laboratory. Use of the cellular phone is authorized **ONLY** in case of emergency.

Garments for general protection

1. Before starting any activity, it is mandatory that you wear a laboratory coat, tie your hair up, wear eye shields if you don't already wear glasses, and disposable gloves.
2. The gloves must cover the lab coat cuff.

Emergency procedures

1. There are two emergency exits: for Mechanobiology Labs, the first one is the main entrance of the laboratory and the second one is at half the laboratory length, on its side; for Technobiology labs the first one is in Athena lab close to the main entrance and the second one is in Minerva lab, opposite to lab main entrance.
2. In case of any emergency that you can handle without putting your safety at risk (such as power failure, malfunctioning of an equipment or injury of a colleague) immediately, call the Safety Officer for assistance.
3. In case of any emergency that you cannot handle without putting your safety at risk (such as a fire, spilling of dangerous agents, intoxication of a colleague) leave the laboratory as soon as possible through the safety exit nearest to you. Close the laboratory door after leaving, reach a safety distance and call the Safety Officer for assistance.
4. Never put your safety at risk to assist others, you are not trained to do so. In case of any emergency, you must call the Safety Officer.

Laboratory rules

1. In the laboratory it's strictly forbidden to smoke, light flames, drink, eat, store alimentary products in the refrigerators/freezers and any other place.
2. It is forbidden to wear personal garments, which do not cover the body entirely.
3. Never touch your eyes and mouth with your hands.
4. Label any sample with: 1) name and surname, 2) date, 3) content, before storing it in the refrigerator/freezer, or any other place.
5. Manipulate biological materials only inside the biological cabinet.
6. Use solvents or toxic materials only inside the chemical cabinet.
7. Never block the access to doors, windows, and emergency exits with equipment or personal belongings.

Waste disposal

1. Never throw waste in the paper bin or in the sink.
2. Never take waste out from the laboratory.
3. In the laboratory, you must follow the specific procedures for waste disposal. Solid waste must be disposed in the biohazard waste container.
4. Liquid waste must be disposed either in the biohazard liquid waste container or in the chemical hazard waste containers.
5. Sharp objects, such as needles or scalpels, must be disposed in the relevant container.

How to end activity and leave the laboratory

1. The procedure to exit the laboratory is the following.
2. Empty the biological safety cabinet and put in order all the materials. Connect the pipette controllers to the charger. Clean the biological safety cabinet with a disinfectant do not use corrosive detergents such as bleach. Close the front panel and shut down the fan and the light.
3. Empty the chemical safety cabinet and put in order all the materials. Clean the chemical safety cabinet with a disinfectant do not use corrosive detergents such as bleach. Close the front panel and shut down the cabinet.
4. Empty the water bath and put in order all the materials. Turn the water bath off.
5. Remove the gloves using the correct procedure and place them in the biological solid waste bin.
6. Wash your hands thoroughly using a disinfecting soap.
7. Now you can safely leave the laboratory.

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MANUAL part I: BIOSAFETY PROCEDURES

BIOLOGY LABS

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INDEX MANUAL PART I: BIOSAFETY PROCEDURES

1. INTRODUCTION

As a part of the long-standing debate on safety and prevention of risks related to working activities, the ***Legislative Decree No. 626/94*** (and following modifications or additions) defined the precautions to be taken in working environments. The ***Legislative Decree No. 363/98*** adapted the rules of the Legislative Decree No. 626/94 to universities and institutes of higher education, while the ***Legislative Decrees No. 91/93*** and ***No. 206/01*** referred to the use of genetically modified microorganisms.

The current rules are contained in the ***Legislative Decree No. 80/81*** (and subsequent additions), that fuses and replaces the previous ones. The legislation considers the protection of workers from biological risks in its own complexity, proposing the setting of safety and prevention on a new basic philosophy. Researchers (and workers in general) are not passive beneficiaries, but authors and direct responsible for their own safety. They are required to deeply know and control their working environment, cooperate and actively participate to the implementation of safety procedures. Aiming at a new organization where knowledge and mastery of operating systems are translated into certainty of the results and research quality, the legislation forces workers to continuous information and training. It states that concrete results can be achieved only by systematic and planned activities, not by occasional sporadic interventions due to contingent safety needs.

1.1 Biological agents: definition and classification

A "**microorganism**" is defined as "any cellular or non-cellular microbiological entity capable of replicate or transfer genetic material, including viruses, viroids, animal cells and plant cells in culture".

"**Cell cultures**" are the result of *in vitro* growth of cells from multicellular organisms.

A "**biological agent**" is defined as "any biological organism (also genetically modified), cell culture or human endoparasite which could cause infections, allergies, intoxication". **Biological agents** are classified into four groups (Group 1, 2, 3 or 4), according to a hazard criterion and the prevailing conditions in the geographical area under consideration.

In particular, some of the parameters under consideration are:

- infectiousness (the capacity of the microorganism to penetrate and multiply in the host);
- pathogenicity (its ability to produce a disease after infecting);
- transmissibility (the capacity of the microorganism itself or a host microorganism to be transmitted from an infected to a susceptible subject;
- neutralizability (the availability of effective prophylactic or therapeutic measures to prevent or cure any disease).

The classification includes only agents that can cause infectious diseases in humans, and the consequent effects have been evaluated exclusively on healthy subjects, in the absence of particular or recurrent situations. In the case of the biological agent under examination cannot be attributed unequivocally to one of the four groups, it must be classified in the highest risk group.

The list DOES NOT include genetically modified agents.

Biological agents of Group 1 (no risks or low individual/collective risks)	An agent that is unlikely to cause diseases in humans or animals.
Biological agents of Group 2 (moderate individual risks, limited collective risks)	A pathogen that can cause diseases in humans or animals, but that is unlikely to represent any serious dangers for users, the community, animals (e.g. livestock) and environment. Laboratory exposures can cause diseases, but effective treatments/preventive measures are available and the risk of spreading is limited.
Biological agents of Group 3 (high individual risks, low collective risks)	A pathogen that usually causes serious pathologies in humans or animals and represents a serious risk for workers. It hardly propagates to the community.

	However, effective therapeutic and preventive measures are available.
Biological agents of Group 4 (high individual and collective risks)	A pathogen that normally causes serious pathologies in humans and animals, represents a serious risk for workers and can spread quickly to the community. In general, effective therapeutic or preventive measures are not available.

Table 1: Classification of biological agents

Annex A shows the list of biological agents.
Annex B gives the classification criteria and guidelines to work with genetically modified microorganisms.

1.2 Biosafety laboratories: measures of containment and equipment

Depending on the risk group of microorganisms used, laboratories need adequate levels of containment. According to their design specifications, construction details and available safety equipment, they are divided into:

- basic laboratories - biosafety level 1 (suitable for microorganisms of group 1);
- basic laboratories - biosafety level 2 (suitable for microorganisms of group 2);
- safety laboratories - biosafety level 3 (suitable for microorganisms of group 3 or large volumes/high concentrations of microorganisms of group 2);
- laboratories of maximum safety - biosafety level 4 (for microorganisms of group 4).

The barriers of containment are differentiated into two categories:

- primary barriers of containment (biological safety cabinets, safety mixers, safety containers for centrifuge, fermenters, various closed containers);
- secondary barriers of containment (floors, walls, ceilings, doors, differential pressures, exhaust air filters, and any equipment to treat waste materials).

The more effective the primary barriers are, the less necessary the secondary ones are; in any case, the observance of safety laboratory procedures must never be ignored (good microbiological technique, GMT).

Laboratory	**Risk group of microorganisms**			
	1	**2**	**3**	**4**
Biosafety Level	biosafety level 1 (basic laboratory)	biosafety level 2 (basic laboratory)	biosafety level 3 (safety laboratory)	biosafety level 4 (maximum safety laboratory)
Examples of Laboratories	Basic training	Primary health services; hospitals of primary level; analysis laboratory; training laboratories for public health	Special diagnosis	Working units with dangerous pathogens
Laboratory Procedures	Good microbiological technique	Good microbiological technique; protective clothing; symbols of biological risks on the door	As for level 2; special clothing; controlled access; controlled airflow	As for level 3; entrances with sealed rooms; mandatory shower at the exit;

				special precautions for waste disposal
Safety Equipment	None - you work on standard counters	Standard counters, class I or II biological safety cabinets; filtered air at the outlet	Class II or III biological safety cabinets; other primary barriers of containment for all activities; filtered air at the inlet and outlet	Class III biological safety cabinets; pressurized suits; autoclaves with double opening; filtered air at the inlet and outlet

Table 2: Classification of biosafety levels of laboratories

1.2.1 Biosafety laboratories: measures of containment

The measures containment for the working area must be defined according to the nature of the activities, the risk assessment and the group of the biological agents in use.

Measures of containment		Biosafety levels of laboratories			
		1	2	3	4
Safety systems (fire protection, emergency electrical system, showers, eye-washing equipment, first-aid devices)		yes	yes	yes	yes
Direct control of the local health authority		no	no	no	yes
Emergency program with intervention by local health authorities, national authorities, public emergency services		no	no	no	yes
Isolation of the working area from other areas of the building		no	no	preferable	yes
Ventilation	**Incoming airflow**	no	preferable	yes	yes
	Mechanical, from the central system	no	preferable	preferable	yes
	Mechanical, independent	no	no	preferable	yes
Working areas: air filtration at the inlet/outlet by particle filters (HEPA)*		no	preferable (input)	yes (input/output)	yes (input/output)
Cabinets: air filtration at the outlet by particle filters (HEPA)*		no	yes	yes	yes
Smooth and easily cleanable surfaces (walls, ceilings, floors, working benches)		yes	yes	yes	yes
Surfaces impermeable to liquids, resistant to acids, alkalis, solvents, and disinfectants		yes	yes	yes	yes
Water supply separate from drinking water		yes	yes	yes	yes
Water supply preventing return flows		no	preferable	yes	yes
Access restricted to authorized people		no	preferable	yes	yes
Rule of "two people"		no	preferable	yes	yes
Wearing of laboratory clothing		no	preferable	yes	yes
ventilated positive pressure suits Chemical shower for decontamination (at the exit)		no	no	no	yes, if necessary
Total change of clothing (at the entrance) Mandatory shower (at the exit)		no	no	no	yes

Table 3 (to be continued): Measures of containment in biosafety laboratories

Measures of containment	Biosafety levels of laboratories			
	1	2	3	4
Decontamination of laboratory clothing before washing	no	preferable	yes	yes
Signal of biological risk (at the entrance and on the doors of the systems for the conservation of infected materials (see page 29, Figure A)	no	yes	yes	yes
Name of the Laboratory Manager on the signal of biological risk	no	yes	yes	yes
Indication of the type of biological agents in use on the signal of biological risk	no	no	yes	yes
Fire doors	yes	yes	yes	yes
Doors equipped with an inspection window (or other devices allowing to see people inside the laboratory)	no	preferable	preferable	yes
Doors closing by themselves, with the possibility of locking	no	no	yes	yes
Double door entry	no	no	yes	yes
Hermetically sealed areas with a shower	no	no	no	yes
Sealed windows	no	no	yes	yes
Washbasins with running water in the laboratory	yes	yes	yes	yes
Working areas with negative pressure (with respect to atmospheric pressure)	no	no	preferable	yes
All necessary equipment is available in the laboratory	no	no	preferable	yes
Handling of infected materials in safety booths, insulators or suitable containers	no	preferable	yes	yes
Biological safety cabinets	no	class I or II	class II, preferable class III	class III
Safe deposits for biological agents	no	yes	yes	yes (watertight doors)
Safe deposits for reagents, radioactive materials, compressed gases	yes	yes	yes	yes
Washbasins with running water operated by foot or elbow at the exit of the laboratory	no	no	yes	yes

Table 3 (to be continued): Measures of containment in biosafety laboratories

Measures of containment	Biosafety levels of laboratories			
	1	2	3	4
Specific procedures for disinfection	no	yes	yes	yes
Equipment and procedures for waste disposal	no	yes	yes	yes
Availability of autoclaves	yes (building)	yes (building)	yes (laboratory, double opening preferred)	Yes (laboratory, double opening)
Incinerators for waste disposal	no	preferable	yes	yes (on site)
Treatment of wastewater	no	no	preferable	yes

Table 3: Measures of containment in biosafety laboratories

***(HEPA):** "High Efficiency Particulate Air". High efficiency dry filtration system (minimum 99.97%, maximum 99.99% with a particle size of 0.3 µm) that allows the protection of the environment from spilling microorganisms. They consist of sheets of glass microfibers folded several times to increase the filtering surface.

1.2.2 Biosafety laboratories: safety equipment

The most frequent ways of transmitting biological agents during laboratory activities are:

- incidental ingestion (oral transmission);
- inoculation (incidental punctures and wounds with sharp objects);
- contamination of the skin (or mucous membranes).

The risk of infections can be minimized by equipment, equipment safety and safe laboratory practices.

Equipment	Avoided risks	Safety properties
Biological safety cabinets (Class I)	Aerosols and spills	Ventilated cabinet, with front opening, designed to protect the operator by an airflow at the inlet, filtered at the outlet by a HEPA filter
Biological safety cabinets (Class II)	Aerosols and spills	Ventilated cabinet, with front opening, designed to protect the operator, the products inside the cabinet and the surrounding environment, characterized by an airflow at the inlet (with filtration of both the intake air and the expelled one). They are distinguished in: IIA (recirculating 70% of the air), suitable for microorganisms of groups 2 and 3 (low to moderate risk), small amounts of toxic chemical agents and trace of radionuclides; IIB1/IIB2 (recirculating 30% of the air), suitable for greater amounts of toxic, volatile or radioactive substances.

Table 4 (to be continued): Safety equipment

Equipment	Avoided risks	Safety properties
Biological safety cabinets (Class III)	Aerosols and spills	Ventilated cabinet with the maximum level of containment. It is totally closed, airtight, kept at negative pressure, with inlet air filtered by HEPA filters and outlet air filtered by two HEPA filters in series. Working is possible by rubber sleeve gloves attached to the cabinet. They are used to work with microorganisms of group 3 and 4 and provide a total barrier between the operator and the working area
Sealed containers for centrifuges	Aerosols, leaking and spills	Sealing has to be guaranteed Autoclavable
Sealed containers to collect/transport infected Materials	Aerosols, leaking and spills	Sealing has to be guaranteed Durable Autoclavable
Autoclave	Infected materials	Effective sterilization by heat
Screw cap bottles Resealable sealed containers	Aerosols and leaking	Effective containment
Protection of the vacuum circuit	Contamination of the laboratory vacuum system with aerosols and excess liquids	Cartridge filter (pore size of 0.45 µm) to shield from aerosols. Disinfectant for excess liquids in the collection bottle

Table 4: Safety Equipment

1.3 Good laboratory practice

Devices and procedures for individual protection (DPI)

Before starting experimental activities, it is necessary to identify the suitable Personal Protective Equipment (PPE). The term PPE indicates any equipment to be worn by workers to reduce one or more risks. After using, they must be stored in the reserved area of the laboratory and immediately replaced in case of damage or breakdown.

Before starting experimental activities, it is necessary to identify the suitable individual protection to be worn. The main PPEs are:

- **Safety glasses:** to protect the eyes from splashes/sprays of biological and chemical agents; shock resistant, they must also be worn over the glasses;
- **Face shields:** to protect the whole face when there is the risk of spills; easily removable in case of accident;
- **Specific safety glasses:** to be worn when working with ionizing radiations (e.g. UV radiation, laser beams);
- **Masks and/or respirators:** to be used when there is the risk of aerosol production;
- **Disposable gloves:** to be always worn before performing any laboratory activity with biological or chemical materials;
- **Heat resistant gloves:** to be worn when using stoves, ovens, mittens or other hot devices/equipment;
- **Cryogenic gloves:** to be worn where when using freezers, deep-freezers and liquid nitrogen;
- **Cut resistant gloves:** to be worn when cutting objects or when there is the risk of breaking glass objects;
- **Gloves and lab coats resistant to specific products:** to be worn when using or transferring infected (or potentially infected) materials.

Protective clothing from biological agents

Protective clothing must be chosen taking into consideration the experimental activities. Protective clothing must be and worn while exposing to biological agents. Such clothing must not only be comfortable but also protect exposed anatomical parts (which may include the base of the neck, the torso, the arms, and the legs).
Protective clothing composed of several parts must guarantee protection along the closure parts and in all supposed working positions; in general, it must cover at least the knee.

- For biological agents in group 1: the use of a laboratory coat with long sleeves is advisable;
- For biological agents in group 2: the use of a laboratory coat with long sleeves with elastic cuffs adhering to the wrists is required to prevent exposure of the inside part of the arms. Alternatively, it is possible to wear a jacket (with long sleeves and elastic cuffs) and pants;
- For biological agents in group 3: the use of a full laboratory suit with long sleeves and closing on the back is required; if necessary it is possible to wear a water-repellent overcoat in TNT (non-woven fabric);
- For biological agents in group 4: the use of a full laboratory suit with long sleeves and closing on the back is mandatory; if necessary it is possible to wear a water-repellent overcoat in TNT;
- All protective clothing must be periodically (or in case of accident) cleaned and the owner's name must be clearly indicated with a permanent marker.

Disposable Gloves

They can be made up of latex or vinyl (vinyl gloves must be preferred because they do not cause allergies).

- Gloves should be removed (and not reused) when finishing the specific laboratory activity. Before wearing gloves, it is necessary to check their integrity and make sure your hands are dry and clean;
- If necessary, use sterile disposable gloves.

The collective protection devices available are:

- **Chemical cabinets**, with adequate levels of containment to be used when working with chemicals (even though moderately toxic);
- **Laminar flow cabinets**, suitable for the manipulation of microbiological agents (in laboratories of microbiology);
- **Detection system in case of gas leaking**;
- **Explosion-proof screens and protections**, to be used when operating with pressured atmosphere, depression or exploding agents.

The emergency devices and systems available in the corridors where the laboratories are located (or, in addition, in the laboratories themselves) are:

- Emergency showers and eye showers;
- Fire extinguishers;
- Alarm systems.

1.3.1 Good microbiological technique

The "*Good microbiological technique*" relates to the process of organisation and the conditions in which laboratory studies are planned, performed, monitored, stored and disseminated.

General rules:

- Only people specifically authorized and informed about the potential risks are allowed to access laboratory working areas. However, access to the laboratory is restricted to the discretion of the Lab Manager;
- Children are excluded from laboratory working areas;
- While working, laboratory doors must be kept closed;
- Smoking, eating, drinking, keeping food or tobacco, chewing, using cosmetics, applying or removing contact lenses is forbidden in areas deputed to the storage or manipulation of infected materials;

- Do not wear wristwatches, jewels, open shoes, garments that can be in the way (e.g. scarves, fringed clothing, etc.) or that may be hazardous when working (e.g. fire, shocks, reversal of reagents and glassware);
- Tie long hair (keep hair away from the face);
- Prefer using glasses instead of contact lenses;
- During laboratory activities it is always necessary to wear the required protective clothing (coats/suits); such clothing musts be worn only in the areas of containment and never out of the laboratory;
- When handling biological liquids or any other material from human or animals wear disposable gloves, glasses/face shields, protective clothing and use the biological safety cabinet;
- Spray non sterile disposable gloves with 70% ethanol (v/v) before starting working in the laboratory, before operating in biological safety cabinets or before opening the incubator;
- Before starting working, check that all equipment is perfectly working, that no alarms are on, that the levels of gas tanks and liquids are optimal;
- If necessary, wear safety glasses, face shields or other devices of individual protection to protect eyes and face from splashes, aerosols or sharp objects;
- Technical procedures must be conducted in such a way to minimise aerosol and droplet production: any manipulation involving the risk of aerosol production musts be carried out in biological safety cabinets after wearing the suitable protections;
- Avoid touching the mouth and eyes during laboratory activities;
- Do not bring any object to the mouth, do not dampen the labels by licking;
- Pay attention when using sharp or pungent objects. If possible, restrict their use;
- Use heat-protective gloves when handling hot materials and equipment (e.g. after sterilization in autoclave) and cryogenic gloves when handling materials in freezers or liquid nitrogen;
- Comply with hygienic requirements, e.g. wash your hands frequently: before starting working; after contamination; immediately after removing gloves; at the end of the work and before leaving the laboratory;
- Do not touch the door handles and other objects in the laboratory with the same gloves used for the manipulation of potentially infected materials;
- The laboratory must be kept clean, in order, and free of any object not required to work;
- Decontaminate working surfaces (e.g. benches, cabinet tops) and instruments at the end of activities; at the end of the working day and after every possible shedding/spill of potentially hazardous materials with suitable disinfectants;
- Keep all specimens properly labelled in the most appropriate containers and conditions (e.g. temperature, humidity). The label has to contain the indication of: date; owner's name; biohazard or other risks, if present) and be written with permanent markers;
- All reagents must be permanently marked and stored at the conditions recommended by the producers. The label has to contain the indication of: date of first use/reception; user's name and be written with permanent markers;
- All cultures, biological samples and other potentially infectious liquids to be disposed, must be placed in sealed containers and decontaminated with disinfectants before disposing with laboratory waste;
- After contact with biological samples, glassware and heat-resistant reusable materials must be autoclaved before washing. Heat-sensitive materials must be placed in special containers with disinfectants for 18-24h before washing;
- Remove the protective clothing and gloves when leaving the laboratory and store in the dedicated areas inside the laboratory;
- After using, gloves must be aseptically removed and the hands have to be washed according to the recommended procedure;
- Spilling of infected liquids, accidents and evident or suspected exposure to infected materials, must be immediately notified to the Lab Manager, that will record the accident;
- Do not take out of the laboratory area any object used during the activities or that has been in contact with potentially hazardous/infected materials.

Hand washing

Washing your hands in case of accidental contact with biological liquids; before and after wearing gloves; before and after eating; after using the toilets is mandatory.

- Open the taps with disposable paper, if there are no washbasins with foot or elbow taps;
- For proper washing procedure, remove bracelets, rings, wristwatches (note: it is not allowed to wear these objects while working in the laboratory), soap hands carefully (fingers, palms, back, wrists, fingernails) for at least 10 seconds (use liquid soap, do not use solid soap), rinse with tap water in a complete and accurate way; dry with disposable paper;
- Only in special cases (e. g. presence of organic liquids, damage to the gloves during risky procedures or if antiseptic cleaning is necessary for highly risky procedures or sectors) wet your hands with antiseptic liquid (fingers, palms, back, wrists, and nails) for at least 30 seconds, then washing as previously described.

Removal of Disposable Gloves

Disposable gloves must be removed as follows:

1. Remove the first glove by knocking it over, starting from the wrist to the fingertips, collecting in the other hand still protected by the glove;
2. Remove the second glove in the same way by introducing the exposed hand between the skin and the inside of the glove; overturning to close the first glove inside the second one;
3. Remove the gloves in the appropriate container;
4. Wash your hands thoroughly.

1.3.2 How to use instruments and equipment

This section completes without replacing section "*Good Laboratory Practice"*, related to the criteria to be followed to choose the suitable PPE and the rules to be observed during laboratory activities.

Hypodermic needles

Risks: accidental injection of infected liquids; aerosol formation during use.

- Use the biological safety cabinet for all operations on infected materials;
- When possible, replace needles with plastic chamfered *cannulae* or prefer syringes with lockable or disposable needles;
- Do not cut the needles;
- Do not recap the needles after use, but store in the container for the disposal of sharp objects;
- Fill the syringe carefully, minimizing the formation of air bubbles or foam;
- Avoid the use of syringes to mix liquids or biological samples;
- Make sure that the needle tip is kept under the surface of the liquid;
- When using a syringe, avoid applying excessive force;
- To avoid spills when extracting a needle from a bottle with a rubber stopper, wrap the needle and cap in an appropriate disinfectant swab and then pull the needle out; then eject both the excess liquid and the air bubbles into a cotton swab moistened with an appropriate disinfectant or in a bottle containing cotton by holding the syringe in vertical;
- Ensure safe preservation and appropriate disposal of used needles.

Bacterial loops

Risks: Formation of spills.

- The ring of the loops must be completely closed and the rod not longer than 6 cm;
- Sterilize the loop with a micro-incinerator (not with a Bunsen beak);
- Disinfect the loop after using;
- If possible, use disposable loops.

Biological Safety Cabinets

Risks: The effectiveness of these cabinets is related to: the airflow; the level of containment; the integrity of HEPA filters. In the case of cabinets of class I and II, the activity is also affected by their positioning with respect to draughts and people movements. For this reason, to avoid turbulence that could interfere with air flows and therefore with their normal operation, they are preferentially placed away from the passage areas and from air currents from doors, windows and ventilation system.

Their efficiency must be checked periodically, in particular: speed and direction of the air flow and suction speed, with the mobile front panel raised to the safety level (maximum 30 cm height); level of containment (e.g. the ability to retain particles released within the working space); penetration factor (linked to the efficiency of HEPA filters).

These cabinets are ineffective against chemical risks and do not protect the operator's hands from spills, breakages or bad working techniques.

It is necessary:

- Before starting the experimental activities, disinfect the working surface by spraying a disinfectant solution and drying with a disposable paper;
- Start the forced ventilation system, let it work for at least 10 minutes and make sure it is working properly, otherwise do not use the cabinet;
- When the cabinet is in use, the glass closure panel must be lowered at least until the level of protection indicated on the cabinet (approx. 30 cm height): during the self-testing, lift the front closure of the panel up to the block to carry out operations inside the cabinet and lower it as soon as possible. Remember that if the closing panel is raised over the security lock there is no guarantee of containment;
- The equipment and materials in the cabinet must be kept to a minimum and placed far from the working area;
- Minimize the movements of non-sterile materials next to sterile material;
- Do not use Bunsen beaks in the cabinet as heat causes imbalances in the airflow and damage the filters. If necessary, use micro-incinerator or sterile loops;
- Do not use flammable substances and solvents under cabinets equipped with gas supply;
- Do not use acids, bases, volatile reagents, corrosive substances inside biological cabinets not specifically intended for this use;
- Do not leave reagents inside the cabinet at the end of activities; if you have not finished and you need to leave reagents inside the cabinet leave the ventilation on;
- If the cabinet is not provided of suction at the height of the working top, do not use substances that trigger the emission of dangerous gases that are denser than air;
- All operations must be carried out in the middle or at the bottom of the working top and be visible from the glass panel;
- The passage of objects or people behind the operator must be reduced to a minimum;
- The operator has to avoid to perturb the air flow by repeatedly introducing or removing the arms from the cabinet;
- Clean any deposit (even though small) inside the cabinet with a disinfectant solution and dry with disposable paper;
- At the end of experimental activities, clean the cabinet with a suitable disinfectant and dry with disposable paper;
- The cabinet fan must be left on for at least 10 minutes after finishing working;
- When finishing working, make sure that the cabinet is perfectly clean, off and closed. Operate the UV light supplied with the cabinet at the end of the working day.

Centrifuges and ultra-centrifuges

Risks: formation of aerosols; spills of infected liquids due to the breaking of centrifuge tubes.

- Follow the rules and suggestions provided by the instrument manual;

- The centrifuge containers and accessories have to be carefully positioned (be sure to have a perfect view while positioning the rotors or samples);
- After inserting the rotor, balance (pairing by weight) the samples before centrifuging. It is possible to use water or, if necessary, 70% (v/v) propanol in water. Do not use salt solutions or sodium hypochlorite because they can corrode metals;
- If available and suitable for the rotor, use watertight centrifuge tubes;
- Secure the sample tubes before placing in the slots;
- Use thick-walled or plastic glass tubes and check their integrity before using;
- Open the centrifuge only after stopping;
- Check if the tubes have not broken before removing from the slots;
- Check if the centrifuge is fully functional. The rotors and tubes should be inspected to ensure that they are not fractured or corroded.

In case of samples containing biological agents of class 2 or potentially contaminated, it is also necessary to:
- Fill and empty the containers in a biological safety cabinet;
- Do not use angle heads except in high speed centrifuges as it has been found leakage of liquids even from intact and closed test tubes;
- Leave at least 2 cm between the liquid level and the edge of the tube, except in ultra-centrifuges and in small prothrombin tubes;
- The containers, rotors and internal parts of the centrifuge must be decontaminated regularly.

In the case of handling samples containing biological agents of classes 3 or 4 (or potentially contaminated), it is also necessary to:
- Centrifuge the materials/samples separately from the others;
- Use sealable centrifugal containers (or safety cups) to house the tubes;
- The tubes must have screw caps and be clearly marked with the class of risk of their content.

Homogenisers and tissue crushers
Risks: formation of aerosols or loss of infected material.

- Use and open the equipment in the biological safety cabinet or in other measures of primary containment;
- Use only laboratory mixers and stomachers;
- Caps and gaskets must be in good conditions and guarantee the sealing;
- Glass shredders must be wrapped in absorbent material and used while wearing gloves: if available, it is better to use poly(tetrafluoroethylene) (PTFE) crushers;
- While using cover the device with a transparent plastic case. When finished, disinfect;
- Before opening a mixer, wait for 10 minutes to allow the aerosols to settle. Cool to condense the aerosols.

Stirrers and shakers
Risks: formation of aerosols, spills, drippings, leakage of infected material.

- Use the equipment in a biological safety cabinet or in another measure of primary containment;
- Use well closed flasks/containers with screw caps, equipped with filters at the openings (if necessary); the caps and gaskets must be in good conditions and guarantee the seal;
- The containers must be preferentially in PTFE, if possible avoid glass;
- While using cover the instrument with a transparent plastic case and disinfect after use.

Sonicators, ultrasonic baths
Risks: aerosol formations and loss of infected material; potential damage to hearing and dermatitis.

- Use the equipment in a biological safety cabinet or in another measure of primary containment;
- While using cover the instrument with a transparent plastic case and disinfect after use;
- Insulate the instrument properly and/or use acoustic protections to protect users from sub-harmonics;
- Wear gloves to protect the skin against both high frequencies and detergents;
- The screens and the external surface of the sonicator must be decontaminated after use.

Refrigerators and Freezers

Risks: possible source of sparks, that could cause ignition of vapours from the conversion of flammable solvents

- During periodic cleaning and disinfection or during decontamination procedures, use a face protection and heavy gloves;
- All stored containers must be clearly identified with the scientific name of the content, the name of the user and the date of preparation. Containers not labelled and/or obsolete will be removed;
- Flammable solutions should not be stored in the refrigerator, unless the refrigerator is explosion-proof: in this case, make a warning on the refrigerator door.

Lyophilizers

Risks: formation of aerosols; leakage of infected material for breakage of the containers used; contamination by direct contact with potentially infected material.

- Use ring seals to completely seal the equipment;
- Use air filters to protect the vacuum lines;
- Use appropriate decontamination methods;
- Use a moisture trap and a metal vapour condenser;
- Check all the glass vacuum containers carefully to verify the absence of superficial nicks;
- Use only glass materials designed for vacuum.

Dryers

Risks: implosion of containers with dispersion of glass fragments and infected material; contamination by direct contact with potentially infected material.

- Place the instrument in a sturdy metal basket.

Electrophoretic cells

Risks: connection of the equipment with electrical cables.

- Place the electrophoretic cells away from water sources;
- Before connecting the electrical cables, make sure that your hands are dry and the instrument is switched off;
- Switch off the instrument before removing or inserting the cover;
- Do not tamper with the safety devices;
- Check the instrument during operation.

Autoclaves

Risks: explosions; burns for the leakage of high-temperature vapours; burns by direct contact with the surfaces of very hot metal; burns by direct contact with the materials in the chamber that are still hot after the thermal cycle.

- Before using this equipment, read the operating instructions carefully;
- Loosen the caps of all containers before autoclaving

- The materials and objects to be sterilized do not have to be placed in close contact with the chamber to ensure free circulation of the steam and easily remove air;
- Plastic bags must be open to allow steam penetration;
- Materials must be placed in small, shallow containers to facilitate air removal and allow a good penetration of the heat;
- An indicator of sterility or a thermocouple must be placed in the centre of the sterilization chamber;
- Check if the water level is optimal. If necessary, add water to the required level;
- When closing the autoclave be sure that the seals are intact, the cover and the valves are correctly closed and the drain valves are not blocked by paper or other loaded materials;
- Check the operation of the autoclave during heating, pressurization and sterilization: a possible malfunction could result in ineffective decontamination, with possible serious inconveniences and risks to operators;
- If the autoclave is not equipped with a safety valve to prevent opening while the chamber is under pressure, at the end of the cycle wait until temperature drops below 80 °C and pressure reaches normal levels. The door can be opened, but left ajar a few millimetres to allow the steam to escape securely and left in such a position for 5 minutes before draining the autoclave;
- Wear heat-resistant gloves and visors to protect your arms, hands, face and neck during the opening of the autoclave and the removal of autoclaved materials;
- The liquid discharge filter on the bottom of the chamber must be removed and cleaned daily.

Compressed gas cylinders
Risks: explosions.

- Check the filling level daily;
- Not properly trained users do not have to touch compressed gas cylinders and valves;
- Do not twirl or drag the compressed gas cylinders, use the cylinder holder trolley to transport compressed gas cylinders;
- Never grasp the cylinders for the valve;
- Avoid any impact of the cylinders against the walls.

Incubators
Risks: infections of cell cultures by bacteria, yeasts, fungi, that can spread easily and quickly within the chamber and sometimes are difficult to eliminate.

- Cultured must be checked periodically by an optical microscope; in case of infection, the culture has to be treated with sodium hypochlorite and eliminated;
- The incubator chamber must be periodically cleaned and disinfected;
- In case of massive infections, the chamber must be cleaned and disinfected.

Pipettes
Risks: ingestion or aspiration of infected liquids; inhalation of aerosols; contamination by spilled liquids.

- Never pipette by the mouth;
- To avoid spilling of liquids, when using automatic pipettes make sure that the power supply is adequate or the battery is charged;
- To reduce the risk of contamination of the pipette boy, avoid wetting the cotton swab placed in the contact area between the pipette and the pipette boy;
- Never blow air into liquids containing infectious agents;
- Do not mix infected solutions by alternating suction/expulsion;
- Do not force liquids out of pipettes;
- Do not use syringes with hypodermic needles as pipettes, but prefer cannulae with blunt needles;

- In case of use of glass pipettes place the container inside the biological cabinet before starting operating; do not use pipettes with cracked or chipped ends; after use, glass pipettes must be immersed in a disinfectant solution for 18-24h before the final decontamination by autoclave.

Micropipettes

Risks: formation of aerosols; contamination by spilled liquids.

- Ensure good sealing of the tips to avoid spilling of liquids by dislocation of the tip;
- Avoid vacuuming the liquids too quickly to reduce the risk of contamination of the pipette itself;
- Never blow air into liquids containing biological agents;
- Do not mix by alternating suction/expulsion with an infected liquid pipette;
- Do not force liquids out of micropipettes.

1.3.3 Interventions of maintenance

An *ordinary* program *of maintenance* must be studied for each equipment defining at least the frequency of the necessary operations and indicating who must carry them out. According to these indications, the interventions will be scheduled and registered by indicating the operation, the date and the operator.
On the same register, all the *extraordinary* operations of *maintenance* and the interventions carried out after failure of laboratory equipment must be reported systematically and precisely. Before maintenance/repair, it is necessary to decontaminate every equipment with a suitable disinfectant solution.

1.4 Safe reception, handling and shipment of infectious materials

Receiving potentially infected materials

- The staff receiving the samples and removing from the shipping packages must be informed about the potential health risks and looked for a qualified assistance in case of breakage or loss of materials;
- Disinfectants must be available;
- Sample packaging must be opened on trays.

Opening glass vials containing lyophilized infected materials

- Any sample with the label "danger of infection" must be opened in a safety biological cabinet;
- Decontaminate the outer surface of the vial;
- Nick the vial more or less halfway of the swab (usually made up of cotton or cellulose);
- Place a glowing iron on the notch to break the glass;
- Gently remove the upper part, treating it as an infected material;
- If it is still above the contents of the vial, remove the cotton swab with sterile forceps;
- To avoid foaming, add the resuspension liquid slowly.

Preservation of samples

- Use suitable containers for samples: glass or plastic, sturdy and able to prevent leaking when properly closed;
- Make sure that there is no leakage outside the container;
- Label the samples clearly to allow their recognition;
- Where necessary, store in plastic bags not closed with staples;
- If samples are suspected of belonging to the risk group 3 or being contaminated by hepatitis B virus or HIV, containers should be identified with a special warning/label of danger of infection;
- Be sure of the integrity of the metal containment gauze capsule: anaerobic containers can explode with dispersal of infected materials.

Storage of vials containing lyophilized infected materials

- Vials must be stored in the gaseous phase above the liquid nitrogen (not immersed in the liquid) or, if possible, in freezers or in solid carbon dioxide (dry ice);
- Wear eye and hand protection when removing vials from the place of storage and refrigeration;

- The outer surfaces of the vials must be disinfected when removing from the place of storing and refrigeration.

Manipulation of blood and other body fluids

The diagnostic work must be carried out in basic laboratory-biosafety level 2, while research and development involving the propagation or concentration of microorganisms must be carried out in basic laboratory-biosafety level 3.

- The samples must be opened in a safety biological cabinet, wrapping the cap with a piece of paper and holding firmly;
- Wear gloves, protective clothing, a plastic apron, safety glasses or a face mask;
- Preferentially use plastic or sturdy glass objects (borosilicate, Pyrex, Duran or similar), taking care to eliminate all objects with cracked or chipped ends;
- Do not use scalpels and knives with unfixed tissue; prefer plastic *cannulae* to hypodermic needles;
- Manipulate slides and samples with tweezers;
- The tubes to be centrifuged must be safely closed. To avoid potential infections with biological agents of class 2 (mandatory with biological agents of classes 3 and 4), it is preferable to use sealed containers;
- Blood and serum must be carefully pipetted, never spilled or pipetted by mouth;
- Waste liquids must be collected in closed containers or discarded by the appropriate drain;
- Used tubes should be dipped in hypochlorite or other suitable disinfectants for at least one night before elimination or decontamination, placed in leak-proof containers and then autoclaved or burnt;
- The worktops must be cleaned with sodium hypochlorite solution at the end of the working day or in case of splashes, spills;
- If possible, disinfect the equipment with glutaraldehyde or hypochlorite at the end of the working activity.

Shipment of materials from and to the laboratory

To avoid accidental loss or spillage during transport, special secondary metal or plastic containers must be used (e.g. trays, boxes). They must be autoclavable or resistant to disinfectants and equipped with supports to keep the samples in a vertical position. The supports must be regularly decontaminated.

Safe shipment of infected samples and materials

Shipment procedures must not affect the samples, they must prevent contaminations of shippers due by improper closure or breakage of the containers, causing the spreading of microorganisms.

The requests vary according to the **class** of biological agents to be shipped:

- **Infected substances:** substances containing viable microorganisms (bacteria, viruses, rickettsial, parasites, fungi, recombinant, hybrids or genetically modified organisms) that are known or reasonably suspected of causing disease in animals and humans. Toxins not containing infected substances are excluded;
- **Diagnostic samples:** human or animal materials that may be excreted or secreted, as well as blood and its components, tissues and fluids that are shipped for diagnostic purposes. Living infected animals are excluded;
- **Biological products:** finished biological products for human or veterinary use, manufactured according to the rules of national and public health authorities and traveling with their approval or license; finished biological products shipped before obtaining the license for research or study purposes, for human or animal use; products for the experimental treatment of animals manufactured according to the rules of national authorities and public health; unfinished biological products prepared according to the procedures of specialized government agencies; living vaccines (animals and humans);
- It is necessary to be informed about current regulations as they are subjected to periodic revision;

- For international shipments, the recipient must consult the national authorities to make sure that the substances can be legally imported and that delivery times are respected; he/she must also obtain the required documents (import permits, accompanying documents, etc.);
- Make arrangements in advance with the carrier to make sure the sample will be received in the necessary time;
- The infected substances and the diagnostic materials must be placed in a three-pack layers: a first waterproof box containing the sample; a second waterproof box containing the first box and absorbent material to absorb all the fluid eventually leaking from the first; and finally a third outer box to protect all the packages from physical-chemical damages;
- Packages containing infected substances must show the label "*infected substance*" (see p. 29, Figure B);
- A copy of the information form for sample identification and description must be taped outside the secondary box; another copy must be sent to the recipient laboratory by airmail (so that workers can identify the sample and decide its manipulation); while a third copy must be kept by the sender;
- If the sample must be transported in liquid nitrogen or not at room temperature, it is necessary that all the packaging is appropriate to storage conditions. Moreover, the primary and secondary packaging must withstand a differential pressure of 95 kPa and a temperature between -40 and 50° C;
- The delivery note must indicate if the substance is perishable.

1.5 Disinfection and sterilization

Disinfection is defined as the inactivation of one or more specific groups of microorganisms, while *sterilization* is the inactivation of all microorganisms.

1.5.1 Chemical Disinfectants

Most disinfectants have toxic effects, so it is necessary to wear gloves, lab coat and eye protection when diluting before use.
"*Dirty situations/conditions*" are those concerning blood shedding or before tissue removal, while "*clean situations/conditions*" are those following the removal of most potentially infected material.

Chlorine (sodium hypochlorite)
It is an active disinfectant against many microorganisms. **A solution for general use must have a concentration of 1 g/L (1000 ppm) of available chlorine; a solution for emergencies involving pathogenic viruses 5 g/L (5000 ppm)**. It is normally available as sodium hypochlorite (for industrial, laboratory or bleach for houseworks) with a concentration of 50 g/L (50000 ppm) to be diluted 1:50 (final: 1 g /L) or 1:10 (final: 5 g/L). Granules or tablets of calcium hypochlorite contain 70% of chlorine available; the resulting aqueous solutions may be cloudy (solutions containing 0.7-1.4 g/L will contain 500-1000 ppm, respectively, while solutions of 7 g/L contain 5000 ppm). It is a very strong and corrosive oxidizing agent for metals. Hypochlorite solutions gradually lose their strength, so they must be prepared freshly.

Sodium dichloroisocyanurate (NaDCC)
It is available in powder containing 60% of available chlorine or in tablets containing the equivalent of 1.5 g/L of available chlorine: 1 or 4 tablets dissolved in 1 liter of water will provide the necessary concentrations.

Chloramine
Chloramine powder contains about 25% of available chlorine and releases chlorine slower than hypochlorites, therefore a higher initial concentration is required to achieve the same effectiveness. It is not inactivated by organic matter as hypochlorites, therefore a concentration of 20 g/L is suitable for both "clean" and "dirty" situations.

Formaldehyde
Formaldehyde (toxic, irritant and suspected to be carcinogenic: avoid contact with eyes, skin and respiratory tract) is a gas active against all microorganisms, except at low temperatures (below 20° C). It requires a relative

humidity of 70%. It is available in the form of p-formaldehyde (flakes or tablets) or formalin (a solution of formaldehyde in water) of about 370 g/L (37%), containing methanol (100 mL/L) as stabilizer. For viral contaminations, use 18.5 g/L formaldehyde (5% formalin in water).

Glutaraldehyde

Glutaraldehyde (toxic, irritant and mutagenic: avoid contact with eyes, skin and respiratory tract) is active against all microorganisms. It is available as a solution at a concentration of 20 g/L (2%), possibly to be activated (with the addition of bicarbonate, making the solution alkaline). The active solution must be used in 2 weeks and eliminated if it becomes cloudy.

Phenolic compounds

They are active against all vegetative forms of microorganisms, but not against spores. Their activity against non-lipid viruses is variable.

Alcohol and alcoholic mixtures

Ethyl alcohol (ethanol) and isopropyl alcohol (2-propanol) have similar disinfectant properties. They are active against vegetative bacteria, fungi, lipid viruses, but not against spores. Their action on non-lipid viruses is variable. For greater effectiveness, they must be used at a concentration of 70% in water: higher or lower concentrations may not have the same germicidal power. They can be mixed with other agents: 70% alcohol with 100 g formaldehyde/L; 70% alcohol with chloramine at a concentration of 2 g/L (2000 ppm of available chlorine).

Iodine and iodine derivatives

Its action is similar than chlorine. Clean surfaces or small amounts of contaminating proteins can be treated with 0.075 g/L (75 ppm) of iodine available. For hand washing, iodine derivatives diluted in ethanol (0.45 g/Ll, 45 ppm of iodine available) can be used. Povidone iodine (Betadine) has a multiple activity and it contains an inhibiting agent. For use it can be diluted 1: 4 v/v with boiling water, it must be prepared fresh and not to be used with copper and aluminium.

Hydrogen peroxide

Thanks to its oxidizing action, it is useful to decontaminate the equipment, but it has not to be used with aluminium, copper, zinc or bronze. The commercial solution (30% in water), must be diluted 1: 5 (v/v) with boiled water, can be used in hot environments, but needs to be protected from light.

Toxicity	Phenols	Hypochlorite	Alcohols	Formaldehyde	Glutaraldehyde	Iodophors
Skin	Yes	Yes	No	Yes	Yes	Yes
Eyes	Yes	Yes	Yes	Yes	Yes	Yes
Lungs	No	Yes	No	Yes	Yes	No

Table 5a: Toxicity of the described chemical disinfectants.

Activities against	Phenols	Hypochlorite	Alcohols	Formaldehyde	Glutaraldehyde	Iodophors
Fungi	Good	Low	Good	Good	Good	Good
Gram + Bacteria	Good	Good	Good	Good	Good	Good
Gram - Bacteria	Good	Good	Good	Good	Good	Good
Mycobacteria	Discrete	Discrete	Good	Good	Good	Good
Spore	None	Discrete	None	Good above 40 °C	Good above 20 °C	Low
Lipid Viruses	None	Low	Low	Low	Low	Low
Non-Lipid Viruses	Variable	Low	Variable	Low	Low	Low

Table 5b: Spectrum of action of the described chemical disinfectants.

Inactivation	Phenols	Hypochlorite	Alcohols	Formaldehyde	Glutaraldehyde	Iodophors
Protein	Low	High	Low	Low	Low	High
Natural materials	Discrete	Low	Low	Low	Low	Low
Synthetic materials	Discrete	Low	Low	Low	Low	Low
Hard Water	Low	Low	Low	Low	Low	Low
Cleaning	Cationic	Cationic	None	None	None	Anionic

T**able 5c:** Inactivating agents for the described chemical disinfectants.

1.5.2 Decontamination of spaces and equipment

Decontamination of spaces and surfaces

Contaminated or potentially contaminated surfaces can be treated with a solution of sodium hypochlorite containing 5 g/L (5000 ppm) of available chlorine or other antiviral disinfectants. Spaces and equipment can be decontaminated with fumigation with formaldehyde (by heating p-formaldehyde 10.8 g/m^3 or by boiling formalin 35 mL/m^3) or formaldehyde solution (by adding the right amount of formaldehyde to two parts of potassium permanganate and then by adding water. Warning: the mixture boils violently and generates formaldehyde gas!). Wear face-filled respirators with air supply, applying the two-people rule.

Seal doors and windows with adhesive tape before releasing the gas. The fumigation must be carried out at room temperature (at least 21 ° C), at a relative humidity of 70%, for at least 8h. It is necessary to ventilate the rooms before accessing: during the execution of this operation or in case it is necessary to enter the room during fumigation, use the face respirators completely covered with air supply, applying the two-people rule.

Decontamination of biological safety cabinets

Class I and II: put the required amount of p-formaldehyde into a container and place on a heating plate inside the cabinet, so that it is visible from the outside. Seal the front glass of the cabinet with adhesive tape then light the plate. Turn off the plate 1 h after the formaldehyde has completely vaporised and leave the cabinet in this condition one night. In the morning, operate the cabinet fan, raise the front glass a few millimeters; after a few minutes open the front glass completely and leave the fan on for 1 h, to completely expel the formaldehyde.

Decontamination of incubators and thermostatic baths

To prevent the growth of microorganisms, clean and disinfect interiors regularly with 70% ethanol in distilled water. During disinfection, do not use sodium azide to prevent the growth of microorganisms, since it forms explosive compounds in contact with some metals.

1.5.3 Sterilization

All contaminated but reusable materials (beaker, bottles, etc.) must be sterilized before washing.
The best sterilization method are high pressure water vapours. There are three types of autoclaves:

- **Gravity autoclaves:** after heating time, they reach 121°C. Their cycle lasts 30' (cycle time). At the end of the cycle, let the internal temperature drop to 80 °C (cooling time) before letting steam escape and opening the lid;
- **Vacuum autoclaves:** they can reach 134 °C and the cycle duration can be reduced to 5';
- **Pressure boilers heated by fuel:** to be used only in case of lack of autoclaves. They are heated by gas or electrically. The cycle is similar than that described for gravity autoclaves.

Autoclave sterilization (pressure gravity circulation) is the best method for all decontamination processes. The use of dry heat, ultraviolet rays, microwaves and ionizing radiation is not recommended due to unpredictable variations in results.

1.6 Collection, decontamination and disposal of infectious waste

Collection and separation

The elimination of laboratory waste, including materials contaminated with human or animal blood; microorganisms (even though genetically modified) and cell cultures; human or animal tissues; human or animal organic liquids (sanitary waste), is regulated by national regulations establishing the procedures for their elimination. Anyone who uses, handles or disposes hazardous biological waste materials is responsible for their proper disposal.
The materials to be wasted must be identified and separated into different categories:

- Uncontaminated waste can be assimilated to normal one;
- Sharp waste must be stored in rigid containers with impenetrable walls, to be filled to the maximum for ¾ and when filled disposed in the container for waste to be incinerated;
- The contaminated material to be autoclaved and then reused must be autoclaved directly without any previous cleaning;
- The contaminated material to be eliminated after autoclaving, must be placed in airtight containers and identified according to a specific code before incineration;
- Anatomical waste.

The European Waste Catalogue includes defined identification codes for dangerous biological materials, including the following:

a) **CER 18.01.03*:** waste of human origin (generic, microorganisms, cell cultures, sharp objects);
b) **CER 18.02.02*:** waste of animal origin (generic, microorganisms, cell cultures, sharp objects).

In the absence of contracts with companies specialized in the transportation and final disposal of waste or in the case that it is foreseen for the class of the laboratory, the provisions listed below must be followed:

Decontamination

Materials for decontamination and disposal must be placed in "biohazard" plastic bags, autoclavable, coloured or identified according to a code indicating whether they should only be autoclaved or subsequently incinerated. Before transporting in the autoclave area, make sure that bags are closed tightly to prevent leakage of infected material.

To ensure sterilization of the contents, open the "biohazard" bags before autoclaving. After autoclaving, put the material to be disposed in biological waste containers.

In general, next to each workplace it is advisable to place small pails in unbreakable material (preferably plastic) containing a disinfectant solution (freshly prepared every day), where the waste material is completely immersed for 18-24h before autoclaving/incinerating. Then the disinfectant must be poured into a special drain and the autoclaved well must be washed before reusing. The choice of the disinfectant depends on the contaminant agent.

Elimination

If allowed by the class of the laboratory, the incinerator is a valid alternative to autoclaving only if: it is controlled by laboratory personnel, it is provided with temperature control and a secondary combustion chamber. The temperature of the primary chamber must be at least 800° C, while the secondary chamber must reach 1000° C. The retention time of the gas in the secondary chamber must be at least 0.5 seconds.

The materials to be removed with incineration, even though already autoclaved, must be transported to the incinerator preferably in plastic bags, placed inside cardboard containers or reusable waterproof transfer containers with airtight lids, which must be disinfected and cleaned before bringing them back to the laboratory. Generally, ashes from incinerators are treated as household waste and removed by local authorities, while autoclaved waste can be removed by incineration away from the laboratory or in authorized landfills.

1.7 Emergency procedures in case of accidents

1.7.1 Laboratory Accidents

In case of accidents, the laboratory must be evacuated and the access forbidden for at least 30 minutes or until the Lab Manager gives the authorization to come back.

Depending on the accident, the procedures must be applied in combination/sequence.

Each incident must be reported promptly to the Lab Manager, who in turn will warn the Prevention and Protection Service.

Broken or spilled cultures: shedding of contaminated material

1. Warn other people in the laboratory, hold your breath and leave the laboratory immediately taking care to close the door;
2. Carefully remove the protective coat and store in a bag suitable for infected material and resistant to autoclaving;
3. Wash your hands and expose your skin with disinfectant soap (preferably based on iodine) and warm water for 15 minutes (possibly under the emergency shower);
4. Wait at least 30 minutes or until the Lab Manager gives the authorization to go back before entering again;
5. Wear cut resistant gloves (if necessary, covered with disposable or single-use sterile gloves), protective clothing (if necessary disposable coat and shoes) and suitably protect the face (with glasses, surgical mask, visor) before disinfection;
6. Crushed or overturned cultures must be covered with absorbent paper. A concentrated disinfectant solution (e.g. povidone iodine or sodium hypochlorite) must be poured in centripetal direction to avoid widening of the contamination area. Leave for at least 30 minutes. The absorbent paper and any broken material can be collected with a scoop (possibly disposable), while glass fragments must be handled with pliers; everything must be stored in a suitable needle-resistant container. Excess liquids must be dried

with absorbent paper soaked in a concentrated disinfectant solution. Disposable paper must be thrown into the container for infected waste;
7. Disinfect the surrounding contaminated areas with a disinfectant solution and dry. The areas must be sprayed with a sodium hypochlorite solution and dry (or clean with absorbent paper soaked in hypochlorite after 15 min of contact). The absorbent paper must be disposed as infected material;
8. In case of contamination of sheets of paper (notes etc.), data must be copied to another sheet and the original disposed as infected material;
9. All reusable materials used for disinfection must be either placed in an autoclavable biohazard bag or dipped for 24h in a disinfectant solution;
10. At the end of each of these operations, wash your hands with soap.

Exposure to biological aerosols

1. Warn other people in the laboratory, hold your breath and leave the laboratory immediately taking care to close the door;
2. Carefully remove the protective coat and store in a bag suitable for infected material and resistant to autoclaving;
3. Wash your hands and expose your skin with disinfectant soap (preferably based on iodine) and warm water for 15 minutes (possibly under the emergency shower);
4. Immediately ask for medical assistance for people exposed;
5. Wait at least 30 minutes or until the Lab Manager gives the authorization to go back before entering again.

Breakage of test tubes containing potentially infectious materials into centrifuges without safety containers

1. If you suspect the breakage of test tubes during running the centrifuge, stop the machine and leave the centrifuge closed for 30 minutes, to allow larger particles to settle. If the centrifuge has already been stopped and opened, the lid must be closed immediately and left closed for 30 minutes. Then follow the instructions as in the point "exposure to biological aerosols";
2. Wear sturdy gloves (made up of thick rubber) and, if necessary, cover with suitable disposable gloves;
3. Use pliers or cotton balls kept with pliers for the recovery of any glass fragments;
4. All broken tubes, glass fragments, containers, accessories and rotor involved in the accident must be immersed for about 24h in a non-corrosive disinfectant (not hypochlorite) or autoclaved;
5. The healthy and plugged tubes may be immersed in disinfectant in a separate container and recovered after 1 h;
6. The interior of the centrifuge must be left in disinfectant solution all night and then disinfected again, washed with water and dried;
7. All used tore ups must be treated as infected waste.

Breakage of test tubes containing potentially infectious materials into centrifugal seal containers

1. Open the container under the biological safety cabinet;
2. In case of breakage, partially close the cap and autoclave the container.

Fires, floods and natural disasters

1. Follow the procedures established by Politecnico di Milano;
2. In the case of a natural disaster, local or national emergency services must be notified of the potential risks within the laboratory buildings;
3. The members of the protective services must enter only with a trained staff member;
4. Cultures and infected materials must be collected in sealed boxes or sturdy disposable bags;
5. The final recovery or elimination must be decided by the security staff according to their knowledge of the local situation.

1.7.2 Injuries: first aid procedures

Call the porter's lodge, explain the dynamics of the event and indicate the specific place where help is needed. In case of no response from the porter's lodge, alert the Lab Manager immediately. Then provide the first aid, if you are able to.

If necessary (in particular if the risk of aerosol formation is suspected) the laboratory must be evacuated and access forbidden for at least 30 minutes or until the Lab Manager authorizes to go back. In any case, as soon as the emergency is over, report the incident to the Lab Manager, who will notify the accident to the Prevention and Protection Service.

APPENDIX 1, Annex A: List of classified biological agents

Biological agents are divided into the following four groups, depending on the risk of infection:

- **Biological agent of Group 1:** an agent that is unlikely to cause disease in humans or animals;
- **Biological agent of Group 2:** an agent that can cause diseases in humans or animals and poses a risk to workers. It is unlikely that it will propagate in the community; effective prophylactic or therapeutic measures are normally available;
- **Biological agent of Group 3:** an agent that can cause serious illnesses in humans or animals and constitutes a serious risk to workers. The biological agent can spread to the community, but effective prophylactic or therapeutic measures are generally available;
- **Biological agent of Group 4:** an agent that can cause serious illnesses in humans or animals, constitutes a serious risk to workers. It may present a high level of risk of spreading to the community; effective prophylactic or therapeutic measures are not normally available.

1. If the biological agent to be classified cannot be uniquely assigned to one of two of the above groups, it must be classified in the highest risk group between the two possibilities;
2. Only agents known to cause infectious diseases in humans are included in the classification. The toxic risks that may be present are indicated alongside each agent. Pathogens from animals and plants that are known not to have any effect on humans have not been considered. In this list of biological agents, genetically modified microorganisms have not been considered;
3. The classification of biological agents is based on their effect on healthy workers. It does not consider the particular effects on workers whose sensitivity could be modified by other causes, such as pre-existing diseases, use of medicines, impaired immunity, pregnancy or breastfeeding;
4. Biological agents not included in groups 2, 3 and 4 are not implicitly included in group 1. For agents including many species that are pathogenic to humans, the list shows only the species that are most frequently implicated in diseases. When an entire genus is mentioned in the list, it is implied that non-pathogenic strains are excluded from classification;
5. When a strain is attenuated or has lost its virulent genes, the level of containment required by the classification of the parental strain is not necessarily applied, unless the assessment of the risk it represents does not require it.
6. All viruses that have been already isolated in humans and are not yet listed in Annex A should be considered as belonging to group 2 at least, unless it is proved that they cannot cause diseases in humans.
7. Agents in Group 3 highlighted by a double asterisk (**), entail a limited risk of infection because they are not carried by the air normally.
8. The measures of containment deriving from the classification of parasites, apply only to the stages of the parasite cycle that can be infectious to humans.
9. The list contains information on biological agents that may cause allergic or toxic reactions. Those for which an effective vaccine is available and those for which the list of potentially exposed workers should be kept for at least ten years.

These indications are:
A: Possible allergic effects;
D: The list of potentially exposed workers must be kept for at least ten years after the ending of the last activity with risk of exposure;
T: Production of toxins
V: An effective vaccine is available

APPENDIX 2, Annex B: Use of genetically modified microorganisms

The legislation regarding the use of genetically modified microorganisms applies to the storage, transport, destruction and disposal of genetically modified microorganisms.
Genetically modified microorganisms are divided into two groups: I and II.

Group I	Receiving or parental organism	not pathogenic
		non-adventitious agent
		proven and extensive experience of safe use or embedded biological obstacles that, without interfering with the optimal development in the reactor or fermenter, confer a limited survival and reproduction capacity, without negative consequences for the environment.
	Vector/Insert	well characterized, its dangerousness is known
		as far as possible, limited in size to genetic sequences necessary for the required function
		It must not increase the stability of the construct in the environment (unless this is a specification of the required function)
		poorly mobilizable
		It must not transfer any resistance to microorganisms, apart from those they can acquire in a known natural way (apart if such acquisition may compromise the use of medicines aiming to control pathogens)
	Genetically modified microorganism	not pathogenic
		in the reactor or fermenter they show the same degree of safety as the recipient or parental organism, but with limited survival and/or reproduction capability, without negative consequences for the environment
		those constructed entirely from a single receiver procariota (including its endogenous plasmids and its viruses) or by a single eukaryotic recipient (including its chloroplasts, mitochondria and plasmids, but excluding its viruses)
		those constructed entirely from genetic sequences of different species which exchange such sequences according to known physiological processes
Group II	microorganisms different from those of Group I	

1. Type A transactions are operations performed for teaching, research, development or other non-industrial, non-commercial and small-scale purposes; Type B operations are those other than type A.
2. Users must take all necessary measures to avoid any negative effect deriving from the use of genetically modified microorganisms on human health and the environment. Therefore, they must provide a preliminary risk assessment to the competent Ministries.
3. The users of genetically modified microorganisms belonging to Group I must apply the principles of good microbiological practice and observe the following rules of safety and hygiene while working:

- Maintain at the lowest technically practicable level the exposure of working areas and environment to physical, chemical and biological agents;
- Apply technical measures of control and, if necessary, integrate with personal protective clothing and equipment.
- Verify and maintain efficient control measures and equipment;
- Take care of staff training;
- If necessary, monitor the presence of viable microorganisms outside the primary physical confinement;
- Constitute special committees for biosafety;
- Prepare and apply internal regulations for staff safety.

4. Before using for the first time genetically modified microorganisms belonging to Group I, users must inform the Ministry of Health and provide these information:

- Name(s) of the person(s) responsible for the contained use of genetically modified microorganisms, including those responsible for supervision, control and safety. Users must also provide information on their education and training;
- Plant location and grid reference;
- Description of the installation sections;
- Description of working activities, classification of the microorganism (s) to be employed (Group I or Group II) and probable amount of microorganisms required;
- Summary of the risk assessment (art. 6 paragraph 2).

5. For type A operations, users of genetically modified microorganisms classified in Group I must prepare a register of the activities, to be kept at the disposal of the Ministry of Health.
6. If the contained use is related to genetically modified microorganisms of Group I, in the absence of a contrary indication by competent authorities, users may start working 90 days after the submitted notification (or less, after agreement with the competent authorities).
7. Users must inform the Ministry of Health and amend the notification in the following situations:

- Changes to the contained use of genetically modified microorganisms might lead to significant consequences in terms of nature and severity of risks;
- Modifications to the category of genetically modified microorganisms;
- Acquisition of new scientific and technological knowledge leading to significant consequences in terms of risks.

8. When an accident occurs, users must immediately inform the President of the Region, the Prefect and the Auditors, and provide the following information:
- Causes of the accident;
- Identity of the genetically modified microorganism(s) released and amount of the release;
- Information to assess the effects of the accident on human health and environment;
- Emergency measures to be taken.

9. Users of genetically modified microorganisms in Group II must choose the containment measures to ensure the protection of human health and environment according to the microorganism(s) and operations to be carried out. Before starting working, they must inform the Ministry of Health, and provide these information:

- Date of presentation or authorisation of the plant notification, as referred to article 7;
- Users' names and their curriculum vitae;
- Receiving or donor microorganisms and, if applicable, host-vector systems;
- Sources and functions provided for the genetic materials;
- Identity and characteristics of the genetically modified microorganism;
- Approximate cultivation volumes;
- Description of the containment measures and other protective measures to be applied, Information on the waste management, treatment and disposal;
- Purposes and duration of the contained use, including expected results;
- Summary of the risk assessment, as referred to article 5, paragraph 2;
- Information requested to predispose the contingency plans, as referred to article 15;
- Proof of the communication of the user notification to the plant's owner (the users and the plant's owner must be different people)
-

Figure A: Example of safety signs (basic combination) for biochemical/biological laboratories

Figure B: Safety sign to be used to ship potentially infectious substances.

POLITECNICO
MILANO 1863

MANUAL part II: ASSAYS AND PROTOCOLS

BIOLOGY LABS

Dept. Chemistry, Materials and Chemical Engineering “G. Natta”

Building 6, Politecnico di Milano

Version: October 2019

INDEX MANUAL PART II: ASSAYS AND PROTOCOLS

Assays and Protocol manual

Part I-INSTRUMENTATION

Version: October 2019

LABORATORY INSTRUMENTATION - GENERAL RULES

For **any** instrumentation in the laboratory, the following rules are generally valid:

1. The copies of the manuals and test certificates of all instruments and equipment mentioned below are available upon request to the senior staff.
2. In case of alarm (audio, visual) of any device, stop using and immediately inform the Safety Officer of the laboratory.
3. The laboratory equipment have forms to be filled in case of scheduled periodic maintenance. The operators are required to record the transactions indicating date, name, surname and signature. The forms must not be removed from their position near the instrument.
4. The laboratory's Safety Officer must agree and approve the cleaning and maintenance operations of the instrumentation.
5. The cleaning and maintenance operations must always be performed just after the instrument has been turned off and plugged off from the electric supply.
6. For cleaning operations, follow as reported in the instrument's manual, by using the indicated detergents and when not available, as a general rule, only use deionized water.
7. For the disinfection, to be performed after the cleaning, use solutions of ethanol 70% V/V in distilled water or isopropanol 60% V/V in distilled water.
8. If an instrument has to leave the instrumentation's laboratory (e.g. for external maintenance), clean and decontaminate the equipment before handing it; moreover, it must also be accompanied by an informing module about the potentially infectious or toxic substances (serum, blood, biological material in general, toxic chemicals) the equipment is exposed to, and the procedures adopted for decontamination.
9. When the laboratory is closed for long periods (e.g. Christmas or summer holidays) power off nonessential equipment and perform their decontamination.
10. All containers in the Laboratory must be labelled in a clear and understandable way to all operators. The labels must include: name of the operator, content description, indications of risk, opening date and term of preservation, information on the storage temperature (+ 4 ° C, -20 ° C, etc.).
11. In case of accidents in the laboratory that causes damage or malfunction to the instrumentation, supplied kit, infrastructure and the furniture is required immediate alert to the laboratory's Safety Officer.

MECHANOBIOLOGY LAB INSTRUMENTATION

AUTOCLAVE

Brand: Golmar

Model: NT 80

Provenance: Lab. Prof. Boschetti, Area 3 of LaBS

Technical assistance:

- Company: Golmar, Milan, phone: 02 38 10 34 56

- Sale representative: Sig. Faravelli, mobile phone: 335 6916327

Year of fabrication: 2008

Maintenance plan: upon notification; annual decontamination

DESCRIPTION

The autoclave allows sterilizing materials at 130°C (including glass, stainless steel, PTFE, PDMS) using pressurized water vapor. **Check if the material to be sterilized can bear autoclave processing**.

STANDARD PROCEDURE

Switch on the autoclave.

Check the control panel. If any of the alarms "minimum", "maximum" and "max used" is on, do not use the autoclave. Call the Safety Officer who will restore the correct operative condition.

For cleaning and filling of the tank, use **only** demineralized water.

Insert the materials in the sterilizing envelope and seal it.

Open the autoclave by pulling the handle upwards;

Insert the envelope and lock the autoclave.

Select the correct sterilization cycle by pushing the button “Cycle”

121°C not enveloped: cycle for inserting a not enveloped solid

121°C enveloped: cycle for inserting an enveloped solid

134 °C special: cycle with rapid drying

134 °C not enveloped: cycle for inserting a not enveloped solid

134 °C enveloped: cycle for inserting an enveloped solid

134 °C Prion: sterilization of objects at risk for CJD (Creutzfeld Jacob Disease)

Sterilization procedure for surgical equipment:

Sterilization of **potentially infected** instruments:

Put them in the autoclave without cleaning or disinfection.

Sterilization procedure of **non-potentially infected** instruments:

Disinfection of the instruments (in a bowl with an adequate disinfectant).

Cleaning with distilled water.

Drying and packaging in heat-sealed or manually sealable envelopes.

NOTE

It is necessary to place the envelopes with the paper side facing upwards, without overlapping them.

Press “start” by pushing the button “I”.

When the cycle ends, an acoustic alarm turns on and the digital display reports "end cycle".

Check the sterilization cycle data on the paper report.

Unlock the door by pushing the button “door”.

Open carefully the autoclave using heat protection gloves.

When the temperature is low enough, extract the envelopes leaving the door open.

Check the sterilization color bar on each envelope.

Do not touch autoclaved materials without heat gloves.

Switch off the autoclave by pushing the green button on the side.

CLEANING AND MAINTENANCE OF THE AUTOCLAVE

Water filling

Switch on the autoclave with the green button on the right side.

Connect the appropriate tube with its filter to the water entrance connector.

Insert the water filter in the bowl of demineralized water.

Press the button “Pump” and hold it for at least one second, in order to activate the loading pump.

The pump stops when the maximum value is reached (if the maximum value is not reached within 180 seconds the pump stops and you have to press again the button).

Drainage of utilized water

A led on the front panel alerts you when it is necessary to drain used water off the tank (in this case the autoclave stops working).

Switch on the autoclave with the green button on the right side.

Connect the drainage tube to the exit connector and drain water.

Substitution of bacteriological filter

The bacteriological filter is placed in the upper left, outside the cylindrical chamber and is visible once you open the door. Substitute the filter every three months or in a shorter interval if its color becomes darker.

Door parameters control

To set the closing pressure of the door, use the provided key to act on the specific register placed behind the gasket. Rotate counterclockwise to increase the closing pressure or clockwise to decrease it.

Substitution of printer paper

Use rolls of thermographic paper of 57 mm width.

Open the printer door.

Insert the roll holding its final part while closing the door.

In order to clean the thermic head of the device, use a cloth lightly wet by ethanol. If the printer door is not correctly closed, the letter “D” appears on the display; in this case open and close again the door. If paper is missing, the letter “P” appears on the display.

Always refer to the user manual.

SHAKING THERMOSTATIC WATER BATH

Brand: Stuart scientific

Model: SBS 30

Provenance: dismissed by “Istituto Mario Negri”, Bergamo

Maintenance plan: weekly decontamination

DESCRIPTION

A thermostatic water bath with integral shaking is a device, which can provide linear or orbital shaking action at a certain speed. Moreover, the presence of a heater allows setting and maintaining the desired temperature by sensing the water temperature constantly.

STANDARD PROCEDURE

Fill the bath, use ½ volume of distilled water and ½ volume of tap water.

Change water from the bath every week to avoid contamination.

In order to switch on the device, push the green button in the bottom right.

The heating and the shaking actions have two distinct starting buttons (both in orange).

In order to set the temperature, firstly switch on the relative system, then keep pushed the white button and rotate the right handle (indicated as “set”) until you read on the display the desired value of temperature. With the handle on the right corner, you can set the maximum temperature beyond which the device gives an alert signal.

In order to set the shaking speed, rotate the handle next to the relative starting button.

Always refer to the user manual.

ANALYTICAL SCALE

Ohaus Analytical Plus

Modello: AP250D

Maintenance program: ordinary cleaning and annual calibration.

- Avoid carrying out weighings greater than the maximum weight declared by the instrument (210 g).
- Do not place containers containing liquids in the weighing chamber.
- Always place an aluminum foil or paper on the scale pan to avoid direct contact with the material to be weighed.
- Periodically and in case of spreading of substances inside the weighing cabin, proceed with cleaning with the appropriate brush.
- The calibration of the instrument is automatic and must be performed daily at the beginning of the laboratory activity.
- If the value on the instrument display does not stabilize (the scale "counts"), do not proceed with weighing the material and notify the Manager.
- Never disconnect the power supply plug of the instrument at the end of the weighing process but deactivate it with the appropriate "Off" button.

CHEMICAL FUME HOOD

Brand: Vetrotecnica

Model: GS800 base

Bought at: Ghiaroni & C. S.A.S. Piazza Galvani 9 20090 Buccinasco (MI). Phone: 02-45708618 (R.A.) - Fax 02-45708619 -e-mail: ghiaroni@ghiaroni.it - www.ghiaroni.it

Purchase year: 2013

Maintenance program: annual decontamination and revision of active carbon filters.

DESCRIPTION

Ductless (recirculating) fume hoods have a fan mounted on the top (soffit) of the hood. Air is sucked through the front opening of the hood and through a filter, before passing through the fan and being fed back into the workplace. With a ductless fume hood, it is essential that **the filter medium** be able to remove the particular hazardous or noxious material being used.

STANDARD PROCEDURE

Wash hands with soap and water

Wear protective garments (gloves, lab coat)

Turn on the cabinet by pressing the green button on the panel.

Check if the red LED is on.

If the **red LED is on, stop** any procedure. The filters need to be changed by the supplier.

If the red LED is off, go on working

Leave the chemical cabinet surface free.

Once you have finished working, close all the chemicals and store them properly.

Do not leave any open/closed bottle of reagents in the chemical hood.

Clean the cabinet with detergent.

Clean the cabinet with ethanol 70%.

Turn off the cabinet.

NOTES

Turn on/off the chemical hood only if needed to preserve active carbon filters.

Do not put anything on the top of the chemical hood to avoid damage to the microfiltration system.

Do not clean with bleach to avoid damages. Always refer to the user manual.

BIOLOGICAL SAFETY CABINET WITH LAMINAR FLOW
Class: II

Brand: EuroClone

Model: Safemate Eco 1.2

Year: 2018

Purchased by: EuroClone SPA, V. Figino, 20/22, 20016, Pero (Mi), Tel. +39 02 382951, Fax. +39 02 38195250

Complete overhaul and testing: June 2019

Maintenance Schedule: annual decontamination; annual revision.

DESCRIPTION

A biological safety cabinet is an enclosed, ventilated laboratory workspace for safely working with materials contaminated with (or potentially contaminated with) pathogens requiring a defined biosafety level. The biological safety cabinet allows manipulation cells under sterile conditions and protecting the operator from biohazards. The biological safety cabinet is designed to provide personnel, environmental and product protection when appropriate practices and procedures are followed.

STERILIZATION

1. Turn the key to switch on the cabinet: press the button with the down oriented arrow and the UV light button twice- 20 minutes.

NOTE do not look at the UV light to avoid damage to your eyes.

2. Turn the key to switch off the cabinet.

STANDARD PROCEDURE

1) UV Sterilization (refer to the above section)
2) Wash hands with soap and water
3) Wear protective garments (gloves, lab coat)
4) Disinfect gloves with ethanol 70% v / v in water.
5) Turn on the biological safety cabinet - press button in the upper left of the key lock button.
6) Remove the front closure panel.
7) Wait until the fan is on.
8) Clean the surfaces with suitable cleaning products from the top to the bottom.
9) Remove after 30 seconds the excess detergent with a clean absorbent paper.
10) Disinfect surfaces with 70% ethanol in water.

NOTES on Good Laboratory Practice (GLP)

Do not insert your hands in the cabinet without gloves.

Disinfect your hands thoroughly with 70% Ethanol.

Do not work near the cabinet aspirator, where the airflow is not sterile.

Do not fill the cabinet because it affects the laminar flow.

Keep the biological safety cabinet working surface tidy and clean.

In case of spills, dry immediately with a clean paper towel, and disinfect with 70% ethanol.

At the end of the work, remove all objects except for tip boxes and sterile equipment.

Disinfect surfaces with 70% ethanol. Wait for 10 minutes.

Switch off the cabinet: turn the key. The fan will stop and the cabinet will close automatically.

NOTES

Every month, or sooner if necessary, lift the perforated top of the hood and clean the collection tank.

Be extremely careful not to touch or wet the HEPA filter placed on the upper part of the hood, because it is very delicate and expensive.

Do not use chlorine, bleach and derivatives for cleaning, because they corrode metals;

Wash first with detergents and then with ethanol 70%.

The maximum UV usage is 30 min per day – the lamp will last about 1 year.

Do not touch the HEPA filter while cleaning to avoid damages. Always refer to the user manual.

CENTRIFUGE WITH CONTROLLED TEMPERATURE (FROM -10 TO +40 °C)

Brand: SIGMA

Model: 3-16PK

Purchased at: EuroClone, Via Figino 20 Pero (Mi), Phone +39 02.38195.1

Year of fabrication: 2010

Maintenance plan: annual decontamination

DESCRIPTION

Centrifuges are designed to rotate at high-speeds around a central axis in order to separate fluids of different density, such as cells from suspensions. Some of them may also have a refrigeration function, which allows adjusting the temperature at desired values.

STANDARD PROCEDURE

Switch on the centrifuge (push the button on the bottom right side).

Open the top panel

Check the integrity of the test tubes

Close the tubes carefully before insertion in the centrifuge.

Insert water-filled tubes symmetric to all samples to balance weights.

Set the centrifugation parameters on the digital control panel: use the right handle to select the parameter to be modified (e.g. timer, temperature, rounds per minute, gravity) and the left handle to modify its value.

NOTE

To confirm the chosen value, you have to press softly the corresponding handle.

Close the lid

Press the start button.

At the end of the centrifugation process, an alarm rings and the centrifuge can be opened.

CLEANING AND MAINTENANCE OF THE CENTRIFUGE

1) Use only mild detergents; if necessary, disinfect with paper wet by 70% ethanol and leave the centrifuge lid open to avoid condensation formation.
2) Lubricate periodically with the provided oil (SG70104) the contact parts between rotor and compartments and between containers and their compartments; then lubricate also the rotor pin with the provided grease (SG70284).

3) Do not use grease in place of oil for the lubrications mentioned above.
4) Every 20 cycles of centrifugation circa, tighten the rotor pivot with the specific provided key.
5) In the case of scatter of liquids or other substances inside the centrifuge, refer to the section
6) “Modalità di utilizzo di strumenti ed apparecchiature” of the manual.
7) Always refer to the user manual.

ULTRAREFRIGERATOR -80°C

Brand: Sanyo

Purchased from: Analytical Control S.p.A., Via Copernico, 15, 20092 Cinisello Balsamo (MI), Phone. +39 02 6129241

Sales representative: Dott. Vercesi mobile+39 320 86 07 797

Maintenance schedule: monthly filter cleaning and checking the seals.

OPERATIONS, CLEANING AND MAINTENANCE

Never insert unlabeled materials. **Label any** sample with 1) operator name, 2) description of the biological origin of the material stored, 3) indications on potential biohazard and chemical hazard, and 4) insertion date. Incorrectly labelled materials are periodically disposed as waste by the Safety Officer.

Always wear cryogloves before opening the freezer door because ultra-low temperature is harmful in case of direct contact with body parts.

Always wear cryogloves when collecting materials because ultra-low temperature is harmful in case of direct contact with body parts.

In case of spill occurs, refers to the Safety Officer. Once you are allowed, clean up all surfaces with 70% ethanol. It is mandatory to stay inside the work plans and far from probes or sources of sparks.

To avoid efficiency losses or failures of the instrumentation, monthly cleaning of the filter of the capacitor, from the deposited dust, should be performed. **Refer to the user manual**.

Make sure that no ice is formed on the two seals of the outer door and outside the inner door of the freezer. Ice formation on these components prevents the maintenance of the internal temperature and in this case, the device will continue to operate without interruption, unable to reach -80 ° C.

Always refer to the user manual.

DEWAR FOR LIQUID NITROGEN

Purchased by: Analytical Control S.p.A., Via Copernico, 15, 20092 Cinisello Balsamo (MI)

Phone. +39 02 6129241

Sales representative: Dott. Vercesi (tel cell. 320 86 07 797)

Maintenance schedule: weekly check of the content level

DESCRIPTION

The dewar is used to store cells in liquid nitrogen at temperatures between -210 °C and -196 °C.

STANDARD PROCEDURE

Operating is allowed **only** in the presence of the Safety Officer.

Liquid nitrogen (-196°C) provides risk of severe frostbite and permanent damage in the event of massive contact with not adequately protected body surfaces.

If you need to cryopreserve cells

Label any cryovial with the following data: 1) cell type, 2) date, 3) cell number, and 4) operator name.

Incorrectly labelled vials are periodically disposed as waste by the Safety Officer.

Check an available position for your vials in the paper form.

Fill the paper form with the same information before storing the vials.

Do not move for any reason the cryovial containers without the permission of the Safety Officer.

Always wear cryogenic gloves, face shield and a whole body coat before operating.

Liquid nitrogen vapors can cause risk of asphyxiation.

Always operate in a well-ventilated environment. When finished provide proper ventilation of the laboratory by opening doors and windows for 5 minutes.

Check the stored material in the paper form.

Take note of the position of the cell you need.

Open the dewar cap.

Remove the additional cap.

Take out the can, avoiding the liquid nitrogen to spill.

Take the vial.

Insert the cannister again.

Replace the additional cap to avoid Liquid Nitrogen massive evaporation

Close the Dewar

Update the paper form.

OPERATIONS, CLEANING AND MAINTENANCE

Operating is allowed only in the presence of the Safety Officer.

Check the level of liquid nitrogen inside the container weekly.

Use the appropriate wooden rod. For safety reasons, do not use metal rods.

In case the level is below the threshold value (about 15 cm), refer to the Safety Officer and contact

Mr. Oscar Bressan tel. 3210, e-mail obressan@polimi.it. Agree with him for the delivery of Liquid Nitrogen.

Do not fill the dewar containing cells over 35 cm to avoid damage to cells.

If the cryovials are not firmly sealed or damaged, the liquid nitrogen may penetrate within the cryovials. Cryovials may explode once taken out from the dewar.

To avoid the explosion, it is necessary to operate as follows: take the tubes under a sterile safety cap with laminar flow very quickly, open the cryovial to let the nitrogen evaporate before tube pressurization.

CRYOSTAT

Brand: MICROM

Provenance: Istituto Mario Negri Bergamo, year 2010

Maintenance program: annual decontamination

DESCRIPTION

The cryostat aims at cutting histological sections at low temperature. It is a very dangerous instrument.

STANDARD PROCEDURE

Before starting to use it, ask to the Laboratory Safety Officer to be adequately trained and refer to the complete manual of the instrument.

As general rules: turn the handwheel ONLY CLOCKWISE (forward), and close the glass window at the end of operations.

ATTENTION: the use of the cryostat implies risks of injury with the cutting blade, which could have potentially come in contact with not fixed biological tissues, and thus biohazards.

VERTICAL FRIDGE/FREEZER +4°C/-20°C

Brand: Bompani

Provenance: Istituto Mario Negri, Bergamo

Maintenance schedule: annual defrosting, annual decontamination

OPERATIONS, CLEANING AND MAINTENANCE

- Do not introduce food or drinks.
- It is a normal home refrigerator: use a disinfectant cleaner, i.e. Aldek, and eventually, 70% ethanol.
- Put all the contents of the refrigerator in the proper ice packs. Put then, ice packs in a polystyrene box when a massive cleaning is needed.
- Always close the refrigerator doors cautiously: this fridge is not equipped with any alarm.

VERTICAL FREEZER -20°C

Brand: Bompani

Provenance: Istituto Mario Negri, Bergamo

Maintenance program: annual defrosting, annual decontamination

OPERATIONS, CLEANING AND MAINTENANCE

1. Do not introduce food or drinks.

2. The cleaning is performed in the same way as for the refrigerator. Defrosting might be necessary before cleaning, thereupon store all the materials and samples in dry ice during the maintenance operations.

3. Close the door immediately to avoid the warming of the contents and the frost formation.

EGG INCUBATOR – TEMPERATURE CONTROLLED

Brand: Borotto

Model: Real 12

Year of purchase: 2017

Purchased by: Borotto, Via Papa Giovanni Paolo II, 7, 37060, Buttapietra (Ve)

DESCRIPTION

The **incubator** is a device used to grow and maintain egg cultures. The incubator allows maintaining temperature and humidity at physiological ranges.

STANDARD PROCEDURE

Check if the incubator had been cleaned. If not, or if you are not sure, decontaminated it properly (detergent + ethanol 70%).

Check the internal water reservoir. It has to be filled with 1 Litre of distilled water

NOTES

Set the temperature to 37.7°C on the digital control panel

Set the humidity to 45% on the digital control panel

Allow the incubator to stabilize the environmental conditions. For at least 12 hrs.

Any time that the incubator door is opened, culture parameters are affected.

Remove the internal shelves and use sodium hypochlorite to disinfect the inner surfaces

Be careful not to wet the sensors and the crankshaft.

Wash the inner chamber by using deionized water.

Always refer to the user manual.

CELL CULTURE MINI-INCUBATOR – O_2 and CO_2
Brand: EPPENDORF

Model: Galaxy S Series

Year of fabrication: 2010

Last complete revision and testing: June 2019

Contacts for maintenance: Eppendorf via Zante 14 20138 Milano

Maintenance plan: weekly decontamination; annual revision

DESCRIPTION

The **incubator** is a device used to grow and maintain microbiological cultures or cell cultures. The incubator allows maintaining temperature, humidity and carbon dioxide (CO_2) at physiological ranges. The incubator is provided with a nitrogen sensor that allows for the control of the partial pressure of oxygen. By controlling the partial pressure of nitrogen, the pressure of oxygen can be varied between 1% and 19%.

STANDARD PROCEDURE

Check if Carbon Dioxide and Nitrogen cylinders are full.

Check if the Carbon Dioxide and Nitrogen pipelines are open. Carbon Dioxide optimal pressure has to be set at 1 bar. Values set above 1 bar may lead to both detachment of the service pipe and CO_2 asphyxiation.

Nitrogen optimal pressure has to be set at 6 bar. Values set above 6 bar may lead to both detachment of the service pipe and N_2 asphyxiation.

Check if the incubator had been cleaned. If not, or if you are not sure, decontaminate it properly (detergent + ethanol 70%).

Check the internal water bowl. It has to be filled with 200 mL of distilled water

NOTES

Add disinfectant to the water bowl

Auto-zero procedure - REFER TO THE USER’s MANUAL

Set the temperature to 37°C on the digital control panel

Set the Carbon Dioxide pressure to 5% and Oxygen pressure to the desired level.

Allow the incubator to stabilize the environmental conditions for or at least 12 hrs.

Any time that the incubator door is open, culture parameters are affected.

After finishing cell culture experiments, switch off the incubator.

Close the gas tubes in the lab.

Close the gas supply lines (See “Gas line monitoring”).

Remove the water bowl and decontaminate the water inside with a solution of 5 g/L sodium hypochlorite. Take care of its disposal.

Remove the internal shelves and spray disinfectant (e.g. Aldek or Pharmacidal). Wait for about 30 seconds and dry it with a sheet of paper.

Disinfect the incubator. Be careful not to spray on the sensors and to clean the corners of the internal incubator chamber.

Use a paper wet by 70% ethanol or 60% isopropanol solution and clean the incubator, shelves and the water bowl.

Do not use bleach (sodium hypochlorite) to clean the incubator.

Always refer to the user manual.

CELL CULTURE INCUBATOR – O_2-CO_2

Brand: Memmert

Model: ICOmed

Year of fabrication: 2018

Purchased by: EN.CO. Srl Unipersonale Apparecchi Scientifici, V. Filande, 13, 30038, Spinea (Ve), Tel. +39 0415 411133, Fax. +39 0415 411090

Last complete revision and testing: January 2019

Contacts for maintenance: EN.CO., service@encosrl.com

Maintenance plan: weekly decontamination; annual revision

DESCRIPTION

The **incubator** is a device used to grow and maintain microbiological cultures or cell cultures. The incubator allows maintaining temperature, humidity and carbon dioxide (CO_2) at physiological ranges.

STANDARD PROCEDURE

Check if Carbon Dioxide cylinders status.

Check if the Carbon Dioxide pipelines are open. Carbon Dioxide optimal pressure has to be set at 1 bar. Values set above 1 bar may lead to both detachment of the service pipe and CO_2 asphyxiation.

Check if the incubator had been cleaned. If not, or if you are not sure, decontaminated it properly (detergent + ethanol 70%).

Check the external water reservoir. It has to be filled with 2 L of distilled water

NOTES

Add disinfectant properly

Set the temperature to 37°C on the digital control panel

Set the Carbon Dioxide pressure to 5%

Allow the incubator to stabilize the environmental conditions. For at least 12 hrs.

Any time that the incubator door is opened, culture parameters are affected.

After finishing cell culture experiments, switch off the incubator.

Close the gas tubes in the lab

Close the gas supply lines (See “Gas line monitoring”)

Remove the water tube and decontaminate the water inside with a solution of 5 g/L sodium hypochlorite. Take care of its disposal.

Remove the internal shelves and spray disinfectant (e.g. Aldek or Pharmacidal). Wait for about 30 seconds and dry it with a sheet of paper.

Disinfect the incubator. Be careful not to spray on sensors and to clean the corners of the internal incubator chamber.

Use a paper wet by 70% ethanol or 60% isopropanol solution and clean the incubator, shelves and the water bowl.

Do not use bleach (sodium hypochlorite) to clean the incubator.

Always refer to the user manual.

CELL CULTURE INCUBATOR – CO_2

NAPCO

Model: 5420-1

Year of fabrication: 1999

Provenance: dismissed by “Istituto Mario Negri”, Bergamo – June 2010

Last complete revision and testing: May 2019

Contacts for maintenance: Belsar, Bellardini Alessandro, mobile phone: 3346412974

Maintenance plan: weekly decontamination; annual revision

DESCRIPTION

The **incubator** is a device used to grow and maintain microbiological cultures or cell cultures. The incubator allows maintaining temperature, humidity and carbon dioxide (CO_2) at physiological ranges.

STANDARD PROCEDURE

Check if Carbon Dioxide cylinders status.

Check if the Carbon Dioxide pipelines are open. Carbon Dioxide optimal pressure has to be set at 1 bar. Values set above 1 bar may lead to both detachment of the service pipe and CO_2 asphyxiation.

Check if the incubator had been cleaned. If not, or if you are not sure, decontaminated it properly (detergent + ethanol 70%).

Check the internal water bowl. It has to be filled with 1 L of distilled water

NOTES

Add disinfectant properly

Set the temperature to 37°C on the digital control panel

Set the Carbon Dioxide pressure to 5%

Allow the incubator to stabilize the environmental conditions. For at least 12 hrs.

Any time that the incubator door is opened, culture parameters are affected.

After finishing cell culture experiments, switch off the incubator.

Close the gas tubes in the lab

Close the gas supply lines (See “Gas line monitoring”)

Remove the water bowl and decontaminate the water inside with a solution of 5 g/L sodium hypochlorite. Take care of its disposal.

Remove the internal shelves and spray disinfectant (e.g. Aldek or Pharmacidal). Wait for about 30 seconds and dry it with a sheet of paper.

Disinfect the incubator. Be careful not to spray on sensors and to clean the corners of the internal incubator chamber.

Use a paper wet by 70% ethanol or 60% isopropanol solution and clean the incubator, shelves and the water bowl.

Do not use bleach (sodium hypochlorite) to clean the incubator.

Always refer to the user manual.

SPECTROPHOTOMETER AND DNA NANOQUANT

Brand: Tecan

Model: Infinite PRO Monochromator, Plate nanoquant

Bought from: TECAN ITALIA S.r.l. • Via Brescia, 39 • 20063 Cernusco sul Naviglio, Milano, Italy

Bought in year: 2012

T +39 02 92 44 790, F +39 02 92 72 90 47

Reference: Petra.kaltofen@tecan.com, 335 295863

Maintenance program: annual verification and calibration.

DESCRIPTION

The spectrophotometer is also called plate reader, it allows to perform quantitative colorimetric assays on cells cultured in multi-well plates.

Operation of the spectrophotometer is allowed only in the presence of the Safety Officer.

This instrument cannot be moved without a specific procedure.

Always refer to the user manual.

Please note:

The spectrophotometer must be moved using a tray lock and with a specific procedure indicated in the user manual.

The cleaning of the Nanoquant must be performed at each use with a lens cloth (lint, non-releasing fibers). Once a year, it should be soaked in an ultrasonic bath with distilled water.

DESCRIPTION AND USE OF THE INSTRUMENT

Infinite PRO is a multi-function microplate reader compatible with robotic systems and must be switched on with the software installed on the computer in order to work correctly.

The plate holder can move both horizontally (in x and y directions) and vertically (in the z direction), so that for each measurement mode, from the top or from the bottom, it is possible to reach the optimal position, furthermore there are three modes of agitation: linear, orbital and double orbital in addition to the possibility of incubating and / or cooling the plates.

To start a method, you can do it directly from the method editor by clicking on the Start button and, once started, the software will switch to the Dashboard view where you can select the corresponding method box (see the relevant chapter in the Reference Guide).

A method can be started directly by pressing the start button integrated in the instrument: it is necessary to define a method and save it, select *Start* from the instrument via the F*ile menu* of the method editor or open a method, select *Start* from tool via the *File menu* of the method editor. To view the progress of a

measurement initiated using the start button integrated in the instrument, open the *dashboard* and select the *Instrument toolbox* in use.

Infinite PRO allows performing luminescence, absorbance and fluorescence analyzes, moreover it has functions that allow quantifying cell vitality and nucleic acids / proteins present (if possible).

Absorbance: applications using cuvettes can be performed with any wavelength between 200 and 1000 nm. Transparent or UV transparent microplates are usually used for absorbance measurement. For high OD values, black microplates with a transparent bottom are more suitable. In general, to obtain accurate OD values it is preferable to avoid measurements above 3 OD. Wavelength range 200 - 1000 nm, selectable in 1 nm steps

Fluorescence: it is possible to select the wavelength by excitation and emission using the monochromator or thanks to the filter option. The two monochromators and filter modes can be combined separately for excitation and emission (so the detection system is extremely flexible and ensures maximum signal strength). The fluorescence polarization module is part of the Fusion Optics systems and is available exclusively for Cima measurements. The optical filters (band-pass filters) are mounted on the filter slides. The spectral transmission values and the fluorescence bandwidth are optimized to obtain the maximum degree of sensitivity. Contact Tecan for information regarding filters other than those supplied with the filter slides.

The export mechanism writes the files in Office Open XML format (.xlsx). The results are automatically saved and can be consulted in C: Users Public Documents Can Check Control Export xlsx (default path) or in the path defined by the user. Depending on the presented settings Result (see chapter Data Management in the Reference Guide), the results can be opened automatically after the measurement in Excel.

Always refer to the user manual.

OPTICAL INVERTED MICROSCOPE (FOR BRIGHT FIELD, PHASE CONTRAST AND FLUORESCENCE)

BRAND: Olympus

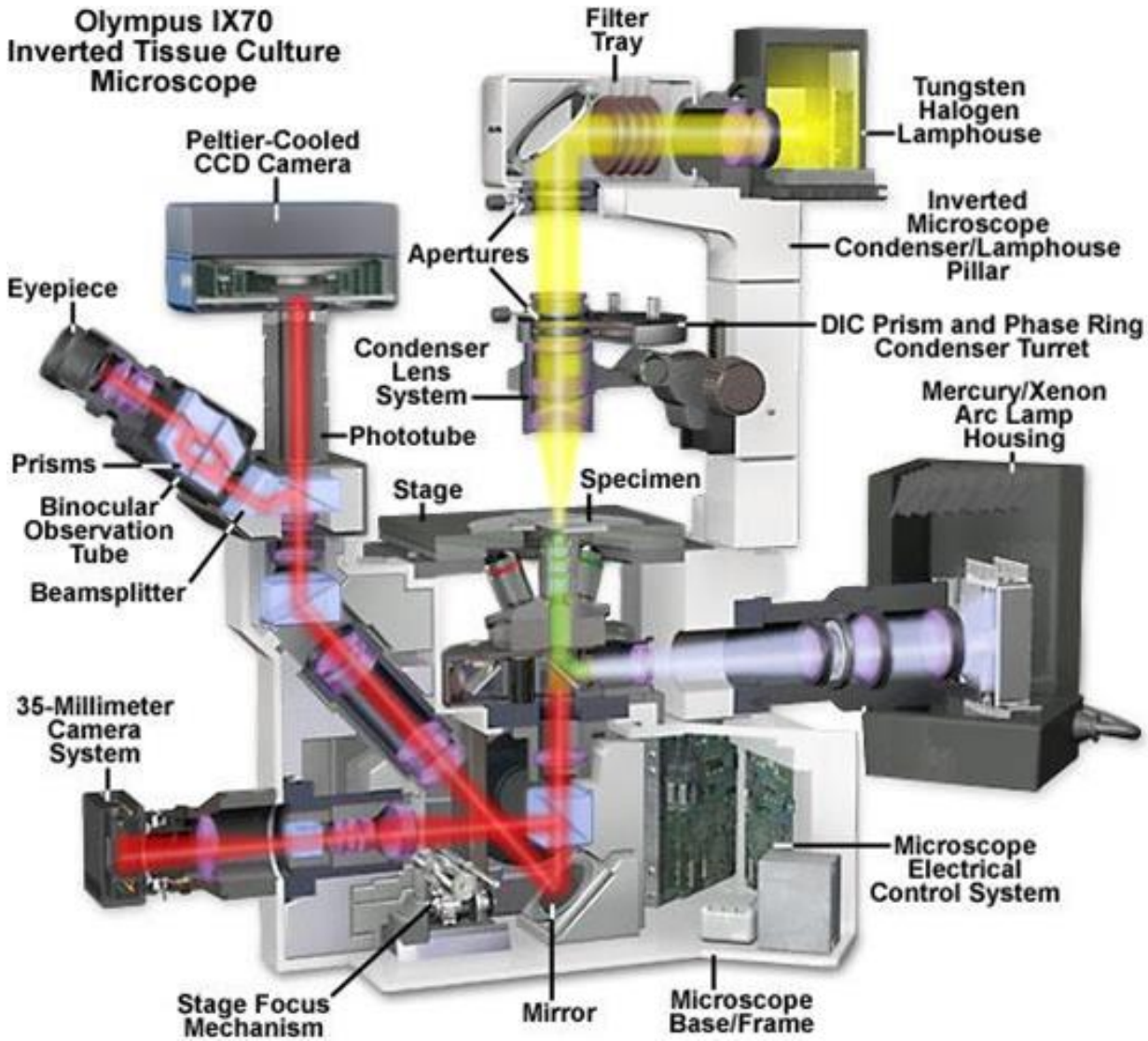
MODEL: IX70

Purchased BY: S.P. Scientific products, Via Aldo Moro, 7 20067 Paullo (MI)

Reference: Andrea Castelli

e-mail:andrea.castelli@olympus-europa.com

Maintenance program: annual phase and fluorescence calibration



DESCRIPTION

The Olympus IX70 is an optical microscope with an inverted-style frame designed primarily for tissue culture applications. It supports phase contrast and fluorescence imaging.

Fluorescence source: Mercury lamp.

Fluorescence filters: DAPI, FITC, TRITC

Objectives: 4x, 10x, 40x.

Camera: RGB Optika View

The microscope does not have motorized systems.

This instrument cannot be moved without a specific procedure.

Always refer to the Safety Officer.

STANDARD PROCEDURE

This microscope is a precision instrument and it is very delicate. Before starting to use it, ask to the Laboratory Safety Officer to be properly trained to operate with it and always refer to the user manual.

1. **ROUTINARY USE** – No Fluorescence (i.e., control of the cell expansion state in culture):

Switch on the microscope to perform Bright Field (BF) and Phase Contrast (PhC) microscopy by a halogen light source lamp.

Lift the yellow filter (otherwise the image will appear as a green one) lifting the left side of the corresponding housing, that is in the tray above the condenser.

The switch is on the right back side of the base

Switch on the microscope halogen and adjust the intensity with the slider that is on the front panel, on the lower left of the base. The push-button above the slider is aimed at stopping the lighting of the sample without switching off the halogen lamp.

Turn the revolving nosepiece to engage the desired objective (usually 10X) and then engage the corresponding light phase filter (Ph0, Ph1, PhC, Ph2, or BF…the phase to be used is printed on the objective turret) rotating the ferret on the upper part of the condenser.

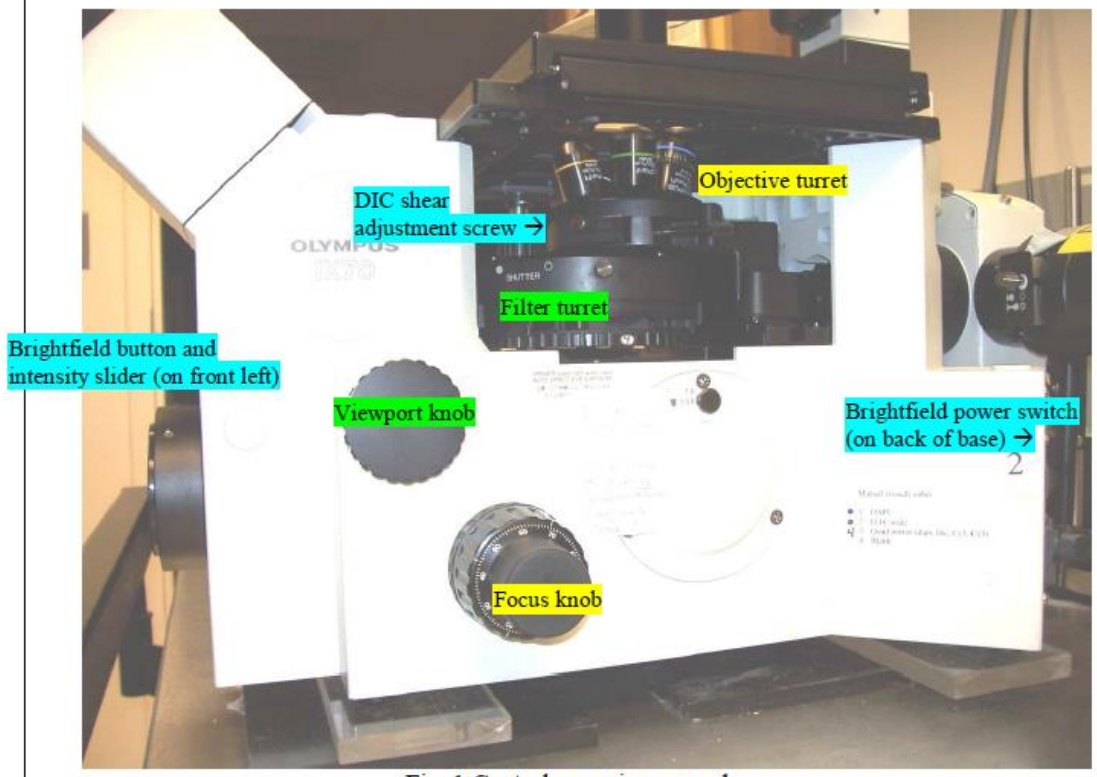


Fig. 1 Controls on microscope base

Put the sample on the microscope table.

Move the sample by operation of the mobile table.

The sample is focused by operation of the focus handle.

Looking through the eyepiece, turn gently the coarse adjustment knob to bring the specimen into focus. This macroscopic regulation has to be done with the big knob on the side of the microscope base. When you have optimized the focus with the coarse adjustment controls, use the fine micrometric adjustment knob (small one, see Fig.3) to improve the focus. Be careful not to clash the objective against the flask surface or against the sample that is examined! Breaking or damaging an objective implies a great damage. For this reason, operate with maximum care.

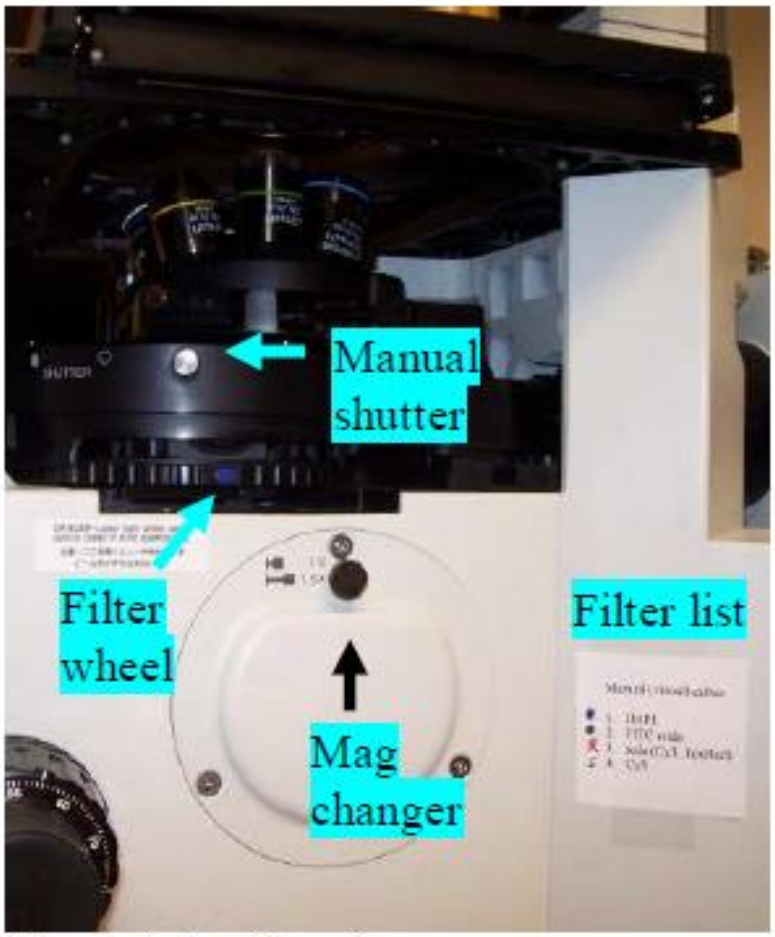


Fig. 2 right side of scope

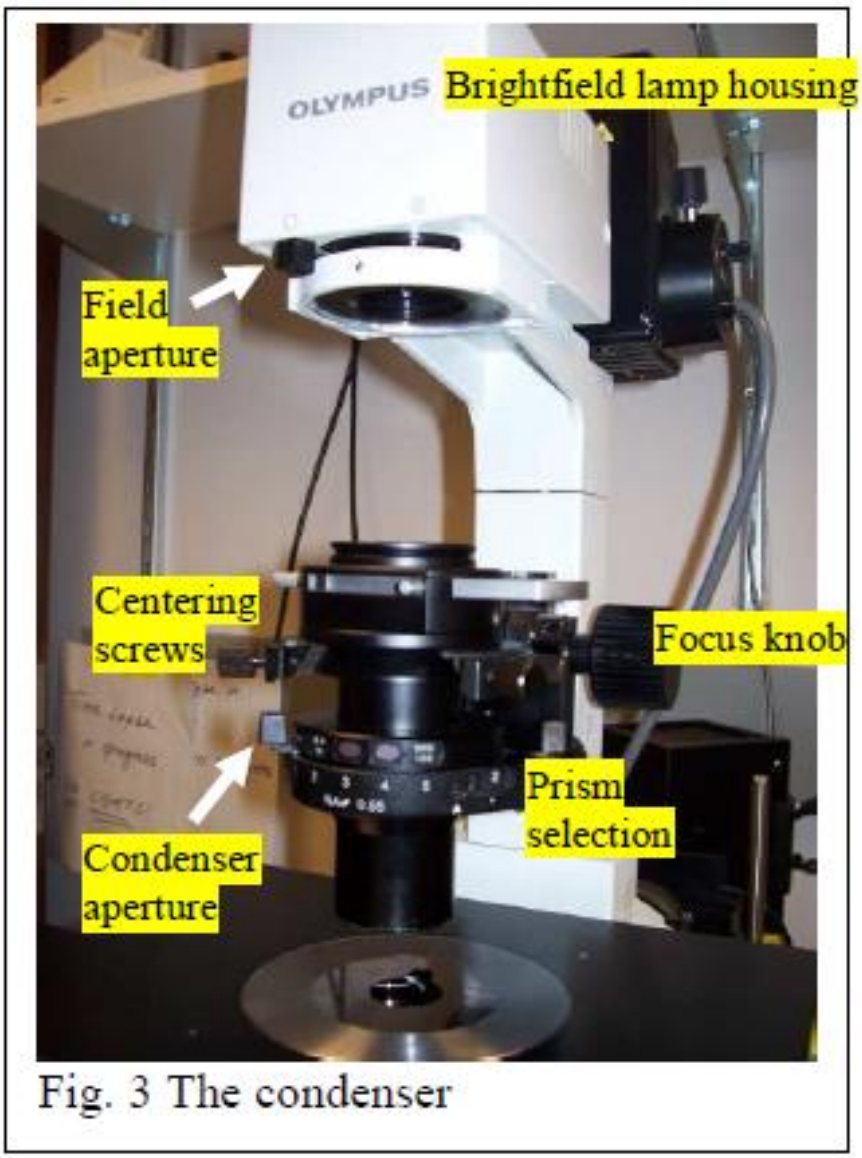


Fig. 3 The condenser

For a better focusing it is possible to operate as follows: with the lever above the condenser completely tighten the octagonal diaphragm, which describes the image field, and adjust the condenser height (knob) in order to see the polygon contour into focus. Then re-open the diaphragm until you are can see the polygon borders inside the image field anymore. In case, it is also possible to center the polygon using the thumbscrews that move the part in which the diaphragm is inserted. Re-bring the desired field in focus using again the macro- and micrometric regulation.

Image acquisition is performed by a high-resolution color video camera and relevant software.

The images and videos acquisition is made through the software "OpticaView", provided with the photo camera installed. On the desktop of the portable computer that accompanies the microscope, there is a link to that software.

After the observation, switch off the halogen lamp (the switch is on the backside). It not enough pushing only the front button: this only interrupts the lighting of the specimen, without switching off the lamp.

Please note that it is compulsory to verify to have switched off the halogen lamp at the end of the equipment usage.

ATTENTION: in case of routine use, the mercury vapor lamp must be never switched on (it is referred to a different power pack, next to the microscope). It is dedicated only to fluorescence analysis. This restriction is due to the fact that this lamp is subjected to consumption and its substitution implies a high expense. To avoid damage, if this lamp is inadvertently switched on, wait for 15 minutes before switching it off.

FLUORESCENCE – (i.e., immunohistochemistry, fluorescence life assays, etc.):

Please refer to the complete manual of the microscope and the Laboratory Safety Officer.

Refer to the instructions given in "**ROUTINARY USE**", but in this case switch on the mercury vapor lamp instead of the halogen one.

ATTENTION: to avoid damages, wait 20 minutes before re-switch on the lamp after its shutdown.

References

V. Bindokas, Olympus IX70 Multi-parameter Fluorescence Microscope Operations Manual

STIRRER IKA

Brand: Topolino Mobil

Year of fabrication: 2018

Purchased by: UNIFO SRL, VIA E. MATTEI 20 -22, 31059, ZERO BRANCO (TV)

PHONE. 0422/485910 FAX. 0422/485900

DESCRIPTION

Mini magnetic stirrer for mixing quantities up to 250 ml for battery and mains operation. Robust and brushless motor. Continuously adjustable speed range. High magnetic adhesion. Portable operation with long operating time (8-12 h). Short recharge time (2-3 h). Standard replaceable AA rechargeable batteries.

Optional power mode:

a) Without mains with standard batteries

b) With the supplied power supply unit (without batteries)

c) Combined battery / network operation (with batteries installed)

STANDARD PROCEDURE

Cover the plate with a foil sheet.

(Connect the external sensor and mount the specific support on the back of the machine) - optional

Adjust the desired stirring and / or heating parameters by turning the knobs on the front of the machine

Remove the foil sheet and clean the plate.

NOTES

Take care of hot surfaces.

STIRRER/HOT PLATE IKA

Brand: IKA

Model: RH-Basic

Year of fabrication: 2018

Purchased by: UNIFO SRL, VIA E. MATTEI 20 -22, 31059, ZERO BRANCO (TV)

PHONE. 0422/485910 FAX. 0422/485900

DESCRIPTION

Stirrer and hot plate with aluminum alloy plate. The strong magnetic field and wide speed range ensures usage for volumes up to 15 liters. External temperature control is possible by connecting the temperature sensor (PT 100). The digital display of the PT1000 module allows for an optimal overview of the temperature.

STANDARD PROCEDURE

Cover the plate with a tinfoil sheet

(Connect the external sensor and mount the specific holder to the backside of the machine) - optional

Adjust the desired stirring and/or warming parameters by turning the knobs on the front-side of the machine

Remove the tinfoil sheet and clean-up the plate

NOTES

Take care of hot surfaces.

PIPETBOY EUROCLONE

- Brand: EuroClone
- Purchased by: EuroClone S. p. A., via Figino 20/22, 20016 Pero (Mi), Italia
- Maintenance schedule: cyclic decontamination, filter change every 6 months, annual calibration
- Primo® mate is designed for reproducible pipetting and dispensing of liquids.
- Light: lightweight and ergonomically shaped handle
- Control: smooth pushbuttons effectively control the input and output of liquids in pipettes
- Precise: Easily accessible switches allow choosing different operation mode depending on the volume of pipette and viscosity of liquid.
- For liquid aspiration, the user can choose HIGH or LOW work speed and additionally adjust the suction by the pressure applied to the trigger button.
- Dispensing can be carried out by gravity (GRAV) or supported by pump (BLOW) which empties the pipette with blow out.
- Versatile: suitable for all types of pipettes (glass or plastic pipettes) 0.5-100 ml
- Safe and secure: PTFE filter protects from liquid contamination and damage;
- filter and pipette holder can be easily removed and autoclaved.
- Battery indicator: red led shows when the battery needs to be charged

MICROPIPETTE SET (0.5-10;10-100;100-1000;1000-5000 µl)

Brand: Gilson

Purchased by: Gilson Italia, via G. Matteotti 98, 20092, Cinisello Balsamo, Italy

Maintenance program: cyclic decontamination, annual calibration.

- Do not pipette by any means with your mouth.
- The micropipettes must always be kept vertical in the appropriate housings and never placed horizontally in the drawers.
- If the micropipettes accidentally fall or are subjected to shock, they may lose the calibration. Therefore it is advisable to carry out the calibration test by taking known volumes of deionized water and weighing them on the analytical balance.

THERMOSTAT HEATER

Brand: DISA Raffaele e F.lli , Via N.D'apulia 7, 20125 Milano, Tel. 02 28 92 698

Provenance: Istituto Mario Negri, Bergamo

Maintenance schedule: annual decontamination

OPERATIONS, CLEANING AND MAINTENANCE

Switch on the oven using the power switch on the front panel

Regulate the temperature using the handle located just above the power switch. There are two indicator lights, a green and a red one. The green one indicates that the heater is on while the red one remains on during the heating phase and then turns off when the desired temperature is reached.

Check the temperature by an analog thermometer located on the upper surface of the oven.

To enable the ventilation in the oven, use the openings located on the top and on the right side of the oven.

It is good practice to keep the internal plane of the heater on water level ("in bolla"). To do this, use a common spirit level (available in the workshop) shimming adequately all the sides of the metal plane.

Wear heat protection gloves when material is inserted.

Wear heat protection gloves when material is extracted.

Switch off the oven at the end of use – **No alarm equipment**.

NOTES

In case of malfunction, check the status of the fuse. The fuse is next to the power cable and enables to protect the electric circuit of the heater.

Always refer to the user manual.

GLASSWARE, CONSUMABLES AND SOLUTIONS

The laboratory is equipped with glassware, disposable polymeric material, salt solutions, culture media, cells, antibiotics, cellular nutrients, various types of reagents.

The aforementioned material is subject to consumption, therefore it is necessary to order it periodically; in this regard it is advisable to always inform the laboratory manager about the exhaustion of the stock of the single article. Report to the responsible if the stocks of the various materials / reagents / cells are running out.

When the ordered material arrives it is advisable:

Check the actual presence of the declared material in the transport document.

Delivery of transport document to the laboratory security officer.

PIPETBOY

Brand: Biohit

STANDARD OPERATION

Clean the tip holder with paper moistened with ethanol at 70%

Regulate the aspiration rate using the handle. The regulation of aspiration flow rate is done using the thumbwheel that is located on the top of the handle.

The top button is used to aspirate, the lower one to eject.

Insert the pipette carefully in order to avoid breakage and harm to hands

Insert to pipette into the liquid container

Press the upper button to aspire liquids and the lower button to eject liquids

Do not fill the pipette beyond the security level.

Maintain a vertical orientation of the pipette during operation, to avoid liquid penetration and damage to the instrument.

Lay down the pipette controller in a horizontal position to maintain pipette sterility, if needed.

When not in use, remove the bracket from the handle to put the PIPETBOY vertically.

After the usage, thoroughly clean the PIPETBOY with paper moistened with 70% ethanol.

Put the PIPETBOY charging in a vertical position.

NOTE

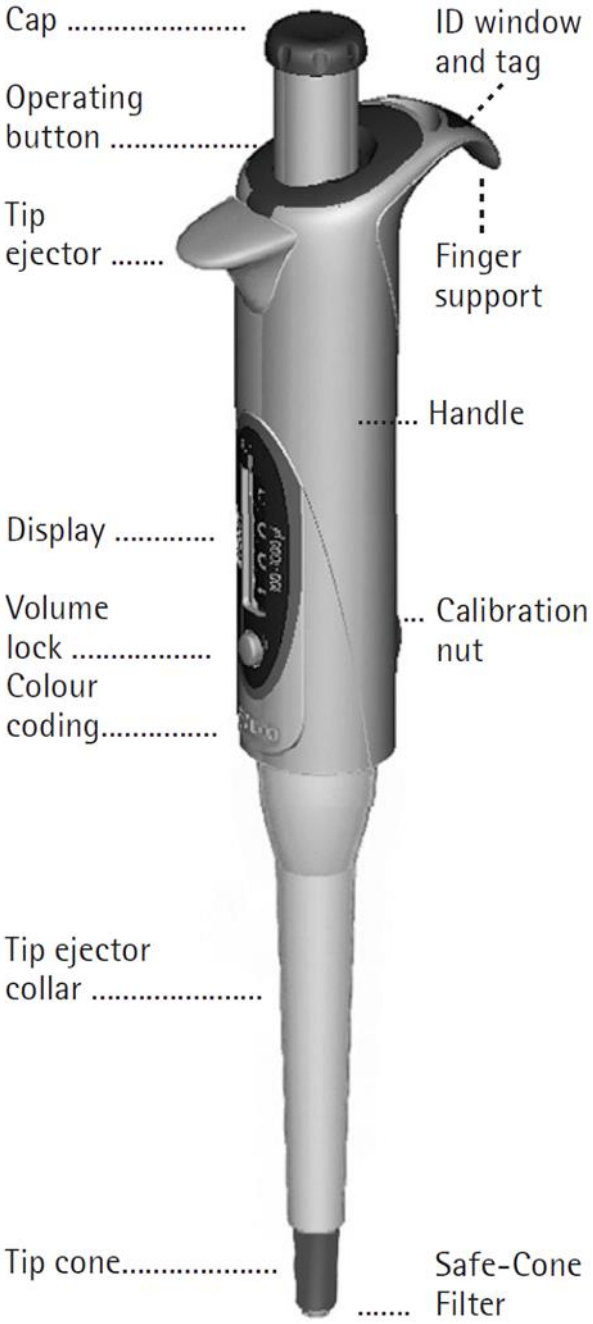
Never fill the pipette beyond the safety edge: this wets the filter, and the PIPETBOY can be damaged. However, there is an interchangeable safety filter, which is able to prevent damage caused by small amounts of liquid in the PIPETBOY.

If you realize that you have wet this filter (the PIPETBOY makes an effort to aspire), change it immediately. To do this, press the little button placed near to the pipette holder and simultaneously turn the blue ferrule, remove the filter (similar to that for the syringes) and replace it with a new one.

MICRO-PIPETBOY
Brand: Biohit, model: m-line

DESCRIPTION

The micro-PIPETBOY is a very delicate and sophisticated instrument, to be used for handling very low and controlled volumes of fluids (down to 1 µl). Be careful that they must never be let fall.



Parts of the Biohit m-line micro-pipettor

STANDARD PROCEDURE AND OPERATION

To select the volume that you want to aspire hold down the blue button (volume lock) located next to the display and simultaneously rotate the ferrule that is on the button used for aspiration (operating button).

NOTE: Do not try to select the volume of intake without first pressing the blue button! It is aimed at releasing the ferrule.

Engage the suitable tip for the pipette size that you are using by pressing lightly on the tip cone coupling.

Fill the tip.

NOTE: Push the button and carefully regulate the aspiration volume. Insert the sterile tip carefully in order to avoid damage to the instrument and in order to avoid contamination of the other tips. Press slightly the upper button to aspire. Do not plunge the tip beyond a security level and maintain a vertical orientation of the pipette during operation, to avoid liquid penetration and damage to the instrument.

Release the liquid properly.

Press slightly to eject liquids. Push firmly to empty the tip.

To release the tip press the appropriate button located next to the inlet one.

When you are not using the micro-PIPETBOY, keep it upright with the appropriate support (to avoid internal contamination).

CLEANING AND MAINTENANCE

These PIPETBOYs have an interchangeable filter to protect from contamination inside of the pipette. To replace them, remove the rubber plug on the aspiration button and press it all the way, until the expulsion of the filter.

Clean the micro-PIPETBOYs with ethanol at 70% before storing them in a vertical position. However, these PIPETBOYs can be put in an autoclave, if necessary.

Please note that you can recalibrate the micro-PIPETBOYs. In case you need to do it, refer to the corresponding manual.

REFERENCE

Sartorius, *mLINE pipette User Manual*, available from http://www.sartorius.com

MECHANOBIOLOGY LAB (NICHOID)

DIGITAL ULTRASONIC BATH Mod. DU-100
Brand: ARGO LAB

Model: DU-32

Bought from: Euro glass SRL (06/2031943)

DESCRIPTION

Argo Lab ultrasonic cleaner baths with housing made of top quality stainless steel. Timer for countdown and built-in heating to 60°C for DU series and 80°C for AU series.

DU series with digital controller and display with indication of actual and set temperature (no mod. DU-06), timer and ultrasonic power control (no mod. DU-06).

Applications:

Cleaning of technical glassware like pipettes, petri dishes and laboratory flasks.

Disinfection and cleaning at the same time.

Degassing of wine or beer samples for analysis of alcohol contents, original worth, color, pH value.

Degassing of food sampled from cans for analysis of stannous contents.

Extraction of soil samples for determination of hydrocarbons

Technical specification:

Max capacity 10 L.

Temperature range to 80°C.

Timer 1-99min,

Ultrasonic power control 1-5.

Accessories:

Included stainless mesh basket, stainless lid and 1x60ml concentrate universal cleaner solution.

NOTE: At least once per years use the concentrate cleaner solution to clean the bath.

Operation of the Bath is allowed only with the permission of the Safety Officer.

This instrument cannot be moved from the Nichoid lab.

Always refer to the Safety Officer

BIOLOGICAL SAFETY CABINETS EuroClone Topsafe Class II (Type A2)

Brand: Top safe

Model: 1.2 Bioair

Bought from: Euroclone

Maintenance program: annual maintenance

DESCRIPTION

The TOPSAFE™ Class II Biological Safety Cabinets incorporate a new microprocessor based control for the handling of low-moderate risk-hazardous agents. Called MAC (Microprocessor Automatic Control air volume monitoring) this new and innovative microprocessor control system provides automatic control of the pre-set airflow volumes throughout the usable life of the installed filters. When a deviation from the set airflow is detected, be it from supply voltage increase-decrease or filter loading the automatic control adjusts the airflows accordingly.

- Ergonomic design with sloped front for user comfort
- LED display, monitoring airflow parameters ; elapsed hour meter
- Three operating modes: normal, stand-by, calibration
- Splash proof power outlet, Gas and Vacuum taps. Gas with solenoid valve
- Audible and visual alarms are activated in case of ventilation parameters out of safety limits, front window open, fan failure
- Rear work zone mounted UV light (as an option) with safety interlock and timer

The exhaust and recirculating flow rates ensure 25 air changes/min in the working area (30% 70% split) and the front barrier air speed is ≥ 0,5 mt/sec.

The outer surfaces are of cold rolled steel with stove paint finish RAL 7035 and RAL 7036, the inner exposed surfaces and spilt liquid sump of grade 3 stainless steel and 2B finish. The front screen is made up of safety laminated glass.

Operation on the cabinet is allowed only in presence of the Safety Officer.

This instrument cannot be moved without a specific procedure.

Always refer to the user manual.

VERTICAL FRIDGE/FREEZER +4°C/-25°C

Brand: KW Apparecchi Scientifici

Purchased from: Analytical Control S.p.A., Via della Resistenza, 119, 53035 Monteriggioni-Siena (Si), Tel. +39 0577 309144, Fax. +39 0577 309142

Maintenance schedule: annual defrosting, annual decontamination.

OPERATIONS, CLEANING AND MAINTENANCE

Do not introduce food or drinks.

It is a normal home refrigerator: use a disinfectant cleaner, i.e. Aldek, and eventually, 70% ethanol.

Put all the contents of the refrigerator in the proper ice packs. Put then, ice packs in a polystyrene box when a massive cleaning is needed.

Always close the refrigerators doors cautiously: this fridge is not equipped with any alarm.

CULTURE INCUBATOR - CO_2

Brand: Euroclone

Model: Safegrow Pro 188, Series P9699

Year of purchase: 2015

Purchased by: Euroclone, Via Figino, 20/22, 20016, Pero (Mi), Phone. +39 02 381951, Fax. +39 02 38101465

Last complete revision and testing: June 2019

Contacts for maintenance: e.g. Belsar, EuroClone.

Maintenance plan: weekly decontamination; annual revision

DESCRIPTION

The **incubator** is a device used to grow and maintain microbiological cultures or cell cultures. The incubator allows maintaining temperature, humidity and carbon dioxide (CO_2) at physiological ranges.

STANDARD PROCEDURE

Check if Carbon Dioxide cylinders status.

Check if the Carbon Dioxide pipelines are open. Carbon Dioxide optimal pressure has to be set at 1 bar. Values set above 1 bar may lead to both detachment of the service pipe and CO_2 asphyxiation.

Check if the incubator had been cleaned. If not, or if you are not sure, decontaminated it properly (detergent + ethanol 70%).

Check the external water reservoir. It has to be filled with 2 L of distilled water

NOTES

Add disinfectant properly

Set the temperature to 37°C on the digital control panel

Set the Carbon Dioxide pressure to 5%

Allow the incubator to stabilize the environmental conditions. For at least 12 hrs.

Any time that the incubator door is opened, culture parameters are affected.

After finishing cell culture experiments, switch off the incubator.

Close the gas tubes in the lab

Close the gas supply lines (See “Gas line monitoring”)

Remove the water tube and decontaminate the water inside with a solution of 5 g/L sodium hypochlorite. Take care of its disposal.

Remove the internal shelves and spray disinfectant (e.g. Aldek or Pharmacidal). Wait for about 30 seconds and dry it with a sheet of paper.

Disinfect the incubator. Be careful not to spray on sensors and to clean the corners of the internal incubator chamber.

Use a paper wet by 70% ethanol or 60% isopropanol solution and clean the incubator, shelves and in water bowl.

Do not use bleach (sodium hypochlorite) to clean the incubator.

Always refer to the user manual.

MINI MICROCENTRIFUGE Corning® LSE™ 230V, EU Plug

Brand: Corning

Model: LSE™ Mini Microcentrifuge (cod 6766)

DESCRIPTION

Corning's LSE Mini Microcentrifuge is a personal benchtop instrument designed for quick spin downs of micro-samples. Operation is simple and convenient; after loading sample tubes, close the lid and the rotor accelerates quickly to 6000 rpm (2000 x g). This speed range is ideal for bringing small droplets to the bottom of the tubes for micro-filtrations, or basic separations. Press the lid release button and rotor comes to a stop. The LSE Mini Microcentrifuge includes an 8-place rotor that will accept standard 1.5 mL to 2.0 mL microcentrifuge tubes, and adapters are included for 0.5/0.4 mL and 0.2 mL tubes. Also included is a 4-place rotor for PCR strip tubes. Rotors included.

This instrument cannot be moved without a specific procedure.

PIPETBOY EUROCLONE

- Brand: EuroClone
- Purchased by: EuroClone S. p. A., via Figino 20/22, 20016 Pero (Mi), Italia
- Maintenance schedule: cyclic decontamination, filter change every 6 months, annual calibration
- Primo® mate is designed for reproducible pipetting and dispensing of liquids.
- Light: lightweight and ergonomically shaped handle
- Control: smooth pushbuttons effectively control the input and output of liquids in pipettes
- Precise: Easily accessible switches allow choosing different operation mode depending on the volume of pipette and viscosity of liquid.
- For liquid aspiration, the user can choose HIGH or LOW work speed and additionally adjust the suction by the pressure applied to the trigger button.
- Dispensing can be carried out by gravity (GRAV) or supported by pump (BLOW) which empties the pipette with blow out.
- Versatile: suitable for all types of pipettes (glass or plastic pipettes) 0.5-100 ml
- Safe and secure: PTFE filter protects from liquid contamination and damage;
- filter and pipette holder can be easily removed and autoclaved.
- Battery indicator: red led shows when the battery needs to be charged

MICROPIPETTE SET (0.5-10;10-100;100-1000;1000-5000 µl)

Brand: Gilson

Purchased by: Gilson Italia, via G. Matteotti 98, 20092, Cinisello Balsamo, Italy

Maintenance program: cyclic decontamination, annual calibration.

- Do not pipette by any means with your mouth.
- The micropipettes must always be kept vertical in the appropriate housings and never placed horizontally in the drawers.
- If the micropipettes accidentally fall or are subjected to shock, they may lose the calibration. Therefore it is advisable to carry out the calibration test by taking known volumes of deionized water and weighing them on the analytical balance.

VORTEX (VIBRATION SHAKER)

Brand: Velp Scientifica

Model: ZX3

Purchased by: EuroClone,Via Figino 20 Pero (MI), Phone +39 02.38195.1

STANDARD PROCEDURE

Operate only with tightly closed tubes.

Turn on the shaker by means of the black switch in "manual" position and by pressing the tube holder perpendicularly. The shaker will stop automatically when the flask is lifted.

When the switch is in "continuous" mode, agitation is independent of the container position. The shaking frequency can be selected by turning the right handle.

CONFOCAL MICROSCOPE FV10i-LIVE

Brand: Olympus

Model: FV10i-LIV

Bought from: Olympus Italia S.r.l., Via Modigliani 45, 20090 Segrate

Reference: andrea.castelli@olympus-europa.com, luca.cevenini@olympus-europa.com

Maintenance program: annual maintenance

DESCRIPTION

The Olympus FV10i-LIV Confocal with Live-Cell is a self-contained fully motorized laser scanning confocal microscope. It is provided with built-in incubator and a culture pod with recirculation ability designed for live-cell imaging. It supports multi-area and multi-color imaging and, thanks to the automated water dispensing system, enables long-term time-lapse imaging.

Lasers: 405 nm (17.1 mW), 473 nm (11.9 mW), 559 nm (15 mW), 635 nm (9.5 mW)

Objectives 10× phase contrast / NA 0.4 (equivalent to UPLSAPO 10x), 60× phase contrast water immersion / NA 1.2 (equivalent to UPLSAPO 60× W) / with motorized correction collar and motorized nosepiece. Pixel size: 256 × 256, 512 × 512, 1024 × 1024. Scanning speed: 1.1 s / frame (for pixel size 512 × 512, High Speed scanning mode) Imaging mode X, Y, Z (optical slicing), T (time lapse) Tiling Specimen holder for Mat-Teck glass bottom dishes with 35 mm diameter, glass slide, Lab-Lek NUNC II cover glass chamber (8 wells type). Incubator Temperature: 37+0.1°C, 0.5°C (can be switched off) Humidity: more than 90% CO_2 concentration: 5% (recommended).

Operation of the microscope is allowed only in the presence of the Safety Officer.

This instrument cannot be moved without a specific procedure.

Always refer to the user manual.

CONFOCAL MICROSCOPE A1R+ HD - Nikon Instruments

Brand: Nikon

Model: A1R+HD

Bought from: Nikon Instruments S.p.A., Via San Quirico 300, 50013, Campi Bisenzio, Italy

Reference: alberto.zorloni@nikon.com; giacomo.cozzi@nikon.it

Maintenance program: annual maintenance.

DESCRIPTION

The Nikon A1R+ Confocal microscope is a fully motorized laser scanning confocal microscope. The A1R+ is Nikon's powerful fully-automated confocal imaging system, capable of capturing high-quality confocal images of cells and molecular events at high speed and enhanced sensitivity. The A1R incorporates a resonant scanner with a resonance frequency of 7.8kHz that allows high-speed imaging at 420 fps (512 x 32 pixels)

It supports multi-area and multi-color imaging and, thanks to the incubator, fixed and live-cell imaging.

Lasers: 405 nm (17.1 mW), 473 nm (11.9 mW), 559 nm (15 mW), 635 nm (9.5 mW)

Fluorescence filters: DAPI (EX340-380 DM 400 BA435-485) , FITC (EX465-495 DM505 BA515-555), Texas Red (EX540-580 DM 595 BA600-660), Cy5 (EX620/60 DM660 BA700/75)

Objectives:

CFI Pian Apocromatico Lambda l OX NA 0,45 WD 4,0mm

MRD00205 CFI Pian Apocromatico Lambda 20X NA 0,75 WD 1,0mm

MRD77410 CFI Apocromatico LWD 40 Lambda S WI NA 1,15 WD 0,60mm

MRD01605 CFI Pian Apocromatico 60X Lambda Oil NA 1.4 WD 0,13mm

Supported samples:

Glass slide

35mm petri dishes

Multiwell

NUNC Labteck II

+ Universal stage

Operation of the microscope is allowed only by the Safety Officer.

This instrument cannot be moved without a specific procedure.

Always refer to the Safety Officer.

pHMETER

- Handle the electrode carefully, avoiding touching the glass membrane with your fingers;
- Before and at the end of each measurement, as well as at the end of the use, rinse the electrode with distilled water and gently dab with disposable absorbent paper;
- Before making any measurements, calibrate the pH meter as follows:
- Turn on the pH meter by pressing the ON / OFF key
- The writing "in it" appears
- Confirm with <ENTER>
- Immerse the electrode in the first buffer solution - (ph 4).
- Press the <pH CAL> key.
- The display shows the temperature measured by the temperature sensor.
- Confirm with <ENTER>
- Your first buffer solution is now measured. After rinsing the electrode, immerse it in the second buffer solution (ph 7): the symbol of the container at the bottom left of the display flashes and the message "bu 2" appears.
- Continue by pressing <ENTER>.
- The second buffer is measured.
- After calibration, the slope and the asymmetry pH (pHas) are shown on the display and the instrument is ready for pH measurements.
- If you accept the value, press <ENTER>. Otherwise, press <pH / mV / EC>. The electrode is now calibrated.
- The calibration sequence can be terminated at any time by pressing the <pH / mV / EC> key.

SYSTEM FOR BLOTTING

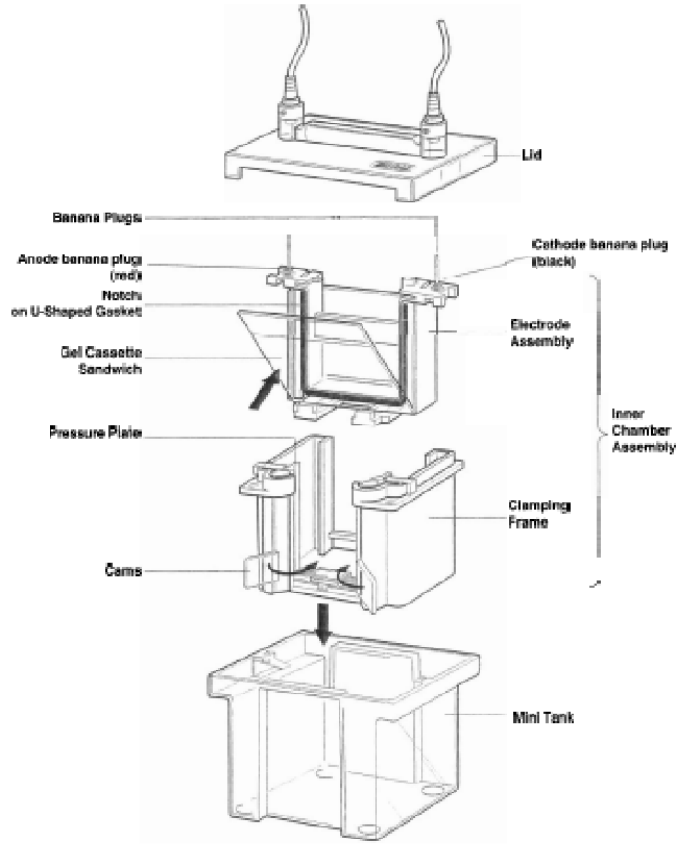


Fig. 2. Assembling the Mini-PROTEAN 3 cell.

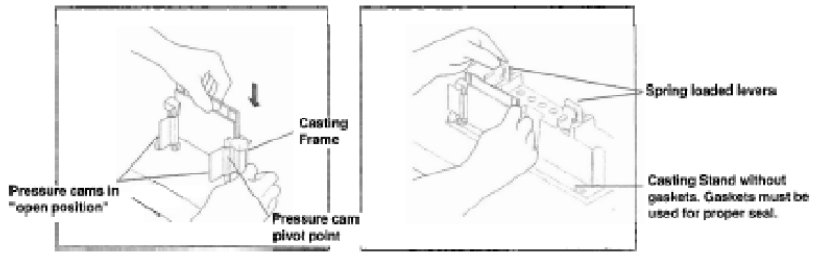


Fig. 3. Assembling the Mini-PROTEAN 3 Casting Frame and Casting Stand.

ASSEMBLY OF "CASTING FRAME and CASTING STAND"

1. Place a "short plate" on the "spacer plate"
2. Insert them in the "casting frame" holding the "short plate" on the side of the closing doors
3. Close the closing doors
4. Insert the "casting frame" in the "casting stand" and hook it with the appropriate mechanism.
5. Repeat the procedure for the second "casting frame".
6. Now load the gel (see the "GEL ELECTROPHORESIS" procedure)

ASSEMBLY OF THE CHAMBER FOR ELECTROPHORESIS

1. Remove the "sandwich" containing the gel from the "casting frames"
2. Place the "sandwiches" in the "electrode assembly" with the "short plate" facing inwards

3. Insert the "electrode assembly" in the "clamping frame".
4. Close the "clamping frame" doors by pressing the "electrode assembly" downwards (thus obtaining the "inner chamber")
5. Insert the "inner chamber" in the "mini tank"

SAMPLES LOADING

1. Fill the running buffer "mini tank" by reversing it inside the "inner chamber", until the level reaches approximately the top of the "spacer plate".
2. Now load the samples (see the "GEL ELECTROPHORESIS" procedure)

ASSEMBLY OF THE MINI TANK AND START OF THE ELECTROPHORETIC STROKE

1. Place the lid ("lid") on the "mini tank" aligning the colors of the sockets.
2. Insert the cables into the generator with appropriate polarity.
3. Set the desired amperage or voltage and start the run.

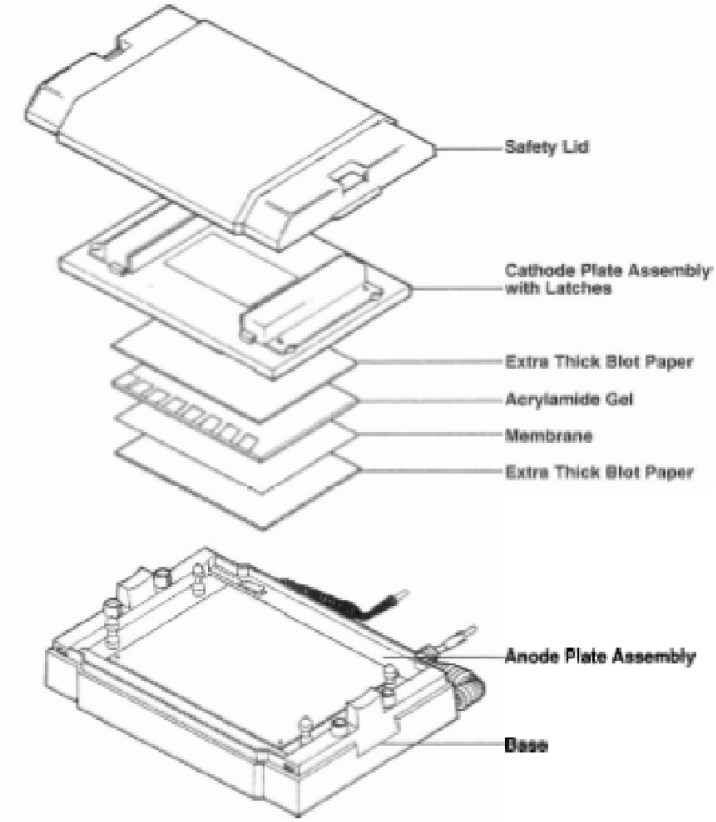
GEL REMOVAL

1. After completing the electrophoresis switch off the generator and disconnect the cables
2. Remove the cover and remove the "inner chamber"
3. Turn the running buffer over in a bowl
4. Remove the "sandwiches" and place them in the bowl
5. Remove the Gel (see "WESTERN BLOT" procedure).
6. Rinse all instruments with deionized water.

NOTES ON SAFETY

- Never invert the polarity of the instrument.
- Always rinse well with deionized water after each use.
- Wash the glass with a special glass cleaner and then rinse it well in deionized water.
- Keep away from water
- Do not work in the presence of water on the bench.
- Do not maneuver with wet hands.

TRANS-BLOT SEMI DRY



Prepare the "transfer buffer".

Cut a membrane and two pieces of filter paper of the size of the gel and wet in the "transfer buffer" for 5-10 minutes.

Remove the safety cover and prepare a "sandwich" with the gel as follows:

- Filter paper (placed directly on the anode)

- Membrane

- Gel

- Filter paper

NB: Remove the air bubbles between the layers

Put the cathode and the safety cover

Connect the "power supply"

Start the "WESTERN BLOT".

Turn off and unplug the power supply and remove the membrane.

NOTES ON SAFETY

- Never invert the polarity of the instrument.
- Do not exceed 25 volts.

- Do not modify the Ph of the transfer buffers during the use of the instrument.
- Do not exceed two hours of use of the instrument.
- Do not use the instrument in environments with temperatures above 50 ° C.
- Keep away from water
- Do not work in the presence of water on the bench.
- Do not maneuver with wet hands.

TECHNOBIOLOGY LAB INSTRUMENTATION: ATHENA LAB

BIOLOGICAL LAMINAR FLOW HOOD
Brand: TopSafe

Model: Class II (Type A2) 1.2

Purchased from: Euroclone SpA, via Figino 20/22, 20016 Pero (MI)

Contacts: sat@euroclone.it; +39.02.38.19.51

Maintenance program: annual

DESCRIPTION

The TopSafe ™ Class II biological laminar flow hood has a new microprocessor control to manage moderate to low risk biological agents. This new and innovative microprocessor control system (MAC: monitoring of the air volume of the automatic microprocessor control) provides the automatic control of the preset air flows for the entire useful life of the filters. When a deviation from the set flow is detected, the automatic control regulates the air flows accordingly, apart from the fact that the voltage or the load of the filter increases or decreases. The hood presents:

- An ergonomic design of the working area to guarantee user comfort;
- LED display, to monitor the flow parameters and the time of use;
- 3 operating modes: normal, stand-by, calibration;
- Anti-splash socket, gas and vacuum taps;
- The audible and visual alarms are activated in the event of: ventilation parameters out of the safety limits, front glass raised above the safety limit, fan failure;
- UV light (with safety lock and timer) mounted in the rear part of the hood, in front of the user;
- The exhaust and recirculation flow rates ensure 25 air changes/min in the working area and the air speed of the front barrier is ≥0.5 m/sec.

The external surfaces are in cold-rolled steel fire painting RAL 7035 and RAL 7036, the internal surfaces are exposed and the liquid collection tank is in grade 3 stainless steel and 2B finish. The front screen is made of laminated safety glass.

The operation of the hood is activated only in the presence of at least one other user besides the operator ("two people's rule").

This instrument cannot be moved without a specific procedure.

OPERATION

To operate:

- Turn the key from position 0 to position 1. At the end of the self-test, the glass will rise to the safety position. If required, to rise more press the arrow pointing upwards (on the control panel); to lower it, press the down arrow (on the control panel). These height changes can trigger the alarm;

- Disinfect the working surface and then the external glass with absorbent paper soaked in ethanol 70% v/v in water and then start the activities.
  NOTE: the area in which it is possible to work in sterility is smaller than the size of the hood (see the drawing on the control panel);
- At the end of the activities, decontaminate the working surface and then the external glass again. Lower the glass completely by pressing the arrow pointing downwards (the alarm will sound) and turn off the hood by turning the key to position 0.

To sterilize the hood (certainly at the beginning and at the end of the working day):

- Turn the key from position 0 to position 1 and press *Mode* before starting the self-test;
- Select the duration of UV sterilization (the minimum is 10 min, it is possible to increase by 10 minutes at a time) and the start time by pressing the key UV;
- Press Socket to start the previously set sterilization program.

In the case of doubt or difficulty, refer to the user manual.

CHEMICAL HOOD

Model: SafeHood 75

Purchased from: Euroclone SpA, via Figino 20/22, 20016 Pero (MI)

Contacts: sat@euroclone.it; +39.02.38.19.51

Maintenance program: annual

DESCRIPTION AND USE OF THE INSTRUMENT

- Turn on the hood flow by pressing the green button on the right side of the instrument and lift the safety glass slightly;
- Leave the hood on for 5 minutes before using it;
- Clean the worktop and safety glass with disinfectants that do not contain chlorine (e.g. ethanol 70% v/v in water);
- As far as possible, avoid keeping the containers open during work;
- Minimize the passages between inside and outside of the hood, to minimize flow turbulence;
- After completing the activities, clean the shelf and the glass with disinfectants that do not contain chlorine;

Leave the hood on for 5 minutes before lowering the glass and turning it off by pressing the green switch.

REFRIGERATED CENTRIFUGE

MODEL: 5804 R

SUPPLIER COMPANY: Eppendorf

ADDRESS: Via Zante 14, 20138, Milan (MI)

CONTACTS: 02 554041; eppendorf@eppendorf.it

ORDINARY MAINTENANCE: annual decontamination

DESCRIPTION

The equipment is designed to rotate at high speed around a central axis and separate materials of different densities, such as cells from liquids. It is refrigerated, i.e. it is possible to adjust the temperature to the desired values.

USE, CLEANING AND MAINTENANCE

- Turn on the centrifuge by pressing the button on the equipment panel;
- Open the top panel;
- Check the integrity of the tubes/tubes and their closure before inserting them in the centrifuge (position them in supports of adequate size);
- Balance the weights, possibly inserting tubes filled with water in a symmetrical position with respect to the samples;
- Set the centrifugation parameters on the control panel: use the right handle to select the parameter to be modified (for example timer, temperature, revolutions) and the left handle to change its value. To confirm the set value, gently press the right handle;
- Close the lid and press the start button;
- At the end of the process, a sound signal will be heard and it will be possible to open the centrifuge;
- Turn off the centrifuge by pressing the button on the equipment panel.
- For cleaning, use only mild detergents; if necessary, disinfect with 70% v/v ethanol-soaked paper and leave the lid open to avoid condensation;
- Lubricate periodically with the supplied oil (SG70104) the contact parts between the rotor and the compartments and between the containers and their compartments; then also lubricate the rotor pin with the supplied grease (SG70284). Do not use grease instead of oil for the lubrications mentioned above;
- Every 20 cycles of centrifugation, tighten the rotor pin with the key provided;
- In case of spillage of liquids or other substances in the centrifuge, consult the relevant section of the manual. Refer to the user manual in case of doubts or problems;

The instrument cannot be moved without a specific procedure. For displacement, refer to the Laboratory Manager.

LIQUID NITROGEN CONTAINER (DEWAR)
MODEL: Cryosystem 750

SUPPLIER COMPANY: BioApp Sa

ADDRESS: Viale del Lavoro 12 / b, 45100, Rovigo (RO)

CONTACTS: 0425. 1547882; info@bioapp.it

Maintenance program: Check the liquid nitrogen level (weekly).

DESCRIPTION

The dewar is used to store bacteria in liquid nitrogen at temperatures between -140 ° C and -196 ° C.

STANDARD PROCEDURE

- The opening of the dewar is allowed only in the presence of at least one other person in the laboratory;
- Wear appropriate PPE: gown, cryogenic gloves and visor.
- In the event of contact with improperly protected body surfaces, liquid nitrogen carries the risk of severe freezing and permanent damage;
- Ventilate the room, liquid nitrogen vapors can cause asphyxia;
- If you need to freeze the bacteria, check that the cryovials are well closed and check that are correctly labeled with at least the following information: 1) strain, 2) freezing date, 3) operator name. Check on the appropriate register where to place them and complete it with the same information shown on the vials. Unlabelled cryovials are disposed of periodically by the Laboratory Manager.
- If bacteria need to be thawed, check the register where the desired cryovials are located and indicate the operation on the appropriate register. If they are not to be thawed, do not move the cryovials without the permission of the Laboratory Manager;
- Open the dewar cap;
- Remove the additional cap;
- If you need to freeze a cryovial, lift the desired tower, lift the rod, remove the desired box and place the cryovial, limiting the leakage of liquid nitrogen. Lower the rod and place the tower in place;
- If you need to thaw a cryovial, lift the desired tower, lift the rod, remove the desired box and take the cryovial, limiting the leakage of liquid nitrogen. Lower the rod and place the tower in place. Before proceeding as indicated in the defrosting protocol, place the vial under a laminar flow biological hood and unscrew the cap to favor the escape of any trapped liquid nitrogen vapors;
- Replace the additional cap;
- Close the Dewar.

OPERATIONS, CLEANING AND MAINTENANCE

- Check the liquid nitrogen level inside the container weekly:
- Wear appropriate PPE (gown, cryogenic gloves and visor);
- Ventilate the room and take the measuring rod. For safety reasons, do not use metal bars;

- Dip the rod in the center of the dewar (be careful! The center has a hole a few cm lower than the rest of the dewar, make sure to position the rod there) and extract it after a few seconds. Shake it to disperse the vapors and mark the freezing level on the appropriate form. The level must be between 15 cm (minimum, the bacteria will be immersed in the nitrogen vapors) and 38 cm (maximum. The liquid nitrogen level will cover all the boxes. Each tower houses 5 boxes). Never exceed 40 cm;
- If the level is low, top up. Contact Oscar Bressan (oscar.bressan@polimi.it) to borrow a 10 liter dewar to withdraw liquid nitrogen from the tank outside the department. This operation is allowed only to authorized personnel. In the absence of authorized personnel, contact Oscar Bressan.

VERTICAL FRIDGE/FREEZER

MODEL: Labor 2T 400 ECT-F

SUPPLIER COMPANY: Fiocchetti (but purchased from Eppendorf)

ADDRESS: Via Zante 14, 20138, Milan (MI)

CONTACTS: 02 554041; eppendorf@eppendorf.it

DESCRIPTION AND USE OF THE INSTRUMENT

- Do not put food and drink in the compartments;
- In case of breakage/damage of the containers with leakage of liquid material, if authorized by the Laboratory Manager, clean all surfaces with paper moisted (not soaked) with 70% v/v ethanol in water ONLY if you are in the middle of the shelves and far from probes or sources of sparks;
- Notify the Laboratory Manager before transferring material to other refrigerators.

INCUBATOR FOR MICROBIOLOGY

MODEL: Innova 42 R

PURCHASED AT: Eppendorf

ADDRESS: Via Zante 14, 20138, Milan (MI)

CONTACTS: eppendorf@eppendorf.it

DESCRIPTION AND USE OF THE INSTRUMENT

• Before switching the instrument on, clean and decontaminate the incubator with a suitable disinfectant (e.g. Aldek or Pharmacidal), to be disposed properly. Do not use bleach (sodium hypochlorite). Wait about 30 seconds and dry with a sheet of absorbent paper;

• Disinfect the incubator with 70% v/v ethanol in water, thoroughly cleaning the corners of the chamber. Allow the solvent to dry/evaporate before reactivating the incubator;

• Check the pressure of the gas mixture required for the bacteria of interest in the gas cylinders. If there is gas, you can open them.

• Open the line intercept in the laboratory and check the adjustment of the gauges;

• Insert the electrical socket and check that the instrument is turned on;

• Set the desired temperature on the control panel;

• Allow the incubator to stabilize for at least 12 hours before inserting bacteria;

• Before experiments, set the desired stirring speed on the control panel, if needed;

• During the experiments, to minimize the passages between inside and outside of the incubator, so as to minimize the chances of contamination;

• After completing the experiments, turn off the incubator and leave the door open to allow for cooling;

• Close the gas line in the laboratory;

• Close the gas supply lines;

• The next day, decontaminate the incubator.

In case of difficulties or doubts, refer to the user manual.

OPTICAL INVERTED MICROSCOPE (FOR BRIGHT FIELD, PHASE CONTRAST)

MODEL: DMi1

SUPPLIER COMPANY: Leica Microsystem Srl

ADDRESS: Via Emilia 28, 20090, Buccinasco (MI)

PHONE NUMBER: 02 574861

MAIL ADDRESS: https://www.leica-microsystems.com/it/contact/contact us-online/country/IT/

ROUTINE MAINTENANCE: calibration (annual)

USE AND MAINTENANCE

Leica DMi1 is an optical microscope with an inverted frame, designed primarily for applications related to cell and tissue culture. It supports light field and phase contrast and is equipped with:

objectives: 4x, 5x, 10x, 20x, 40x;

Leica MC120 HDMI digital camera (2.5 Megapixel) and Leica MC170 (5 Megapixel) for image acquisition (with related software).

It is a precision instrument and is very delicate. Before using it, ask the Laboratory Manager to be adequately trained and refer to the user manual.

For routine use (e.g. control of cells in static conditions):

- Turn on the microscope to view samples in Bright Field (BF) or Phase Contrast (PhC) with a halogen lamp. The switch is located on the right rear side of the base;
- Lift the yellow filter (otherwise the image will appear green) by lifting the left side of the corresponding housing, ie in the tray above the condenser;
- Place the sample on the microscope table;
- Adjust the intensity with the left side wheel. The button on the right side of the microscope interrupts the illumination of the sample without turning off the halogen lamp;
- Gently rotate the swivel lens holder to insert the desired lens and then insert the corresponding light phase filter (Ph0, Ph1, PhC, Ph2 or BF) by turning the wheel on top of the condenser;
- Be careful not to hit a target against the sample, to avoid breaking or damaging the lens;
- Move the sample with the motorized table and focus it with the appropriate wheel;
- Looking through the eyepiece, gently rotate the coarse adjustment wheel on the side of the microscope base to focus the specimen. Once the approximate focus has been optimized, improve it using the fine adjustment knob.
- Operate as follows:

1. Completely tighten the octagonal diaphragm, which describes the image field, with the lever above the condenser and adjust the height of the condenser (knob) to see the contour in focus;
2. Reopen the diaphragm until you see the edges of the outline within the image field. Finally, it is also possible to center the contour with the knurled-head screws that move the part where the diaphragm is inserted;

3. Refocus the desired field using the macro and micrometric adjustment again;
4. Image acquisition is performed via software, supplied with the installed camera. On a laptop desktop equipping the microscope, there is a link to this software;
5. After observation, turn off the halogen lamp (the switch is on the back). Pay attention! It is not enough to simply press the right button, as this only interrupts the switching on of the sample, without turning off the lamp.

- The instrument cannot be moved without a specific procedure. For displacement, consult the Laboratory Manager.

SPECTROPHOTOMETER - PLATE READER

MODEL: Spark

PURCHASED FROM: Tecan Italia S.r.l.

ADDRESS: Via Zante 14, 20138, Milano (MI)

CONTACTS: 02.9244.790; tecan-it@tecan.com

SPARK is a multi-method microplate reader compatible with robotic systems. It allows to perform luminescence, absorbance and fluorescence analyzes, in addition it has functions that allow to quantify cell viability and nucleic acids/proteins present.

The housing for the plates can move either horizontally (in the x, y direction) or vertically (in the z direction). It is possible to measure from above or below and there are three modes of agitation: linear, orbital and double orbital. It is possible to heat and / or cool the samples.

ROUTINE MAINTENANCE: cleaning of injectors and supports (monthly. See user manual, chapter 7). General inspection by a specialized technician (annual).

DESCRIPTION AND USE OF THE INSTRUMENT

- Turn on the software of the instrument installed on the PC and then turn on the instrument with the appropriate rear button;
- To start a method, click on the Start button from the editor. Once started, the software will switch to the Dashboard view, from which you can select the corresponding method box. Alternatively (if the method has been previously saved), open the desired method and select Start from the tool via the File menu of the editor. To view the progress of a measurement, open the Dashboard and select the Instrument panel;
- Absorbance: it is possible to measure the absorbance of the samples for wavelengths between 200 nm and 1000 nm, selectable in steps of 1 nm.
  Use microplates of transparent radiation material in use; for example, in the case of UV radiation, use UV transparent microplates. For high optical density (OD) values, black microplates with a transparent bottom are more suitable. In general, to obtain accurate results it is preferable to avoid measurements above 3 OD;
- Cell counting module: it is possible to perform cell counting and determine the percentage of live cells. The confluence value indicates the percentage of surface (for example, of the wells of the 6 to 96-well plate) covered by the adherent cells;
- Module for the evaluation of proteins and nucleic acids: the NanoQuant plate is designed for the quantification of nucleic acids and proteins in a volume of 2 µl, using absorbance as a detection mode;
- There are two applications optimized for routine analysis of nucleic acids:
  - the NanoQuant quantification application, used for the quantification of nucleic acids at 260 nm and to obtain information on the concentration and purity of the sample;

  - the labeling efficiency application also provides information on the concentration of the marker or markers used;

- The results are automatically exported to Office Open XML format (.xlsx) and saved in C: Users Public Documents CanControl Export xlsx (default path) or in the path defined by the user. Depending on the Result presentation settings (see chapter Data Management in the user manual), the results can be opened automatically after the method is executed;

For more details, refer to the user manual.

TECHNOBIOLOGY LAB INSTRUMENTATION: MINERVA LAB

VORTEX MIXER

MODEL: Vortex Mixer
COMPANY: Gilson
ADDRESS: via Matteotti 98, Cinisello Balsamo (MI)
CONTACTS: 02.6607.041; supporto-it@gilson.com; service-it@gilson.com

- Operate only with tightly closed tubes;
- Turn the instrument on using the black switch in the "manual" position and pressing the tube support perpendicularly. The shaker stops automatically when the tube is lifted;
- When the switch is in "continuous" mode, the agitation is independent of the position of the tube. The stirring frequency can be selected by turning the right handle.

NOTES: Avoid placing the instrument on the shelf where the scale or multi-method plate reader are located.

MEA
MultiChannelSystems

Model: MEA1060 + TC02 + PH01 + MEAS2/1 + PS20W

Year of fabrication: 2003

Purchased by: Multi Channel Systems MCS GmbH, Reutlingen, Germany. TPhone: +49 7121 90925 25, Fax: +49 7121 90925 11, Email: sales@multichannelsystems.com / support@multichannelsystems.com

Contacts for maintenance: Multi Channel Systems, support@multichannelsystems.com

DESCRIPTION

The MEA60-System is a complete system for in vitro recording from microelectrode arrays (MEAs), MEA perfusion, and data acquisition from up to 64 channels. Raw data from up to 60 MEA electrodes is amplified by a MEA1060 amplifier, and then acquired by the MC Card of the connected computer. Recorded data is graphed, analyzed, revised, and exported with powerful and easy-to-use MC_Rack. The temperature controller TC01/02 regulates the MEA temperature. MEA60-System-E is an alternative version of the system with an extended perfusion system, featuring a perfusion cannula PH01 with programmable fluid temperature and a two-channel temperature controller TC02.

STANDARD PROCEDURE

The instrument **cannot** be used by your own. Do refer always to the referent personnel.

BIOLOGICAL LAMINAR FLOW HOOD

Brand: TopSafe

Model: Class II (Type A2) 1.2

Purchased from: Euroclone SpA, via Figino 20/22, 20016 Pero (MI)

Contacts: sat@euroclone.it; +39.02.38.19.51

Maintenance program: annual

DESCRIPTION

The TopSafe ™ Class II biological laminar flow hood has a new microprocessor control to manage moderate to low risk biological agents. This new and innovative microprocessor control system (MAC: monitoring of the air volume of the automatic microprocessor control) provides the automatic control of the preset air flows for the entire useful life of the filters. When a deviation from the set flow is detected, the automatic control regulates the air flows accordingly, apart from the fact that the voltage or the load of the filter increases or decreases. The hood presents:

- An ergonomic design of the working area to guarantee user comfort;
- LED display, to monitor the flow parameters and the time of use;
- 3 operating modes: normal, stand-by, calibration;
- Anti-splash socket, gas and vacuum taps;
- The audible and visual alarms are activated in the event of: ventilation parameters out of the safety limits, front glass raised above the safety limit, fan failure;
- UV light (with safety lock and timer) mounted in the rear part of the hood, in front of the user;
- The exhaust and recirculation flow rates ensure 25 air changes/min in the working area and the air speed of the front barrier is ≥0.5 m/sec.

The external surfaces are in cold-rolled steel fire painting RAL 7035 and RAL 7036, the internal surfaces are exposed and the liquid collection tank is in grade 3 stainless steel and 2B finish. The front screen is made of laminated safety glass.

The operation of the hood is activated only in the presence of at least one other user besides the operator ("two people's rule").

This instrument cannot be moved without a specific procedure.

OPERATION

To operate:

- Turn the key from position 0 to position 1. At the end of the self-test, the glass will rise to the safety position. If required, to rise more press the arrow pointing upwards (on the control panel); to lower it, press the down arrow (on the control panel). These height changes can trigger the alarm;
- Disinfect the working surface and then the external glass with absorbent paper soaked in ethanol 70% v/v in water and then start the activities.

NOTE: the area in which it is possible to work in sterility is smaller than the size of the hood (see the drawing on the control panel);

- At the end of the activities, decontaminate the working surface and then the external glass again. Lower the glass completely by pressing the arrow pointing downwards (the alarm will sound) and turn off the hood by turning the key to position 0.

To sterilize the hood (certainly at the beginning and at the end of the working day):

- Turn the key from position 0 to position 1 and press *Mode* before starting the self-test;
- Select the duration of UV sterilization (the minimum is 10 min, it is possible to increase by 10 minutes at a time) and the start time by pressing the key UV;
- Press Socket to start the previously set sterilization program.

In the case of doubt or difficulty, refer to the user manual.

Purchased from: Euroclone SpA, via Figino 20/22, 20016 Pero (MI)

Contacts: sat@euroclone.it; +39.02.38.19.51

Maintenance program: annual

DESCRIPTION AND USE OF THE INSTRUMENT

- Turn on the hood flow by pressing the green button on the right side of the instrument and lift the safety glass slightly;
- Leave the hood on for 5 minutes before using it;
- Clean the worktop and safety glass with disinfectants that do not contain chlorine (e.g. ethanol 70% v/v in water);
- As far as possible, avoid keeping the containers open during work;
- Minimize the passages between inside and outside of the hood, to minimize flow turbulence;
- After completing the activities, clean the shelf and the glass with disinfectants that do not contain chlorine;
- Leave the hood on for 5 minutes before lowering the glass and turning it off by pressing the green switch.

PCR HOOD
Model: Aura PCR

Purchased from: Euroclone SpA, via Figino 20/22, 20016 Pero (MI)

Contacts: sat@euroclone.it; +39.02.38.19.51

Maintenance program: annual

DESCRIPTION

Aura PCR is a very easy to operate cabinet specifically designed for the containment of DNA carry-over and subsequent UV-based neutralisation.

- The main structure and the front window of the cabinet are made of 6 mm tempered glass
- The top cover is made of cold rolled steel with stove enamel coating
- Under the cover, three powerful UV lights optimize the neutralisation cycle
- The work surface is made of polyethylene 100% UV proof

OPERATING MODE

In order to expose all internal surfaces and tools to UV radiation for a preset period of time, the normal working procedures have to be completed, the tempered glass front panel has to be closed and timer can be activated.

The 6 mm tempered glass body of the Aura PCR cabinet, acting as a filter to UV radiation, is safely protecting the external environment from dangerous exposure of personnel to those radiations. Once the preset count-down time is over, the UV are automatically switched off whereas the fluorescent lights are turned on. The operator can therefore resume his job. Front window opening is interlocked with the UV lights that are automatically turned off in case of unwanted opening for total operator safety.

TERMOCYCLER

MODEL: Mastercycler X50s

PURCHASED AT: Eppendorf

ADDRESS: Via Zante 14, 20138, Milan (MI)

CONTACTS: eppendorf@eppendorf.it

DESCRIPTION:

The block temperature regulation gives rise to the next stage of PCR reproducibility, whereas the adaptable user management and profound documentation capabilities give peace of mind to laboratories working to set standards. It has an intuitive touch display, low noise levels, low power consumption, and the versatile flexlid® concept complete the product to be a powerful PCR cycler. Up to 10 PCR cyclers can be combined – ideal for high throughput applications or labs with a high number of users running different assays. Should you feel you need more flexibility or throughput, up to 50 PCR cyclers can be combined in a computer-controlled network.

NOTE: Use only PCR tubes (capacity 0.2 mL) or 96 well-PCR plates.

POWER PRO 3AMP

MODEL: 3AMP, CLEAVER

SUPPLIED BY: AUROGENE

ADDRESS: Via dei Lucani 51-55, 00185, Roma (RM)

CONTACTS: phone: 06/98185510; mail: technical@aurogene.eu

DESCRIPTION:

At 300V, 3000mA, 300W, the PowerPro 3AMP is designed for virtually all high current electrophoresis applications. The PowerPro 3AMP's higher current output capability is perfect for electroblotting units with high-intensity plate electrodes.

Electrotransfers may be performed as timed runs in constant or programmable mode to prevent overheating and buffer depletion. A run time extendable to a maximum 999 minutes in constant mode is also useful for overnight transfers undertaken at constant low current in wire electrode systems.

The PowerPro 3AMP shares the built-in protocol and constant parameter features of the other

REAL TIME-PCR INSTRUMENT

MODEL: LineGene 9600 Series, BIOER

SUPPLIED BY: AUROGENE

ADDRESS: Via dei Lucani 51-55, 00185, Roma (RM)

CONTACTS: phone: 06/98185510; mail: technical@aurogene.eu

DESCRIPTION:

LineGene 9600 is Bioer technology fluorescence quantitative PCR detection system of the new products. It offers a large sample size, detection channel and wider temperature range.

LineGene 9600 has a large sample size of up to 96 samples and the ultrafast two-color scanning speed. New design of up to eight channels of the LineGene 9600 covering the wavelength range of fluorescence detection of all.

NOTE: Use only optical PCR 8-Tube Strips or optical PCR plates with optical adhesive sealing film.

REFRIGERATED CENTRIFUGE

MODEL: 5804 R

SUPPLIER COMPANY: Eppendorf

ADDRESS: Via Zante 14, 20138, Milan (MI)

CONTACTS: 02 554041; eppendorf@eppendorf.it

ORDINARY MAINTENANCE: annual decontamination

DESCRIPTION

The equipment is designed to rotate at high speed around a central axis and separate materials of different densities, such as cells from liquids. It is refrigerated, i.e. it is possible to adjust the temperature to the desired values.

USE, CLEANING AND MAINTENANCE

- Turn on the centrifuge by pressing the button on the equipment panel;
- Open the top panel;
- Check the integrity of the tubes/tubes and their closure before inserting them in the centrifuge (position them in supports of adequate size);
- Balance the weights, possibly inserting tubes filled with water in a symmetrical position with respect to the samples;
- Set the centrifugation parameters on the control panel: use the right handle to select the parameter to be modified (for example timer, temperature, revolutions) and the left handle to change its value. To confirm the set value, gently press the right handle;
- Close the lid and press the start button;
- At the end of the process, a sound signal will be heard and it will be possible to open the centrifuge;
- Turn off the centrifuge by pressing the button on the equipment panel.
- For cleaning, use only mild detergents; if necessary, disinfect with 70% v/v ethanol-soaked paper and leave the lid open to avoid condensation;
- Lubricate periodically with the supplied oil (SG70104) the contact parts between the rotor and the compartments and between the containers and their compartments; then also lubricate the rotor pin with the supplied grease (SG70284). Do not use grease instead of oil for the lubrications mentioned above;
- Every 20 cycles of centrifugation, tighten the rotor pin with the key provided;
- In case of spillage of liquids or other substances in the centrifuge, consult the relevant section of the manual. Refer to the user manual in case of doubts or problems;

The instrument cannot be moved without a specific procedure. For displacement, refer to the Laboratory Manager.

CENTRIFUGE FOR MINI-TUBES

MODEL: Labor 2T 400 ECT-F touch

SUPPLIER COMPANY: Eppendorf

ADDRESS: Via Zante 14, 20138, Milan (MI)

CONTACTS: 02 554041; eppendorf@eppendorf.it

ORDINARY MAINTENANCE: annual decontamination

DESCRIPTION

The equipment is designed to rotate at high speed around a central axis and separate small volumes of materials of different densities, such as cells from liquids. It is refrigerated, i.e. it is possible to adjust the temperature to the desired values (from -9°C to 40°C).

USE, CLEANING AND MAINTENANCE

- Turn on the centrifuge with the appropriate button on the side of the appliance;
- Open the top panel and lift the lid;
- Check the integrity of the mini-tubes and their closure before inserting them in the centrifuge, positioning the hinge of the cap towards the inside;
- Balance the weights, possibly inserting tubes containing water in a symmetrical position with respect to the samples to be balanced;
- Replace the cover;
- Set the centrifugation parameters on the control panel: use the right handle to select the parameter to be modified (for example timer, temperature, revolutions) and the left handle to change its value. To confirm the set value, gently press the corresponding handle. It is also possible to perform a short, or centrifuge at increasing speed up to the maximum value for a few seconds (for example, to "spin" the samples or recover an antibody on the bottom);
- Press the start button;
- At the end of the process, a sound signal will be heard and it will be possible to open the centrifuge;
- Turn off the centrifuge by pressing the button on the equipment panel.
- For cleaning, use only mild detergents; if necessary, disinfect with 70% ethanol-soaked paper and leave the lid open to avoid condensation;
- Lubricate periodically with the supplied oil (SG70104) the contact parts between the rotor and the compartments and between the containers and their compartments; then also lubricate the rotor pin with the supplied grease (SG70284). Do not use grease instead of oil for the lubrications mentioned above;
- Every 20 cycles of centrifugation, tighten the rotor pin with the key provided;
- In case of spillage of liquids or other substances in the centrifuge, consult the relevant section of the manual. Refer to the user manual in case of doubts or problems;
- The instrument cannot be moved without a specific procedure. For displacement, refer to the Laboratory Manager.

FREEZER -80 ° C

MODEL: Cryocube F740i

SUPPLIER COMPANY: Eppendorf

ADDRESS: Via Zante 14, 20138, Milan (MI)

CONTACTS: 02 554041; eppendorf@eppendorf.it

ROUTINE MAINTENANCE: filter cleaning and control of the formation of ice on the seals (monthly)

USE, CLEANING AND MAINTENANCE

- Never insert unlabeled materials.
- Label any sample with 1) operator's name, 2) description of the biological origin of the preserved material, 3) indications of the potential biological and chemical risk, 4) date of insertion. Materials that are not labeled or incorrectly labeled will be periodically disposed by the Laboratory Manager;
- Wearing cryogenic gloves before opening the freezer door and taking materials, the very low temperature can cause cold burns in case of direct contact;
- In the event of spilled material, notify the Laboratory Manager. Clean all surfaces with 70% v/v ethanol in water, keeping at the center of the shelves and away from probes or sources of sparks;
- To avoid loss of efficiency or equipment failure, it is necessary to perform a monthly cleaning of the condenser filter to remove the deposited dust. To remove the filter, refer to the user manual;
- Make sure that there is no ice on the two seals of the outer door and outside the inside door of the freezer. The formation of ice on these components prevents the maintenance of the internal temperature. The device will continue to operate without interruptions, but will probably not be able to reach -80 ° C;
- For other operations, refer to the user manual.

LIQUID NITROGEN CONTAINER (DEWAR)

BRAND: MVE

MODEL: Cryosystem 750

SUPPLIER COMPANY: BioApp Sa

ADDRESS: Viale del Lavoro 12 / b, 45100, Rovigo (RO)

CONTACTS: 0425. 1547882; info@bioapp.it

Maintenance program: Check the level of liquid nitrogen (weekly)

DESCRIPTION

The dewar is used to store cells in liquid nitrogen at temperatures between -140 ° C and -196 ° C.

OPERATION

- The opening of the dewar is allowed only in the presence of at least one other person in the laboratory;
- Wear appropriate PPE: gown, cryogenic gloves and visor.
- In the event of contact with improperly protected body surfaces, liquid nitrogen carries the risk of severe freezing and permanent damage;
- Ventilate the room, liquid nitrogen vapors can cause asphyxia;
- Before freezing the cells, check that the cryovials are well closed and check that they are correctly labeled with at least the following information: 1) type of cells, 2) date of freezing, 3) number of passages, 4) number of frozen cells, 5) name of the operator. Check on the appropriate register where to place them and complete it with the same information shown on the vials. Periodically, unlabelled cryovials are disposed by the Laboratory Manager.
- Before thawing, check the register to find where the desired cryovials are located and indicate thawing on the appropriate register. If they are not to be thawed, do not move the cryovials without the permission of the Laboratory Manager;
- Open the dewar cap;
- Remove the additional cap;
- If you need to freeze a cryovial, lift the desired tower, lift the rod, remove the desired box and place the cryovial, limiting the leakage of liquid nitrogen. Lower the rod and place the tower in place;
- If you need to thaw a cryovial, lift the desired tower, lift the rod, remove the desired box and take the cryovial, limiting the leakage of liquid nitrogen. Lower the rod and place the tower in place. Before proceeding as indicated in the defrosting protocol, place the vial under a laminar flow biological hood and unscrew the cap to favor the escape of any trapped liquid nitrogen vapors;
- Replace the additional cap;
- Close the Dewar.

MAINTENANCE

- Check the liquid nitrogen level inside the container weekly:

- Wear appropriate PPE (gown, cryogenic gloves and visor);
- Ventilate the room and take the measuring rod. For safety reasons, do not use metal bars;
- Dip the rod in the center of the dewar (be careful! The center has a hole a few cm lower than the rest of the dewar, make sure to position the rod there) and extract it after a few seconds. Shake it to disperse the vapors and mark the freezing level on the appropriate form. The level must be between 15 cm (minimum, the cells will be immersed in the nitrogen vapors) and 38 cm (maximum. The liquid nitrogen level will cover all the boxes. Each tower houses 5 boxes). Never exceed 40 cm;
- If the level is low, top up. Contact Oscar Bressan (oscar.bressan@polimi.it) to borrow a 10 liter dewar to withdraw liquid nitrogen from the tank outside the department. This operation is allowed only to authorized personnel. In the absence of authorized personnel, contact Oscar Bressan.

VERTICAL FRIDGE/FREEZER

MODEL: Labor 2T 400 ECT-F

SUPPLIER COMPANY: Fiocchetti (but purchased from Eppendorf)

ADDRESS: Via Zante 14, 20138, Milan (MI)

CONTACTS: 02 554041; eppendorf@eppendorf.it

DESCRIPTION AND USE OF THE INSTRUMENT

- Do not put food and drink in the compartments;
- In case of breakage/damage of the containers with leakage of liquid material, if authorized by the Laboratory Manager, clean all surfaces with paper moisted (not soaked) with 70% v/v ethanol in water ONLY if you are in the middle of the shelves and far from probes or sources of sparks;
- Notify the Laboratory Manager before transferring material to other refrigerators.

INCUBATOR FOR CELL CULTURES WITH OXYGEN AND CARBON DIOXIDE CONTROL

MODEL: Steri-Cycle i160

PURCHASED BY: Thermo-Fisher Scientific, SS Rivoltana, 20029, Rodano (MI)

CONTACTS: it.fisher@thermofisher.com; +39.02.950.59.479

Maintenance program: monthly decontamination; annual revision

DESCRIPTION

The incubator is a device used to grow and maintain cell cultures. Check the temperature, humidity and level of carbon dioxide and oxygen.

OPERATION

- Before turning it on, clean and decontaminate the incubator. Remove the water tank and the shelves. Decontaminate with a suitable disinfectant (eg Aldek or Pharmacidal), to be disposed properly. Do not use bleach (sodium hypochlorite). Wait for about 30 seconds and dry with a sheet of absorbent paper;
- Disinfect the incubator with 70% v/v ethanol in water, thoroughly cleaning the corners of the chamber. Be careful not to spray alcohol on the sensors;
- It is also possible to perform an automatic sterilization cycle already set in the incubator. At the end of the cycle, let the incubator cool to room temperature before activating;
- Fill the tank with distilled water (about 2 liters) and add a suitable disinfectant (follow the manufacturer's instructions for the disinfectant doses to be added);
- Check the carbon dioxide pressure in the cylinders. If there is gas, open them. In the case that the cylinder is empty, contact lucio.ogliani@polimi.it (02.2399.3233);
- Open the carbon dioxide line interface in the laboratory and check the manometer settings. The optimal pressure of the wall line must be set to 1 bar. Values above 1 bar may lead to the detachment of the service pipe and asphyxia from carbon dioxide;
- If using the incubator in oxygen control, open the intercept of the nitrogen line. Again, the optimal pressure should be set to 1 bar;
- Insert the electrical socket and check that the instrument is turned on;
- Using the control panel, set the temperature to 37 ° C;
- Using the control panel, set the carbon dioxide concentration to 5% and oxygen concentration to the desired level. Perform the automatic zero procedure to calibrate the instrument (REFER TO THE USER MANUAL). NOTE: the incubator does not take carbon dioxide and nitrogen until it has reached 37 ° C;
- Allow the incubator to stabilize for at least 12 hours before storing the cells;
- After completing cell culture experiments, turn off the incubator and leave the door open to cool;
- Close the gas line in the laboratory;
- Close the gas supply lines;
- The next day, decontaminate the incubator.

In case of difficulties or doubts, refer to the user manual.

OPTICAL INVERTED MICROSCOPE (FOR BRIGHT FIELD, PHASE CONTRAST)
MODEL: DMi1

SUPPLIER COMPANY: Leica Microsystem Srl

ADDRESS: Via Emilia 28, 20090, Buccinasco (MI)

PHONE NUMBER: 02 574861

MAIL ADDRESS: https://www.leica-microsystems.com/it/contact/contact us-online/country/IT/

ROUTINE MAINTENANCE: calibration (annual)

USE AND MAINTENANCE

Leica DMi1 is an optical microscope with an inverted frame, designed primarily for applications related to cell and tissue culture. It supports light field and phase contrast and is equipped with:

objectives: 4x, 5x, 10x, 20x, 40x;

Leica MC120 HDMI digital camera (2.5 Megapixel) and Leica MC170 (5 Megapixel) for image acquisition (with related software).

It is a precision instrument and is very delicate. Before using it, ask the Laboratory Manager to be adequately trained and refer to the user manual.

For routine use (e.g. control of cells in static conditions):

- turn on the microscope to view samples in Bright Field (BF) or Phase Contrast (PhC) with a halogen lamp. The switch is located on the right rear side of the base;
- lift the yellow filter (otherwise the image will appear green) by lifting the left side of the corresponding housing, ie in the tray above the condenser;
- place the sample on the microscope table;
- adjust the intensity with the left side wheel. The button on the right side of the microscope interrupts the illumination of the sample without turning off the halogen lamp;
- gently rotate the swivel lens holder to insert the desired lens and then insert the corresponding light phase filter (Ph0, Ph1, PhC, Ph2 or BF) by turning the wheel on top of the condenser;
- Be careful not to hit a target against the sample, to avoid breaking or damaging the lens;
- Move the sample with the motorized table and focus it with the appropriate wheel;
- Looking through the eyepiece, gently rotate the coarse adjustment wheel on the side of the microscope base to focus the specimen. Once the approximate focus has been optimized, improve it using the fine adjustment knob.
- Operate as follows:
  Completely tighten the octagonal diaphragm, which describes the image field, with the lever above the condenser and adjust the height of the condenser (knob) to see the contour in focus;

  Reopen the diaphragm until you see the edges of the outline within the image field. Finally, it is also possible to center the contour with the knurled-head screws that move the part where the diaphragm is inserted;

  Refocus the desired field using the macro and micrometric adjustment again;

Image acquisition is performed via software, supplied with the installed camera. On a laptop desktop equipping the microscope, there is a link to this software;

After observation, turn off the halogen lamp (the switch is on the back). Pay attention! It is not enough to simply press the right button, as this only interrupts the switching on of the sample, without turning off the lamp.

- The instrument cannot be moved without a specific procedure. For displacement, consult the Laboratory Manager.

SPECTROPHOTOMETER - PLATE READER
MODEL: Spark

PURCHASED FROM: Tecan Italia S.r.l.

ADDRESS: Via Zante 14, 20138, Milano (MI)athe

CONTACTS: 02.9244.790; tecan-it@tecan.com

SPARK is a multi-method microplate reader compatible with robotic systems. It allows to perform luminescence, absorbance and fluorescence analyzes, in addition it has functions that allow to quantify cell viability and nucleic acids/proteins present.

The housing for the plates can move either horizontally (in the x, y direction) or vertically (in the z direction). It is possible to measure from above or below and there are three modes of agitation: linear, orbital and double orbital. It is possible to heat and / or cool the samples.

ROUTINE MAINTENANCE: cleaning of injectors and supports (monthly. See user manual, chapter 7). General inspection by a specialized technician (annual).

DESCRIPTION AND USE OF THE INSTRUMENT

- Turn on the software of the instrument installed on the PC and then turn on the instrument with the appropriate rear button;
- To start a method, click on the Start button from the editor. Once started, the software will switch to the Dashboard view, from which you can select the corresponding method box. Alternatively (if the method has been previously saved), open the desired method and select Start from the tool via the File menu of the editor. To view the progress of a measurement, open the Dashboard and select the Instrument panel;
- Absorbance: it is possible to measure the absorbance of the samples for wavelengths between 200 nm and 1000 nm, selectable in steps of 1 nm.
  Use microplates of transparent radiation material in use; for example, in the case of UV radiation, use UV transparent microplates. For high optical density (OD) values, black microplates with a transparent bottom are more suitable. In general, to obtain accurate results it is preferable to avoid measurements above 3 OD;
- Fluorescence: it is possible to select the excitation and emission wavelengths using the monochromator or the filter option. The two modes monochromator and filter can be combined separately for excitation and emission.

Intensità di fluorescenza Cima

Parametri	Monocromatore	Filtro
Intervallo di lunghezza d'onda	Eccitazione: 230 – 900 nm Emissione: 280 - 900 nm, selezionabile a passi di 1 nm	Eccitazione: 230 – 900 nm Emissione: 230 – 900 nm

Intensità di fluorescenza Fondo (monocromatore e opzione filtro)

Parametri	Fibra Fondo standard	Fibra Fondo ottimizzata per UV
Intervallo di lunghezza d'onda	**Monocromatore e filtro:** 350 - 900 nm, selezionabile a passi di 1 nm (solo monocromatore)	**Monocromatore** Eccitazione: 230 – 900 nm Emissione: 280 - 900 nm, selezionabile a passi di 1 nm **Filtro** Eccitazione: 230 – 900 nm Emissione: 230 – 900 nm

- Luminescence: the standard module allows for the integral measurement of a luminescence signal (without distinction between emission wavelengths) and can be used with all microplate formats, including the 384-well plate format. The advanced module allows to perform all the multicolored applications available, as well as fast and high sensitivity luminescence scans and can be used with all microplate formats supported by the instrument.

Parametri	Modulo standard per luminescenza	Modulo avanzato per luminescenza
Intervallo di lunghezza d'onda	370-700 nm	370-700 nm
Intervallo di lunghezza d'onda per scansione in luminescenza	N/A	390-660 nm

- Cell counting module: it is possible to perform cell counting and determine the percentage of live cells. The confluence value indicates the percentage of surface (for example, of the wells of the 6 to 96-well plate) covered by the adherent cells;
- Module for the evaluation of proteins and nucleic acids: the NanoQuant plate is designed for the quantification of nucleic acids and proteins in a volume of 2 µl, using absorbance as a detection mode;
- There are two applications optimized for routine analysis of nucleic acids:
  - the NanoQuant quantification application, used for the quantification of nucleic acids at 260 nm and to obtain information on the concentration and purity of the sample;

  - the labeling efficiency application also provides information on the concentration of the marker or markers used;

- The results are automatically exported to Office Open XML format (.xlsx) and saved in C: Users Public Documents CanControl Export xlsx (default path) or in the path defined by the user. Depending on the Result presentation settings (see chapter Data Management in the user manual), the results can be opened automatically after the method is executed;
- For more details, refer to the user manual.

UV IMAGING SYSTEM
MODEL: FIRE-READER© V10

PURCHASED FROM: UVItec Limited - Cambridge

ADDRESS: Unit 36, St. John's Innovation Center, Cowley Road, Cambridge CB4 oWS - UK

CONTACTS: +39 3519238600; uvitec.italia@uvitec.co.uk

The FireReader V10 is a multi-applications, full imaging platform, offering up to 7 different illuminations. Boasting huge 6- megapixel sensor, the FireReader geldoc system delivers up to 65,535 gray levels, ideal for publication-level images and research-level data.

Its main applications are fluorescence imaging for DNA and RNA gels probed with fluorescent stains (e.g. GelRed, Ethidium Bromide), epi fluorescence (Gels and Blots with GFP, FITC, Alexa 488), and visible imaging for colorimetry and protein gels (e.g. Coomassie Blue, Cooper, Silver-stained, Red Ponceau, Petri dish colonies).

ROUTINE MAINTENANCE: clean gel support with water after each use.

DESCRIPTION AND USE OF THE INSTRUMENT:

- Turn on the instrument and UVITEC 1D software before use.
- There are two possible Application Selectors for imaging analysis: UV-gel for UV excited gel samples, and White Light for colorimetric samples.
- There is a safety triangle button outside the instrument chamber, that is for safety UV use. If requested, it is possible to inactivate the safety system in order to directly see the gel result without the use of the monitor.
- The instrument allows a live visualization of the image ("Live mode").
- During image acquisition, the software allows to adjust zoom, focus and exposure.
- Acquire and save the image.
- For details, refer to the user manual.

CHEMILUMINESCENCE IMAGING SYSTEM
MODEL: ALLIANCE ADVANCED

PURCHASED FROM: UVItec Limited - Cambridge

ADDRESS: Unit 36, St. John's Innovation Center, Cowley Road, Cambridge CB4 oWS - UK

CONTACTS: +39 3519238600; uvitec.italia@uvitec.co.uk

The Alliance Q9 Advanced is a chemiluminescence imaging system, boasting the highest optics specifications and sensitivity on the market. The Q9 Advanced is fully automated, expandable and tailored to desired workflow, upgradeable through plug-n-play technology.

Its main application is chemiluminescence analysis of Western Blot/Dot Blot membranes.

ROUTINE MAINTENANCE: clean blot support with water after each use to avoid background signals detection due to contamination of the tray.

DESCRIPTION AND USE OF THE INSTRUMENT:

- Turn on the instrument and ALLIANCE software before use.
- The instrument takes almost three minutes to get ready, to cool the camera.
- The camera starts cooling process when the software is opened.
- Do not switch off the instrument if someone has to use it right after your analysis.
- The software allows two chemiluminescence protocols: the standard protocol and the "weak signal" protocol, for weak signals detection.
- It is possible to acquire the image in automatic, manual or serial mode.
- After parameters setting, the software shows a preview, after which it is possible to start the ECL acquisition.
- After image acquisition, the software allows to obtain 3D representation of signals, background adjustment, merging of molecular weight marker, and comparison of two different acquisitions.
- The ALLIANCE software provides for the quantitative Western Blot analysis and electrophoretic distance analysis.
- For details refer to the user guide.

Assays and Manual Protocols

Part II-CULTURE OF BIOLOGICAL AGENTS

Version: October 2019

CELL CULTURE

The **primary cells** are obtained from tissues using a wide range of procedures, which depend on the nature of the tissue and the cells:

1) migration of cells from tissue fragments and their adhesion to a substrate (usually
2) polystyrene for cell cultures);
3) mechanical disintegration of fabrics for shredding;
4) enzymatic breakdown of tissues.

The cells obtained using these techniques constitute a heterogeneous population: the culture conditions (type of media, additives, serum concentration etc.) can be manipulated to optimize the system and promote the growth of the type of cells initially required or other types of cells obtained.

Strictly speaking, a cell culture is defined as primary only when the cells are in toto resulting from an initial isolation procedure; once the cells have been passed / completed a cell cycle, (i.e. subcultured), the cultures should be referred to as secondary, tertiary; by convention, all crops that have completed the third division, or those subsequent to the third, are defined as "tertiary".

Non-primary cells include: cells obtained from passage of primary cells using trypsin; cells previously isolated from cryopreserved primary sources under nitrogen and resuscitated for use; cell lines that are commercially available: these cells have previously been immortalized chemically or virally.

The methods described in this manual are basic methods valid for most of the cells used in the laboratory: in any case, ask your coordinator for instructions.

Even though not indicated in every specific protocol, always work in sterile conditions with cells.

When mixing different solutions, change the pipette or tip when moving from a solution to another.

Label everything (solutions, flasks, tubes…). Indicate the content, date and operator’s name.

Unlabelled objects are periodically disposed by the Safety Officer.

PREPARATION OF TRYPSIN OR TRYPSIN/EDTA (ETHYLENEDIAMINETETRAACETIC ACID) SOLUTION

DESCRIPTION

Adherent cells grow as a monolayer or sheet attached to a substrate. Trypsin breaks or gently separates intracellular and cell-to-substrate links by creating a single cell suspension from which new subcultures can be obtained. EDTA is a chelating agent that binds calcium and magnesium ions that may inhibit trypsin activity.

CHEMICALS AND REAGENTS

Reagents	Quantity	Stored
Trypsin 10X (Sigma Aldrich; T4674)	50 ml	Freezer
Sterile solution NaCl 0.9% in water or PBS	500 ml	Cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Stored
500 ml filtering system (Corning, 430769)	1	Cabinet
5ml Pipettes	3	Cabinet
25 ml Pipettes	1	Cabinet
15 ml Tubes	50	Cabinet

PROCEDURE

Estimate the total volume of trypsin or trypsin/EDTA needed. Roughly, to detach cells you will need 0.5 ml of working solution for a T25 flask, 1 ml for a T75 flask or 2 ml for a T150 flask.

STOCK SOLUTION

Usually the stock solution of trypsin/EDTA contains 25 g trypsin/l (we call this “stock solution 10X”).

Check the trypsin concentration on the commercial datasheet and aliquot in 10 ml aliquots indicating the correct value of the dilution needed to obtain the working solution. For example, with the Euroclone M0920D product you can aliquot directly without dilution.

WORKING SOLUTION

The working concentration is 2.5 g trypsin/l (we call this working solution 1X). To obtain 1X trypsin (or trypsin/EDTA) from 10X trypsin (or trypsin/EDTA), the starting solution has to be diluted 1:10 v/v with sterile NaCl 0.9% or PBS. If you have to dilute 1:10 v/v, basically,

1. Thaw 1 aliquot of 10X trypsin to room temperature (trypsin basic solution: 25 g trypsin/l);
2. In a new 15ml tube, put 1 ml of 10X trypsin and add 9 ml of sterile NaCl 0.9% or PBS;
3. Repeat the procedure for the whole volume of 10X trypsin;
4. Store the aliquots of 1X trypsin at -20°C until use. After thawing, store 1X trypsin at 4°C. The refrigerated working solution is stable up to three months.

NOTE

Verify that you are working with 10X before starting diluting. Sometimes, 1X trypsin (or 1X trypsin/EDTA) is purchased.

PREPARATION OF COMPLETE CULTURE MEDIUM

DESCRIPTION

Culture medium is a solution containing the substances required to support the maintenance, proliferation or differentiation of a variety of cells. It is prepared by supplementing the commercial basal medium with suitable additives. The composition of the final culture medium varies with cell type. After preparation, aliquots can be stored at 4°C for frequent use. Stock aliquots can be stored at -20°C.

NOTE

Check the code of basal medium before starting. If the type is the same (e.g. EMEM) but the code or the manufacturer is different, different amounts or types of additives may be required. In this case, refer to the technical datasheet

L-glutamine is usually added to the basal culture medium. If some particles are visible after thawing the aliquot, pipette the suspension until it becomes transparent

Usually the basal medium contains a pH indicator (red phenol). The color of the complete medium will change from red-rose in case of contaminations or pH variations

Aliquots to be stored at -20°C have not to be filled to the maximum level (e.g. a 50 ml tube has to be filled up to 40 ml). They must be labeled with the following information: COMPLETE MEDIUM FOR (cell type), DATE and OPERATOR'S NAME.

CRYOPRESERVATION SOLUTION (FREEZING MIX) – ALL CELL TYPES EXCEPT SH-SY5Y CELLS AND rBMSCS

DESCRIPTION

Cryopreservation is the technique of freezing cells and tissues at very low temperatures to maintain the biological material genetically stable and metabolically inert. Cryopreservation is performed at controlled rate freezing in the presence of cryoprotectants (DMSO) to minimize the effect due to the formation of ice crystals.

Reagents	**Quantity**	**Stored**
FBS (Sigma, F7524)	8 ml	Freezer
DMSO (Sigma, D2650)	1 ml	Cabinet

Materials	**Storage**
Pipettes (2 ml,10 ml)	Cabinet
15 ml tubes	Cabinet

PROCEDURE

- A sterile solution composed of 80% v/v FBS and 20% v/v DMSO has to be prepared.
- For example, for 10 ml solution, 8 ml FBS has to be mixed to 2 ml DMSO.
- Defrost FBS to room temperature in the water bath at 37°C;
- In a biological safety cabinet, transfer FBS in a 15 ml tube;
- In a fume hood, add DMSO;
- In a biological safety cabinet, filter the solution with a 0,2 µm syringe filter in a 15 ml tube;
- After using, store the remaining freezing mix at -20°C.

NOTE

If possible, it is better to prepare the freezing mix just before using

The described freezing mix is not suitable for SH-SY5Y cells

Do not freeze more than 1.5-2 million cells/vial.

About 1.5 ml freezing mix/vial is required.

CRYOPRESERVATION SOLUTION (FREEZING MIX) FOR SH-SY5Y CELLS

Reagents	**Quantity**	**Stored**
FBS (Sigma, F7524)	8 ml	Freezer
DMSO (Sigma, D2650)	1 ml	Cabinet
DMEM (Sigma, D6546)	1 ml	Fridge

Materials	**Storage**
Pipettes (2 ml,10 ml)	Cabinet
15 ml tubes	Cabinet

PROCEDURE

- A sterile solution composed of 80% v/v FBS, 10% v/v DMSO and 10% v/v DMEM has to be prepared. For example, for 10 ml solution, 8 ml FBS has to be mixed to 1 ml DMSO and 1 ml DMEM.
- Defrost FBS in the water bath at 37°C
- In a biological safety cabinet, transfer FBS in a 15 ml tube and add DMEM
- In a fume hood, add DMSO
- In a biological safety cabinet, filter the solution with a 0,2 µm syringe filter in a 15 ml tube
- After using, store the remaining freezing mix at -20°C

NOTE

If possible, it is better to prepare the freezing mix just before use.

Do not freeze more than 1.5-2 million cells/vial.

About 1.5 ml freezing mix/vial is required.

PREPARATION OF OSTEOGENIC MEDIUM

DESCRIPTION

This medium is used to induce the osteogenic differentiation of mesenchymal stromal cells.

CHEMICAL AND REAGENTS

Reagents	Quantity	Characteristics	Location
DMEM (Dulbecco's modified Eagle's medium) (es: GIBCO, Cod product: 10938-025)	500 mL	Con: 4500 mg/L D-Glucose, (NEAA) Whithout: L-Glutamine, Sodium Pyruvate	+4°C
FBS (fetal Bovine Serum) (es: LONZA, Cod product: DE 14-801F)	500 mL		-80°C, aliquote a -20°C
HEPES (1M) (es: GIBCO, Cod product: 15630-056)	100 mL	Biologic Buffer	+4°C
Sodium Pyruvate solution (es: GIBCO, Cod product: 11360-039)	100 mL	Carbohydrates source	+4°C
PSG (100x) (es: GIBCO, Cod product: 10378-016)	1 aliquote of 5mL	200 mM L-Glutamine, 10.000 U pen, 10mg strept in 0,9%NaCl	-20°C
Ascorbic Acid-2P (15mM-100x) (es: SIGMA, Cod product: A8960)	5 g		Powder 4°C, Aliquote -20°C
β-glycerophosphate (1 M-100x) (es: SIGMA, Cod product: G9891)	25 g		Powder at RT, Aliquote -20°C
Colecalciferolo (10 µM-1000x) (es: SIGMA, Cod product: C9756)	1g	Vit D3	Powder 4°C, Aliquote -20°C
Desametasone (100 µM-10000x) (es: SIGMA, Cod product: 2915)	100 mg		Powder 4°C, Aliquote -20°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materiale	**Collocazione**
Falcon 50 mL sterile (es: CELBIO-CORNING CC430829)	Cabinet
Sterile pipettes: 50 mL, 25mL, 10mL	Cabinet
Pipette gun	Cabinet
Eppendorf 1.5 mL sterile	Cabinet
Filters and syringes	Cabinet
Micropipettes	Cabinet

PROCEDURE

This culture medium is prepared by supplementing DMEM with:

- 0.15 mM Ascorbic Acid-2P (if the stock solution is 15 mM, it means 1% v/v 15 mM Ascorbic Acid-2P
- 10 mM β-glycerophosphate (if the stock solution is 1 M, it means 1% v/v 1 mM β–glycerophosphate)
- 10 nM cholecalciferol (if the stock solution is 10 µM, it has to be diluted 1000 times)
- 0.01 µM dexamethasone (if the stock solution is 100 µM, it has to be diluted 10000 times)
- 10% v/v fetal bovine serum (FBS)
- 0.01 M HEPES buffer (if the stock solution is 1 M, it means 1% v/v 1 M HEPES buffer)
- 2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)
- 1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin)
- 1 mM sodium pyruvate (if the stock solution is 100 mM, it means 1% v/v 100 mM sodium pyruvate)

- Defrost the solutions in the water bath at 37°C;
- For 50 ml osteogenic medium, mix the components as follows:
    - 42.0 ml DMEM
    - 5 ml ascorbic acid-2P (stock: 15 mM - final concentration: 0.15 mM)
    - 0.5 ml β-glycerophosphate (stock: 1M - final concentration: 10 mM)
    - 50 µl cholecalciferol (stock: 10 µM - final concentration: 10 nM)
    - 5 µl dexamethasone (stock: 100 µM - final concentration: 0.01 µM)
    - 5 ml FBS
    - 0.5 ml HEPES buffer 1 M
    - 0.5 ml 200 mM L-glutamine

    - 0.5 ml sodium pyruvate 100 mM
    - 0.5 ml penicillin and streptomycin 100X
- Filter the osteogenic medium by a 0,2 µm syringe filter
- The osteogenic medium is stable up to 2-3 weeks and it has to be stored at 4°C.

NOTE

Sometimes a single solution composed of 200 mM L-glutamine and penicillin and streptomycin 100X is purchased (PSG, Sigma, code G1146 or Invitrogen, Gibco®, code 10378-016). In this case, the volume of basal medium to be used is 42.5 ml and 0.5 ml PSG will be added instead of 0.5 ml 200 mM L-glutamine and 0.5 ml penicillin and streptomycin 100X

- For the preparation of differentiation factors, see the specific procedures

ASCORBIC ACID-2P

CHEMICAL AND REAGENTS

Ascorbic Acid-2P (Sigma, code A8960-5g. MW: 289.54 g/mol). Stored at 4°C

MilliQ water

EQUIPMENT, GLASSWARE AND CONSUMABLES

10 ml tube

1.5 ml sterile eppendorf tubes

0.2 µm syringe filter

Syringe

PROCEDURE

- Stock concentration: 15 mM (we call 100X, with respect to the final concentration (0.15 mM))
- Weight 43.4 mg ascorbic acid-2P
- Dissolve in 10 ml MilliQ water
- Filter the solution
- Aliquot in 1.5 ml sterile eppendorf tubes (1 ml/eppendorf) and store at -20°C

BETA-GLYCEROPHOSPHATE

CHEMICAL AND REAGENTS

β-glycerophosphate (Sigma, code G9891-25g. MW: 216.04 g/mol). Stored at room temperature

MilliQ water

EQUIPMENT, GLASSWARE AND CONSUMABLES

10 ml tube

1.5 ml sterile eppendorf tubes

0.2 µm syringe filter

Syringe

PROCEDURE

- Stock concentration: 1 M (we call 100X, with respect to the final concentration (10 mM))
- Weight 2.2 mg β-glycerophosphate
- Dissolve in 10 ml MilliQ water
- Filter the solution
- Aliquot in 1.5 ml sterile eppendorf tubes (1 ml/eppendorf) and store at -20°C

CHOLECALCIFEROL (ALSO REFERRED TO AS VITAMIN D3)

CHEMICAL AND REAGENTS

Cholecalciferol (Sigma, code C9756-1g. MW: 384.64 g/mol). Stored at 4°C

Ethanol

MilliQ water

EQUIPMENT, GLASSWARE AND CONSUMABLES

10 ml tube

1.5 ml sterile eppendorf tubes

0.2 µm syringe filter

Syringe

PROCEDURE

- Stock concentration (B): 10 µM (we call 1000X, with respect to the final concentration (0.01 µM))
- Prepare a pre-stock solution (A, 10 mM) in ethanol by dissolving 3.84 mg cholecalciferol in 1 ml absolute ethanol. Store at -20°C
- Prepare the stock solution to be used (B, 10 µM) by adding 10 µl cholecalciferol 10 mM (pre-stock solution, A) and add 9.99 MilliQ water
- Filter the solution B
- Aliquot in 1.5 ml sterile eppendorf tubes (1 ml/eppendorf) and store at -20°C.

NOTE

For osteogenic medium preparation, use the stock solution B (cholecalciferol 10 µM). Never use the pre-stock solution A (cholecalciferol 10 mM)! This is just an intermediate dilution.

DEXAMETHASONE

CHEMICAL AND REAGENTS

Dexamethasone (Sigma, code D2915-100mg. MW: 392.46 g/mol) stored at 4°C

Ethanol

MilliQ water

EQUIPMENT, GLASSWARE AND CONSUMABLES

10 ml tube

1.5 ml sterile eppendorf tubes

0.2 µm syringe filter

syringe

PROCEDURE

- Stock concentration (B): 100 µM (we call 10000X, with respect to the final concentration (0.01 µM))
- Prepare a pre-stock solution (A, 10 mM) in ethanol by dissolving 3.92 mg dexamethasone in 1 ml absolute ethanol. Store at -20°C
- Prepare the stock solution to be used (B, 100 µM) by adding 100 µl dexamethasone 10 mM (pre-stock solution, A) and add 9.9 MilliQ water
- Filter the solution B
- Aliquot in 1.5 ml sterile eppendorf tubes (1 ml/ eppendorf) and store at -20°C.

NOTE

For osteogenic medium preparation, use the stock solution B (dexamethasone 100 µM). Never use the pre-stock solution A (dexamethasone 10 mM)! This is just an intermediate dilution.

CRYOPRESERVATION SOLUTION (FREEZING MIX) FOR RAT BONE MARROW STROMAL CELLS (rBMSCS)

CHEMICALS AND REAGENTS

Reagents	Quantity	Stored
DMSO (sigma, D2650)	0,5 ml	Cabinet
FBS (Sigma, F7524)	3 ml	Freezer
FBS (Sigma, F7524)	6.5 ml	Fridge

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Stored
2 ml Pipettes	1	Cabinet
10 ml Pipettes	1	Cabinet
15 ml Pipettes	3	Cabinet
15 ml Falcon Tubes	1	Cabinet

PROCEDURE

- A sterile solution composed of 30% v/v FBS, 5% v/v DMSO and 65% v/v Alpha-MEM has to be prepared. For example, for 10 ml solution, 3 ml FBS has to be mixed to 0.5 ml DMSO and 6.5 ml DMEM.
- Defrost FBS in the water bath at 37°C;
- In a biological safety cabinet, transfer FBS in a 15 ml tube and add alpha-MEM;
- In a fume hood, add DMSO;
- In a biological safety cabinet, filter the solution with a 0,2 µm syringe filter in a 15 ml tube;
- After using, store the remaining freezing mix at -20°C.

NOTE

If possible, it is better to prepare the freezing mix just before use.

Do not freeze more than 2-3 million cells/vial.

About 1.5 ml freezing mix/vial is required.

Another possibility is to prepare a freezing mix composed of 90% v/v FBS and 10% v/v DMSO.

CELL FREEZING

DESCRIPTION

As highlighted before, the main problem is to avoid cell damage due to the formation of ice crystals. In this specific procedure this problem is avoided using a cryopreservation solution and a freezing procedure composed of two steps.

CHEMICALS AND REAGENTS

Reagents	Quantity	Stored
Freezing mix	2 ml	Freezer

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Stored
2 ml Pipettes	1	Cabinet
10 ml Pipettes	1	Cabinet
1.8 ml Criovials	5	Cabinet
15 ml Falcon Tubes	1	Cabinet

PROCEDURE

- After trypsinization, transfer cell suspension in a 15 ml or 50 ml Centrifuge tube;
- Withdraw a little aliquot for cell counting (see the specific procedure) and centrifuge the remaining suspension (2000 rpm for 5 min. Reduce centrifugation rate to 1000 rpm for more delicate cells, such as SH-SY5Y cells);
- Check that Mr.Frosty container is filled with isopropyl alcohol to the correct level;
- LABEL THE CRYOVIAL(S). Indicate: cell type, number of passages, number of cells, operator's name and date (e.g. MG63, P10, $2 \cdot 10^6$ cells, MTR-Manuela Raimondi, 25/01/2015). Do not freeze more than 1.5-2 million cells/vial;
- After centrifugation, remove the supernatant with a pipette and suspend the cells in a suitable volume of freezing mix (about 1.5 ml freezing mix for 1.5-2 million cells. See the specific procedure for its preparation);

- Quickly, transfer the cell suspension in the cryovial(s) and place the cryovial(s) in the Mr. Frosty container;
- Quickly, move the Mr. Frosty container at -80°C for at least 2 h;
- AFTER wearing protective clothing (crygloves, face shield, coat), move the cryovial(s) to the Dewar (container filled with liquid nitrogen) and fill the register.
- Defrost Mr. Frosty container at room temperature.

NOTE

Do not forget to label the cryovials and fill the register

Leaving the cells at -80°C for a long period will reduce their viability

Check that Mr-Frosty container is at room temperature before starting the procedure

RESUSCITATION OF FROZEN CELLS

DESCRIPTION

Before being cultured, cells have to be thaw. It is crucial to thaw cells correctly to maintain their viability and enable to recover more quickly. Some cryoprotectants, such as DMSO, are toxic above 4°C, therefore it is essential to thaw the cells as quick as possible and dilute DMSO with culture medium to minimize its toxic effects.

CHEMICALS AND REAGENTS

Reagents	Quantity	Stored
Specific Medium	50 ml	Fridge/Freezer

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Stored
Sterile Pasteur Pipettes: 3 ml	1	Cabinet
Sterile Pipettes: 1 ml, 5 ml, 10 ml	3-4 per each	Cabinet
10-100 µl Micropipettes	1	Bench
Micropipettes tips	1	Cabinet
50 ml Tubes	3-4	Cabinet
Flask 25 or 75 cm^2	To be decided	Cabinet

PROCEDURE

- Transfer 10-15 ml of culture medium in a 50 ml tube and heat at 37°C in a water bath;
- Using cryo-gloves, face shield and protective coat, extract the cryovial from liquid nitrogen container.

- To remove the liquid nitrogen eventually entrapped in the cryovial and avoid explosions caused by gas heating, open the cryovial carefully in the biological safety cabinet;

FIRST OPTION:

- with a sterile pipette, transfer 1 ml of culture medium into the cryovial and pipette gently to dilute the cryoprotectant in contact with the cells.
- As it gradually defrosts, transfer the cell suspension to the tube with culture medium.
- Continue to add culture medium and transfer the cell suspension until the cells are completely defrosted;

SECOND OPTION (better for more delicate cells, such as SH-SY5Y cells and stem cells):

- warm the cryovial at 37°C in a water bath for 1 minute, then go on as in the first option;
- Transfer the tube with the cells in the centrifuge (remember to balance before using) and centrifuge at 2000 rpm for 5 minutes (for more delicate cells, it might be better to reduce centrifugation rate to 1000 rpm and centrifugation time to 3 min);
- Remove the culture medium with a pipette without touching the cell pellet (at the bottom of the tube), add new culture medium (about 20 ml if you would like to plate the cells in a 150 cm^2, 10 ml for 75 cm^2 and 5 ml for 25 cm^2 flasks) and suspend the cells;
- If necessary, count the cells to estimate the number of the vital ones (see the specific procedure);
- Transfer the cell suspension in the suitable flask;
- Check cell density by an optical microscope and move the flask to the incubator.

NOTE

Consider the cell type while choosing the flask for cell culturing. For example, for 1.5-2 million MG63 cells you will need a 75 cm^2 flask, for 1.5-2 million SH-SY5Y cells you will need a 25 cm^2 flask.

CELL COUNTING

DESCRIPTION

Cell counting is a general name for various methods for the quantification of cells in life sciences, including medical diagnosis and treatment. This procedure is really useful because numerous protocols in biology and medicine require the counting of cells. Many times we are interested only in the concentration of cells. In a known volume of culture medium we can calculate the average value of the cells from the concentration using a very simple formula. In this specific case we will use a counting chamber and an optical microscope to evaluate the average concentration and then we will estimate the number of cells.

CHEMICALS AND REAGENTS

Reagents	Quantity	Stored
Trypan Blue (Sigma, T8154)	1	Cabinet

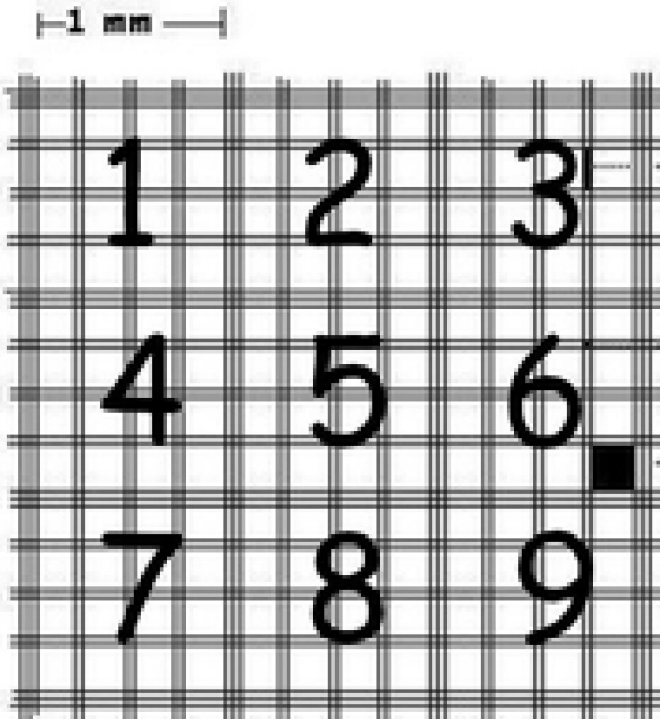
EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Stored
2 ml Pipettes	1	Cabinet
Pasteur pipettes	1	Cabinet
10-100 µl Micropipettes	1	Bench
Neubauer chamber	1	Cabinet

PROCEDURE

- After centrifugation, suspend the cell pellet in a known volume of culture medium;
- To avoid contamination, transfer an aliquot (about 200 µl) to a 2 ml eppendorf tube;
- Transfer an aliquot (100 µl max) from the 2 ml eppendorf tube to a 0.5 ml eppendorf tube;
- Add the same volume of trypan blue dye to the 0.5 ml eppendorf tube and mix;
- Take the Burker or Neubauer chamber and fix the glass slide with the lateral clamps.

- The Burker (or, similarly, Neubauer) chamber is composed of a slide that, observed by an optical microscope, shows 2 similar grids arranged in two opposite areas of the chamber. The sides of the grids are delimited by 3 parallel lines. Inside there are 9 squares containing 16 squares each. The 9 squares have side equal to 1 mm and are indicated in the figure below with numbers from 1 to 9. Above the chamber is placed a thin glass slide to delimit the filling volume. The distance between the chamber and the slide is defined (0.1 mm in the supplied chambers).
- Transfer about 10 µl of the diluted cell suspension in the Burker or Neubauer chamber.
- NOTE: be careful to keep a steady flow and not to create a suction due to the piston return. If there are bubbles, clean the chamber with ethanol and load again the cell suspension.
- By an optical microscope, count the live -white and bright- cells in squares 1, 5 and 9 (stained cells -blue- are not viable). By convention, the cells that pass through, even minimally, the median line of the 3 lines delimiting the main square have not to be considered.
- To obtain the total number of cells in the starting suspension, the average number of cells counted in squares 1, 5 and 9 is multiplied by 10^4, by 2 (dilution with trypan blue) and by the volume of medium (ml) of the starting cell suspension.



NOTE

Use trypan blue under safety hood. In case of pouring give immediate notice!!!!

After using, clean the chamber with ethanol and throw the tissue paper in the biological waste

Reduce the volume of trypan blue to be used to the minimum.

CULTIVATION OF ADHERENT CELLS

DESCRIPTION

Cell culture is a complex process where cells are grown under controlled conditions outside their physiological environment. Adherent cells require a surface, such as cell culture-treated poly(styrene) to survive and eventually proliferate. For adherent cultures, the medium can be replaced by aspiration, while medium changes in non-adherent cultures involve centrifuging the culture and suspending the cells. To subculture adherent cells, they have to be detached (by trypsin or trypsina/EDTA).

CHEMICALS AND REAGENTS

Reagents	Quantity	Stored
PBS or NaCl 0,9% solution (autoclaved)	about 5 ml	Cabinet
Trypsin 2.5 g/l	about 1 ml	Freezer
Specific medium	About 50 ml	Freezer
Trypan Blue (Sigma, 0,4% T8154)	falcon	Cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Stored
2 ml Pipettes	1	Cabinet
10 ml Pipettes	1	Cabinet
15 ml Falcon	…..	Cabinet
Neubauer chamber	1	Cabinet
One use tips	…..	Cabinet

Flasks 25 cm^2 o 75 cm^2	…..	Cabinet

PROCEDURE

1. Check the plate/flask by the optical microscope to assess the confluence level (how much area is covered by the cells. For example, 70% confluence means that about 70% of the growing area is covered by cells) and observe cell morphology;
2. Move to the biological cabinet, bend the flask and aspirate the culture medium by positioning the pipette in a corner;
3. Add about 3-5 ml (for a 75 cm^2 flask) sterile PBS (or NaCl 0.9%), shake gently, then remove;
4. Add 3-5 ml 1X trypsin or trypsin/EDTA solution (2.5 g/l), shake gently to wet all the surface and move to the incubator for about 5 min. The amount of trypsin or trypsin/EDTA may also be reduced, but at least 0.5 ml is needed for 25 cm^2 flasks and 1 ml for 75 cm^2 flasks;
5. Verify cell detachment by the optical microscope. If necessary, gently side taps may facilitate the detachment. If cells have not detached yet, provide additional incubation for a few minutes and then check again by the microscope;
6. Once cell have detached, inhibits trypsin by culture medium (at least, the same volume of culture medium is required. For more delicate cells such as stem cells, it is better to dilute trypsin 1:4 or 1:5 (v/v);
7. Transfer the cell suspension to a 15 ml or 50 ml Centrifuge tube and centrifuge at 2000 rpm for 5 minutes. For more delicate cells, reduce centrifugation speed;
8. Discard the supernatant with a pipette without touching the pellet;
9. Suspend the cells in culture medium and count (see the specific procedure);
10. Cells can now be frozen (see the specific procedure), be cultured or be plated (for example to test materials. See cultivation of adherent cells in contact with materials),

NOTE

It is appropriate not to plate less than 10^4 cells/cm^2 to avoid inhibition of their proliferation due to a low concentration of growth factors

Subculture the cells when they have reached 70% confluence.

CULTIVATION OF ADHERENT CELLS IN CONTACT WITH MATERIALS

Cells can be grown in direct contact (DIRECT CYTOCOMPATIBILITY) with materials to assess the effects of material properties on their growth, proliferation and/or metabolism.

Cells can be also grown in medium previously incubated with the materials (but not in direct contact with the material. This is called INDIRECT CYTOCOMPATIBILITY).

This is useful to assess if the samples release toxic leachable or degradation products and their effect on the cells.

NOTE

Before being in contact with cells or culture medium, it is important to ensure that samples may fit culture plates.

Before being in contact with cells or culture medium, it is important to ensure that samples have been sterilized. Take into consideration that the sterilization process can affect the material properties (and therefore the outcome of the test).

For both INDIRECT and DIRECT CYTOCOMPATIBILITY it is necessary to set the duration of the experiment and chose the necessary controls (negative controls = cells grown in the same test conditions, but without the material or molecules whose effect is under study; positive controls = some material or factor that affects cell behavior in a known and highly reproducible way).

An example of a DIRECT test is shown in the next procedure.

DESCRIPTION

For direct cytocompatibility assays, cells are plated directly on the materials to be tested. With this procedure we can investigate how the cells interact with the material and how the material influences their behavior with respect to a known behavior (control material). In fact, because the results make sense, it is necessary to use suitable controls for comparison with the material tested. Both control and tested materials must be sterilized; in this way the results are independent from the sterilization process. Test parameters (such as time points and culture conditions) must be decided before the beginning of the procedure; the most suitable choices depend on every specific situation.

CHEMICALS AND REAGENTS

Reagents	Quantity	Stored
Specific Medium	about 50 ml	Freezer

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Stored
Micropipettes (10-100 µl)	1	Bench
Multidispenser	1	Cabinet
12.5 ml dispenser tips	…..	Cabinet
Multiwell plates	1	Cabinet

PROCEDURE

- Both test and control materials have to be sterilized and placed in sterile multiwell plates for cell cultures;
- After detachment from the flask and counting (see specific procedures), cells are diluted to a density of $2 \cdot 10^5$ cells/ml (for biochemical assays) or $2 \cdot 10^4$ (for electron microscopy analysis) and a 50 µl drop of cell suspension is plated directly on the materials by a micropipette;
- Move the microplate to the incubator and wait for 1 h, to allow cell adhesion on the materials;
- Add culture medium (e.g., 1 ml/well), then move back the plate to the incubator for the desired number of hours or days.

NOTE

If you are testing round-shaped samples, they can be placed in a 24-well microplate if their diameter is about 12 mm

Periodically, change the cell culture medium;

To obtain valid and acceptable results for industrial/and academic applications, perform your experiments according to defined laws or indications, e.g. ISO 10993-5; 1999 - Biological evaluation of medical devices.

PREPARATION OF COMPLETE MEDIUM FOR MG-63 HUMAN OSTEOSARCOMA CELL LINE

DESCRIPTION

MG-63 cells are a line of immortalized cells. They are osteoblast-like cells and they can be exploited to model the behavior of osteoblasts in bone tissue.

Final composition: EMEM + 2mM Glutamine + 10% FBS + 1% NEAA + (Pen/Strep 1%: 100 unit/ml pen.; 0.1 mg/ml strep. final).

CHEMICAL AND REAGENTS

Reagents	**Quantity**	**Stored**
EMEM (Sigma, M2279)	500 ml	Fridge
Glutamine 200mM (Sigma, G7513)	5 ml	Freezer
FBS (Sigma, F7524)	50 ml	Freezer
Pen/Strept 100x (Sigma, P0781)	5 ml	Freezer
NEAA (Sigma, M7145)	5 ml	Fridge

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Storage**
Pipettes (5 ml, 25 ml)	Cabinet
500 ml filtering system	Cabinet
50 ml tubes	Cabinet

PROCEDURE

This culture medium is prepared by supplementing EMEM with:

-10% v/v fetal bovine serum (FBS)

-2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)

-1% v/v NEAA

-1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin)

- Defrost the solutions in the water bath at 37°C;
- Sum up the volumes of the additives you need to add to EMEM solution and remove this volume from the bottle. For example, for 500 ml complete medium, you need 50 ml FBS, 5 ml NEAA, 5 ml 200 mM L-glutamine and 5 ml penicillin and streptomycin 100X. Therefore, remove 65 ml from EMEM bottle;
- Transfer in two 50 ml tubes and store at -20°C;
- Pour the required amount of each component into EMEM bottle;
- Connect the filter system to the vacuum tube;
- Transfer the final solution into the funnel of the filter system and filter. Check if the flow is constant and uniform;
- The culture medium can be used immediately (up to 2-3 weeks, storage at 4°C) or stored at -20°C.

PREPARATION OF COMPLETE MEDIUM FOR C2C12 CELLS (MURINE MYOBLAST CELL LINE), U87-MG (HUMAN GLIOBLASTOMA-ASTROCYTOMA CELL LINE) AND EAHY CELLS (MURINE ENDOTHELIAL CELL LINES)

Final composition: DMEM + 2mM Glutamine + 10% FBS + (Pen/Strep 1%: 100 unit/ml pen.; 0.1 mg/ml strep. final).

CHEMICAL AND REAGENTS

Reagents	**Quantity**	**Stored**
DMEM (Sigma, D6546-L929; D5671-C2C12)	500 ml	Fridge
Glutamine 200mM (Sigma, G7513)	5 ml	Freezer
FBS (Sigma, F7524)	50 ml	Freezer
Pen/Strept 100x (Sigma, P0781)	5 ml	Freezer

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Storage**
Pipettes (5 ml, 25 ml)	Cabinet
500 ml filtering system	Cabinet
50 ml tubes	Cabinet

PROCEDURE

This culture medium is prepared by supplementing DMEM with:

-10% v/v fetal bovine serum (FBS)

-2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)

-1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin).

- Defrost the solutions in the water bath at 37°C;
- Sum up the volumes of the additives you need to add to DMEM solution and remove this volume from the bottle. For example, for 500 ml complete medium, you need 50 ml FBS, 5 ml 200 mM L-glutamine and 5 ml penicillin and streptomycin 100X. Therefore, remove 60 ml from DMEM bottle;
- Transfer in two 50 ml tubes and store at -20°C;
- Pour the required amount of each component into DMEM bottle;
- Connect the filter system to the vacuum tube;
- Transfer the final solution into the funnel of the filter system and filter. Check if the flow is constant and uniform;
- The culture medium can be used immediately (up to 2-3 weeks, storage at 4°C) or stored at -20°C.

PREPARATION OF COMPLETE MEDIUM FOR L929 CELLS (MURINE FIBROBLAST CELL LINE) AND SH-SY5Y CELLS (HUMAN NEUROBLASTOMA CELL LINE)

DESCRIPTION

L929 and SH-SY5Y cell lines are immortalized cells. L929 cells are fibroblast-like cells and they can be exploited to model the behavior of fibroblasts in soft tissue. SH-SY5Y cells are neuronal-like cells and they can be exploited to model the behavior of dopaminergic neurons (e.g. for studies related to Parkinson's disease).

CHEMICAL AND REAGENTS

Reagents	Quantity	Stored
DMEM (Sigma, D6546-L929; D5671-C2C12)	500 ml	Fridge
Glutamine 200mM (Sigma, G7513)	5 ml	Freezer
FBS (Sigma, F7524)	50 ml	Freezer
Pen/Strept 100x (Sigma, P0781)	5 ml	Freezer

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Storage
Pipettes (5 ml, 25 ml)	Cabinet
500 ml filtering system	Cabinet
50 ml tubes	Cabinet

PROCEDURE

This culture medium is prepared by supplementing DMEM with:

-10% v/v fetal bovine serum (FBS)

-2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)

-1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin)

- Defrost the solutions in the water bath at 37°C;
- Sum up the volumes of the additives you need to add to DMEM solution and remove this volume from the bottle. For example, for 500 ml complete medium, you need 50 ml FBS, 5 ml 200 mM L-glutamine and 5 ml penicillin and streptomycin 100X. Therefore, remove 60 ml from DMEM bottle;
- Transfer in two 50 ml tubes and store at -20°C;
- Pour the required amount of each component into DMEM bottle;
- Connect the filter system to the vacuum tube;
- Transfer the final solution into the funnel of the filter system and filter. Check if the flow is constant and uniform;
- The culture medium can be used immediately (up to 2-3 weeks, storage at 4°C) or stored at -20°C.

NOTE

For SH-SY5Y cells, Invitrogen, Gibco®, code 10938-025 may be also used as basal medium.

PREPARATION OF COMPLETE MEDIUM FOR U937 CELLS (HUMAN LYMPHOBLAST CELL LINE)

CHEMICAL AND REAGENTS

Reagents	Quantity	Characteristics	Storage
RPMI 1640			+4°C
L-Glutamine 200mM (Sigma, G7513)		100X	-20°C
FBS (Sigma, F7524)			-20°C
Pen/Strept 100x		10000 U/ml	-20°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Storage
Pipettes and pipette gun	Cabinet
Micropipettes and tips	Cabinet
Falsk	Cabinet

Reagents	**Quantity**	**Stored**
RPMI 1640		Fridge
L-Glutamine 200mM (Sigma, G7513)	100X	Freezer
FBS (Sigma, F7524)		Freezer
Pen/Strept 100x (Sigma, P0781)	10000 U/ml	Freezer

Materials

500ml filtering system (Corning, 430769)	1	Cabinet
5ml Pipettes	3	Cabinet
25 ml Pipettes	2	Cabinet
50 ml Tubes	15	Cabinet

PROCEDURE

This culture medium is prepared by supplementing RPMI 1640 with:

-10% v/v fetal bovine serum (FBS)

-2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)

-1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin)

- Defrost the solutions in the water bath at 37°C;
- Sum up the volumes of the additives you need to add to RPMI solution and remove this volume from the bottle. For example, for 500 ml complete medium, you need 50 ml FBS, 5 ml 200 mM L-glutamine and 5 ml penicillin and streptomycin 100X. Therefore, remove 60 ml from RPMI bottle;
- Transfer in two 50 ml tubes and store at -20°C;
- Pour the required amount of each component into RPMI bottle;
- Connect the filter system to the vacuum tube;
- Transfer the final solution into the funnel of the filter system and filter. Check if the flow is constant and uniform;
- The culture medium can be used immediately (up to 2-3 weeks, storage at 4°C) or stored at -20°C.

CULTURE

Since U937 cells grow in suspension mode, they can be kept in culture by:

- adding fresh medium to the exhausted one (1:3 dilution, 20 ml of fresh medium should be added to the 10 ml of suspension medium)
- moving a part of the cell suspension to a new flask with fresh medium (1:3 dilution means 3,3 ml of suspension medium in 6,6 ml of fresh medium)

Note: the optimal concentration is 1 or 2 $x10^5$ cells/ml

PREPARATION OF COMPLETE MEDIUM FOR THE EXPANSION OF HUMAN CHONDROCYTES

DESCRIPTION

Chondrocytes are primary cells. They represent the cell component of cartilage tissue.

CHEMICAL AND REAGENTS

Reagents	Quantity	Characteristics	Storage
DMEM (Dulbecco's modified Eagle's medium) (es: GIBCO, Cod product: 10938-025)	500 mL	Con: 4500 mg/L D-Glucose, (NEAA) Whithout: L-Glutamine, Sodium Pyruvate	+4°C
FBS (fetal Bovine Serum) (es: LONZA, Cod product: DE 14-801F)	500 mL		-80°C, aliquote a -20°C
HEPES (1M) (es: GIBCO, Cod product: 15630-056)	100 mL	Biologic Buffer	+4°C
Sodium Pyruvate solution (es: GIBCO, Cod product: 11360-039)	100 mL	Carbohydrates source	+4°C
PSG (100x) (es: GIBCO, Cod product: 10378-016)	1 aliquote of 5mL	200 mM L-Glutamine, 10.000 U pen, 10mg strept in 0,9%NaCl	-20°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Storage
Falcon 50 mL sterile (es: CELBIO-CORNING CC430829)	Cabinet
Sterile pipettes: 50 mL, 25mL, 10mL	Cabinet
Pipette gun	Cabinet

PROCEDURE

This culture medium is prepared by supplementing DMEM with:

-10% v/v fetal bovine serum (FBS)

-0.01 M HEPES buffer (if the stock solution is 1 M, it means 1% v/v 1 M HEPES buffer)

-1 mM sodium pyruvate (if the stock solution is 100 mM, it means 1% v/v 100 mM sodium pyruvate)

-2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)

-1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin).

1. Defrost the solutions in the water bath at 37°C.
2. Sum up the volumes of the additives you need to add to DMEM solution and remove this volume from the bottle. For example, for 500 ml complete medium, you need 50 ml FBS, 5 ml 1 M HEPES buffer, 5 ml 100 mM sodium pyruvate, 5 ml 200 mM L-glutamine and 5 ml penicillin and streptomycin 100X. Therefore, remove 70 ml from DMEM bottle;
3. Transfer in two 50 ml tubes and store at -20°C;
4. Pour the required amount of each component into DMEM bottle;
5. Connect the filter system to the vacuum tube;
6. Transfer the final solution into the funnel of the filter system and filter. Check if the flow is constant and uniform;
7. The culture medium can be used immediately (up to 1 month, storage at 4°C) or stored at -20°C.

NOTE

Sometimes a single solution composed of 200 mM L-glutamine and penicillin and streptomycin 100X is purchased (PSG, Sigma, code G1146). In this case, the volume of basal medium to be removed is reduced to 65 ml.

PREPARATION OF COMPLETE MEDIUM FOR RAT BONE MARROW STROMAL CELLS (rBMSCS)

CHEMICAL AND REAGENTS

Reagents	**Quantity**	**Stored**
alphaMEM (Gibco, 22571-020)	500 ml	Fridge
Glutamine 200mM (Sigma, G7513)	5 ml	Freezer
FBS (Sigma, F7524)	50 ml	Freezer
Pen/Strept 100x (Sigma, P0781)	5 ml	Freezer

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Storage**
Pipettes (5 ml, 25 ml)	Cabinet
500 ml filtering system	Cabinet
50 ml tubes	Cabinet

PROCEDURE

This culture medium is prepared by supplementing alpha-MEM with:

-20% v/v fetal bovine serum (FBS)

-1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin).

- Defrost the solutions in the water bath at 37°C;

- Sum up the volumes of the additives you need to add to alpha-MEM solution and remove this volume from the bottle. For example, for 500 ml complete medium, you need 100 ml FBS and 5 ml penicillin and streptomycin 100X. Therefore, remove 105 ml from alpha-MEM bottle);
- Transfer in three 50 ml tubes and store at -20°C;
- Pour the required amount of each component into alpha-MEM bottle;
- Connect the filter system to the vacuum tube;
- Transfer the final solution into the funnel of the filter system and filter. Check if the flow is constant and uniform;
- The culture medium can be used immediately (up to 1 month, storage at 4°C) or stored at -20°C.

NOTE

When using Alpha-MEM, Invitrogen, Gibco®, code 22571-020 GlutaMAX™, L-glutamine has not to be added. It is already supplied with the basal medium.

PREPARATION OF COMPLETE MEDIUM FOR HUMAN BONE MARROW STROMAL CELLS (hBMSCS)

CHEMICALS AND REAGENTS

Reagente	Quantità	Caratteristiche	Conservazione
α MEM (Minimum Essential Medium) (es: ThermoFisher, Cod product: 22571-020)	500 mL	With: GlutaMAX ™ -I or add L-glutamine at same concentration	+4°C
FBS (fetal Bovine Serum) (es: LONZA, Cod product: DE 14-801F)	500 mL		-80°C, aliquote a -20°C
HEPES (1M) (es: SIGMA, Cod product: HO887)	100 mL	Biologic Buffer	+4°C
Sodium Pyruvate solution (es: SIGMA, Cod product: S8636)	100 mL	Carbohydrates source	+4°C
PSG (100x) (es: SIGMA, Cod product: G1146)	1 aliquote of 5mL	200 mM L-Glutamine, 10.000 U pen, 10mg strept in 0,9%NaCl	-20°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Storage
Falcon 50 mL sterili (es: CELBIO-CORNING CC430829)	Cabinet
Sterile pipette: 50 mL, 25mL, 10mL	Cabinet
Pipette gun	Cabinet

PROCEDURE

This culture medium is prepared by supplementing alpha-MEM with:

-10% v/v fetal bovine serum (FBS)

-0.01 M HEPES buffer (if the stock solution is 1 M, it means 1% v/v 1 M HEPES buffer)

-2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)

-1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin).

-1 mM sodium pyruvate (if the stock solution is 100 mM, it means 1% v/v 100 mM sodium pyruvate)

- Defrost the solutions in the water bath at 37°C;
- Sum up the volumes of the additives you need to add to alpha-MEM solution and remove this volume from the bottle. For example, for 500 ml complete medium, you need 50 ml FBS, 5 ml 1 M HEPES buffer, 5 ml 100 mM sodium pyruvate, 5 ml 200 mM L-glutamine and 5 ml penicillin and streptomycin 100X. Therefore, remove 70 ml from alpha-MEM bottle;
- Transfer in two 50 ml tubes and store at -20°C.
- Pour the required amount of each component into alpha-MEM bottle.
- Connect the filter system to the vacuum tube.
- Transfer the final solution into the funnel of the filter system and filter. Check if the flow is constant and uniform.
- The culture medium can be used immediately (up to 1 month, storage at 4°C) or stored at -20°C.

NOTE

In this case, even though you are using Alpha-MEM, Invitrogen, Gibco®, code 22571-020 GlutaMAX™, L-glutamine has to be added.

Sometimes a single solution composed of 200 mM L-glutamine and penicillin and streptomycin 100X is purchased (PSG, Sigma, code G1146). In this case, the volume of basal medium to be removed is reduced to 65 ml.

PREPARATION OF SERUM-FREE MEDIUM FOR HBMSCS

CHEMICALS AND REAGENTS

Reagente	Quantità	Caratteristiche	Conservazione
DMEM (Dulbecco's modified Eagle's medium) (es: GIBCO, Cod product: 10938-025)	500 mL	Con: 4500 mg/L D-Glucose, (NEAA) whitouta: L-Glutamine, Sodium Piruvate	+4°C
HEPES (1M) (es: GIBCO, Cod product: 15630-056)	100 mL	Biologic Buffer	+4°C
Sodium Piruvate solution (es: GIBCO, Cod product: 11360-039)	100 mL	Source of carbohydrates	+4°C
PSG (100x) (es: GIBCO, Cod product: 10378-016)	1 aliquote of 5mL	200 mM L-Glutamine, 10.000 U pen, 10mg strept in 0,9%NaCl	-20°C
ITS +1 Insulina, transferrina, Selenio (Sigma Cod product: I2521)	5 mL	whithn: 1,0 mg/ml insulin da pancreas bovine, 0,55 mg/ml human transferrin, 0,5 µg/ml sodium selenite, 50 mg/ml bovine serum albumin, 470 µg/ml linoleic acid	+4°C
HSA (100x, es. Sigma, Cod product: A6909)	5 mL	30% in sodium chloride 0,85%	+4°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Material	Storage
Sterile pipettes: 50 mL, 25mL, 10mL, 5 mL, 2mL	Cabinet
Pipette gun	Cabinet
Micropipettes and tips	Cabinet
Flask T75 (CELBIO-CORNING)	Cabinet

PROCEDURE

This culture medium is prepared by supplementing DMEM with:

- 0.01 M HEPES buffer (if the stock solution is 1 M, it means 1% v/v 1 M HEPES buffer)

- 2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)

- 1% v/v HAS 100X (where 100X means that the starting solution is 30% in 0.85% sodium chloride)

- 1% v/v ITS+1 (if the stock solution contains 1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin (substantially iron-free), 0.5 μg/ml sodium selenite, 50 mg/ml bovine serum albumin and 470 μg/ml linoleic acid)

- 1% v/v Penicillin and Streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin)

- 1 mM sodium pyruvate (if the stock solution is 100 mM, it means 1% v/v 100 mM sodium pyruvate)

- Defrost the solutions in the water bath at 37°C;
- For 100 ml serum-free medium, mix the components as follows:
    - 94.0 ml DMEM
    - 1 ml HEPES buffer 1 M
    - 1 ml sodium pyruvate 100 mM
    - 1 ml 200 mM L-glutamine
    - 1 ml penicillin and streptomycin 100X
    - 1ml ITS+1
    - 1ml HSA 100x
- Filter the serum-free medium by a 0.2 μm syringe filter
- The serum-free medium has to be stored at 4°C

NOTE

Sometimes a single solution composed of 200 mM L-glutamine and penicillin and streptomycin 100X is purchased (PSG, Sigma, code G1146 or Invitrogen, Gibco®, code 10378-016). In this case, the volume of basal medium to be used is 95 ml and 1 ml PSG will be added instead of 1 ml 200 mM L-glutamine and 1 ml penicillin and streptomycin 100X.

PREPARATION OF ADIPOGENIC MEDIUM

DESCRIPTION

This medium is used to induce the adipogenic differentiation of mesenchymal stromal cells.

CHEMICAL AND REAGENTS

Reagent	Quantity	Characteristics	Storage
DMEM (Dulbecco's modified Eagle's medium) (e.g.: GIBCO, Product code: 10938-025)	500 ml	With: 4500 mg/l D-Glucose, non- essential amino acids (NEAA) Without: L-Glutamine, Sodium Pyruvate	+4°C
FBS (Fetal Bovine Serum) (e.g.: LONZA, Product code: DE 14-801F)	500 ml		-80°C, aliquots at -20°C
HEPES (1M) (e.g.: GIBCO, Product code: 15630-056)	100 ml	Biological Buffer	+4°C
Sodium pyruvate solution (e.g.: GIBCO, Product code: 11360-039)	100 ml	Carbohydrates source	+4°C
PSG (100x) (e.g.: GIBCO, Product code: 10378-016)	1 x 5ml aliquot	200 mM L-Glutamine, 10.000 U penicillin, 10mg streptomycin in 0.9%NaCl	-20°C
Insulin (2 mg/ml - 200x) (e.g.: Sigma, Product code: I9278)	10 mg/ml (stock solution)		-20°C aliquots + 4 °C stock solution
Indomethacin (40 mM - 200X) (e.g.: Sigma, Product code: I7378 - 5g)			-20°C aliquots
Isobutyl-1-methylxanthine- IBMX (40 mM-80X) (e.g.: Sigma, Product code: I7018-100 mg)			-20°C aliquots
Dexamethasone (100 µM - 100X)			-20°C aliquots

(e.g.: Sigma, Product code: D2915-100 mg)			

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Location**
Sterile pipettes: 50 ml, 25ml, 10ml, 5 ml, 2ml	Cabinet/drawer
Pipettor	Cabinet/drawer
Micropipettes and tips	Cabinet/drawer
Syringes and sterile filters	Cabinet/drawer
1.5 ml Eppendorf tubes	Cabinet/drawer
15 ml Falcon tubes	Cabinet/drawer

PROCEDURE

For adipogenic differentiation, 2 different culture media must be alternatively used to reduce the cytotoxic effect. These media are named **INDUCTION** and **MAINTENANCE** adipogenic medium, respectively.

- Put all bottled and falcon reagents in a 37°C water bath.

Note: if, after the thawing procedure, some solid particles are found in the aliquot of PSG, indomethacin and IBMX, vortex the tube until everything becomes liquid.

- After thawing / heating the reagents, transfer all the containers to the class II hood.

Pay attention to operating in sterile conditions at all times.

INDUCTION MEDIUM

Quantity	**10 ml medium**
DMEM	8.375 ml
10% FBS	1 ml
1% HEPES	100 µl
1% Sodium Pyruvate	100 µl
1% PSG	100 µl
10 µg/ml insulin	50 µl (stock: 2 mg/ml)
200 µM Indomethacin	50 µl (stock: 40 mM)

500 µM IBMX	125 µl (stock: 40 mM)
1 µM dexamethasone	100 µl (stock: 100 µM)

MAINTENANCE MEDIUM

Quantity	**10 ml medium**
DMEM	8.65 ml
10% FBS	1 ml
1% HEPES	100 µl
1% Sodium Pyruvate	100 µl
1% PSG	100 µl
10 µg/ml insulin	50 µl (stock: 2 mg/ml)

- Filter both media by a 0,2 µm syringe filter

Both media are stable up to 2 weeks and they have to be stored at 4°C

The medium contains a pH indicator and the color of the medium will change due to contamination or pH change.

NOTE

Sometimes a single solution composed of 200 mM L-glutamine and penicillin and streptomycin 100X is purchased (PSG, Sigma, code G1146 or Invitrogen, Gibco®, code 10378-016).

In this case, for induction medium, the volume of basal medium to be used is 8.375 ml and 0.1 ml PSG will be added instead of 0.1 ml 200 mM L-glutamine and 0.1 ml penicillin and streptomycin 100X. For maintenance medium, the volume of basal medium to be used is 8.65 ml and 0.1 ml PSG will be added instead of 0.1 ml 200 mM L-glutamine and 0.1 ml penicillin and streptomycin 100X

For the preparation of differentiation factors, see the specific procedures

PREPARATION OF DIFFERENTIATION FACTORS

Reagent	**Initial Conc**	**Final Conc**	**MW**	**Preparation**	**Storage**
Insulin	2 mg/ml 200X	10 µg/ml		Take 1 ml of insulin 10 mg / ml and add 4 ml of sterile PBS, in order to obtain a solution with a concentration of 2 mg/ml.	-20°C
Indomethacin	40 mM 200X	200 uM	357.79 g/mol	Weigh 14.3 mg and dissolve in 1 ml of absolute EtOH in a sterile eppendorf.	-20°C.
IBMX	40 mM 80X	500 uM	222.24 g/mol	Weigh 44.4 mg and dissolve in 5 ml of absolute EtOH. After complete dissolution, filter the solution and aliquot into eppendorf.	-20°C.
Dexamethasone	100 uM 100X	1 uM	392.46 g/mol	(A) dissolve 3.92 mg in 1 mL of absolute EtOH (10mM) (B) take 10 µL solution (A) and add 9.99 mL of H2O MilliQ (filter) (100 uM)	Solution (A) 10 mM (-20°C) ATTENTION: for medium preparation use solution B - never use solution A

ISOLATION AND CULTURE OF HUMAN CHONDROCYTES: CARTILAGE PREPARATION AND DIGESTION

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
DMEM (e.g.: GIBCO, Product code: 10938-025)	With: 4500 mg/l D-Glucose, non essential amino acids (NEAA) Without: L-Glutamine, Sodium Pyruvate	
Complete Medium (C.M)		SEE PROTOCOL for human chondrocytes expansion
PBS + 1% PSG		SEE PROTOCOL
Collagenasi 0.15%		SEE PROTOCOL

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Product example	Location
Micropipettes and tips		Cabinet/drawer
100 uM filter	BD FALCON 352360	Cabinet/drawer
50 ml Falcon tubes	CELBIO-CORNING CC430829	Cabinet/drawer
Petri dishes	CELBIO-CORNING 430597	Cabinet/drawer
Sterile tweezers	Reda	Cabinet/drawer
24G or 22Gblaster scalpel	xinda farmac-zabban s.p.a or paragon ref.p308	Cabinet/drawer

PROCEDURE

- Warm complete medium, PBS, PSG and DMEM in the water bath at 37°C
- Prepare 100 ml PBS supplemented with 1% v/v PSG
- Bring the cartilage sample to the laboratory and put them into the safety class II cabinet, open the jar and remove the physiological solution
- Under sterile conditions, transfer the cartilage biopsy in the Petri dish and cover with PBS + 1% v/v PSG. Use the scalpel to cut tangential chips
- Weight an empty 50 ml tube (tare)
- With tweezers, transfer the chips into the tube
- Weight the Centrifuge tube with the cartilage chips

- Transfer the cartilage chips in the sterile Petri dish and cut them smaller. Beware to maintain the cartilage wet with complete medium
- Prepare the corresponding volume of collagenase II 0.15% w/v (For every gram of cartilage, add 10 ml collagenase 0.15% w/v)
- With tweezers, transfer the chips into the tube (or a sterile jar) containing collagenase II 0.15% w/v solution
- Move the tube (jar) on the orbital shaker for digestion for 22 h) and incubate the shaker at 37°C with 5% CO_2
- Take a new sterile tube and fill with 20-30 ml complete medium to neutralize the collagenase
- Filter in that tube the digested cartilage with a sterile 100 mm cell strainer
- Centrifuge for 5 min at 1400 rpm and remove the supernatant
- Wash the cells with PBS and centrifuge for 5 min at 1400 rpm
- According to the dimension of the cell pellet, suspend in 5 or 10 ml of complete medium for counting (see the specific protocol)

PREPARATION OF DIGESTION SOLUTION: COLLAGENASE II 0.15% W/V

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
Collagenase II powder (e.g.: Worthington, CLS-2)		+ 4°C
DMEM (e.g.: GIBCO, Product code: 10938-025)	With: 4500 mg/l D-Glucose, non essential amino acids (NEAA) Without: L-Glutamine, Sodium Pyruvate	
Complete Medium		SEE REFERENCE PROTOCOL

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Product Example	Location
Pipettes and pipettor	CELBIO-CORNING CC4101	Cabinet/drawer
0.2 uM sterile filter	ALBET JACS0202550	Cabinet/drawer
50 ml Falcon tubes	CELBIO-CORNING CC430829	Cabinet/drawer
Plastic weighing box (square)		Cabinet/drawer
Sterile tweezers	Reda	Cabinet/drawer
Stainless steel palette		
20 ml Syringe or 150 mL filter system	Millipore SGPU01RE	Cabinet/drawer

PROCEDURE

- A solution of collagenase II 0.15% w/v is composed of:

Quantity	10mL medium
DMEM	5 ml
Complete Medium (CM)	5 ml
Collagenase II powder	15 mg

- Warm DMEM and complete medium in the water bath at 37°C
- Weigh the collagenase powder using a plastic ship as a support and a palette (or spoon)
- Bend the ship long one's bias line and put into the 50 ml tube. Pay attention not to put collagenase in the bottom of the tube.
- Move to the class II hood.
- Put the proper volume of DMEM in the centrifuge tube. To collect the collagenase, wash well the ship
- Take away the ship from the 50 ml tube with the tweezers and filter this solution through a 0.2 µm filter.
- Add the proper volume of complete medium to the filtered solution. Not to filter the complete medium to preserve SBF content.

NOTE

Prepare the correct volume of solution according to the cartilage to be processed: 1 g cartilage needs 10 ml collagenase 0.15% w/v solution

Collagenase powder is very volatile: pay attention not to inhale it! Wear the protective mask.

EXPANSION OF CHONDROCYTES

CHEMICAL AND REAGENTS

FGF-2 (warmed)

TGF-b1 (warmed)

Complete medium (see the specific protocol for its preparation)

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Product example**	**Location**
150, 75 or 25 cm^2 flasks	T150 (CELBIO-CORNING CC430825), T75 (CELBIO-EUROCLONE SP70075) or T25 (CELBIO-CORNING CC430639))	Cabinet/drawer
50 ml Falcon tubes	BD	Cabinet/drawer
T75 flasks without vent cap	CELBIO-CORNING CC430720	Cabinet/drawer
Sterile Pipettes (50- 25-10 -5 and 2 ml)		Cabinet/drawer

PROCEDURE

- Warm complete medium, TGF-b1 and FGF2 in the water bath at 37°C
- Calculate the volume of cell suspension and prepare a tube or flask without vent cap with complete medium supplemented with the proper concentration of growth factors (TGFb-1 1 ng/ml and FGF-2 5 ng/ml. See the specific protocol for its preparation).
- Suspend the cells in the medium supplemented with the proper concentration of growth factors (TGFb-1 1 ng/ml and FGF-2 5 ng/ml. See the specific protocol for its preparation)
- Plate the cell suspension in the appropriate flask with the following cell density:
- Cells isolated after cartilage digestion (P0 cells): 10.000 cells/cm^2
- Cells after the first confluence (P1 cells), second confluence (P2 cells) and so on: 5.000 cells/cm^2
- Write date, passage number (P0-P1, P1-P2,...) and sample code on flasks
- Incubate the cells at 37°C and culture them until they are sub-confluent (70% confluence)

NOTE

A 150 cm^2 flask has to be filled with 20 ml cell suspension, a 75 cm^2 flask with 10 ml cell suspension and a 25 cm^2 flask with 5 ml cell suspension.

ENZYMATIC DETACHMENT OF CHONDROCYTES

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
Tripsin/EDTA (1X) (e.g.: GIBCO, code: 25300-054)		Aliquots at +4°C Bottle -20°C
Complete Medium (C.M.)	SEE REFERENCE PROTOCOL	
Collagenase II (e.g.: Worthington, code: CLS-2)	SEE REFERENCE PROTOCOL	Aliquots at -20°C Bottles +4°C
Sterile PBS (e.g.: GIBCO Code: 20012-019)	with monobasic potassium phosphate, sodium chloride and dibasic sodium phosphate	
Trypan blue	1 : 100 in solution	Toxic reagents cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Product example	Location
50 ml Falcon tubes	BD-352070	Cabinet/drawer
Burker chamber	CELBIO-CORNING CC430720	Cabinet/drawer
Sterile pipettes (50- 25-10 -5 and 2 ml)		Cabinet/drawer

PROCEDURE

- Warm a 50 ml tube with 10 ml complete medium in the water bath at 37°C
- Remove the medium from the flasks with the (sub)confluent cells
- Wash each flask with 5 ml PBS (pre-heated in the water bath at 37°C)
- Add 4 ml collagenase solution 0.3% w/v (pre-heated in the water bath at 37°C) in every flask and move to the incubator for 10 min at 37°C
- Shake the flasks to improve the detachment and collect the detached cells in a centrifuge tube
- Add 5 ml trypsin/EDTA 1X (pre-heated in the water bath at 37°C) in each flask and move to the incubator for 5 min at 37°C
- Shake the flasks and collect the detached cells in the same centrifuge tube
- Rinse the flasks with 10 ml complete medium and collect the remaining cells in the same centrifuge tube. Check under the microscope if the flask is “clean” (without any cells), otherwise do other washing steps with PBS or incubate with trypsin again
- Centrifuge for 5 min at 1400 rpm
- Remove the supernatant

- According to the dimension of the cell pellet, suspend in 5 or 10 ml of complete medium for counting (see the specific protocol)

PELLET CULTURE

CHEMICAL AND REAGENTS

Serum-free medium (see the specific protocol for its preparation)

TGF-b1 (see the specific protocol for its preparation)

1% v/v ascorbic acid-2P 10 mM (see the specific protocol for its preparation)

Dexamethasone (see the specific protocol for its preparation)

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Product example**	**Location**
50 ml Falcon tubes	BD-352070	Cabinet/drawer
Conical microtube	CELBIO-EUROCLONE	Cabinet/drawer
Sterile Pipettes (50- 25-10 -5 and 2 ml) and tips		Cabinet/drawer

PROCEDURE

- Warm serum free medium, TGF-b1, ascorbic acid-2P and dexamethasone in the water bath at 37°C
- Suspend the cells at a density of 1 million cells/ml in serum free medium supplemented with TGFb1, ascorbic acid 2P and dexamethasone at the proper concentration in a centrifuge tube
- Distribute 0.5 ml of this cell suspension per tube = 0.5 million cells/pellet
- Prepare at least 9 pellets for cell code:
    - 3 tubes for histology
    - 3 tubes for biochemistry
    - 3 tubes for viability assay
- Centrifuge for 2 min at 1100 rpm
- Unlock the caps to allow for gas diffusion
- Change the medium twice a week. The pellets are usually cultured for 2 weeks

PREPARATION OF ASCORBIC ACID-2P FOR CHONDROCYTES

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
DMEM (es. GIBCO, Code: 10938-025)	With: 4500 mg/L D-Glucosio, amminoacidi non essenziali (NEAA) Without: L-Glutamine, Sodium Pyruvate	2-8°C
Ascorbic Acid-2P (AA) (e.g. Sigma, Code: A-8960)	MW = 289.54 g/mol for Ascorbic Acid-2-phosphate (AA)	2-8°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Product example	Location
50 ml Falcon tubes	BD-352070	Cabinet/drawer
micropipettes		Cabinet/drawer
Sterile pipettes (50- 25-10 -5 and 2 ml)		Cabinet/drawer
Pipettor		Cabinet/drawer
0.2 uM syringe filter	Millipore	Cabinet/drawer

PROCEDURE

- Stock concentration: 10 mM (that is 2.89 mg/ml. We call 100X, with respect to the final concentration (0.1 mM)). MW: 289.54 g/mol
- Weight the proper amount of ascorbic acid-2P (e.g. for 30 ml of 100x solution, you need 30 · 2.89 mg = 87.7 mg ascorbic acid-2P)
- Dissolve in the proper volume of DMEM. Prepare the solution in ice!
- Filter the solution
- Aliquot in 1.5 ml sterile eppendorf tubes (1 ml/eppendorf) and store at -20°C in the dark
- Working solution is 0.1 mM

NOTE

Do not expose ascorbic Acid-2P to direct light

DEXAMETHASONE FOR BONE MARROW STROMAL CELLS (BMSCS)

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
DMEM (e.g. GIBCO, Code: 10938-025)	With: 4500 mg/l D-Glucose, non essential amino acids (NEAA) Without: L-Glutamine, Sodium Pyruvate	2-8°C
Dexamethasone (Dex) (e.g. Sigma, Code: D2915)	MW = 392.5/mol	2-8°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Product example	Location
50 ml Falcon tubes	BD-352070	Cabinet/drawer
micropipettes		Cabinet/drawer
Sterile pipettes(50- 25-10 -5 and 2 ml)		Cabinet/drawer
Pipettor		Cabinet/drawer
0.2 uM syringe filter	Millipore	Cabinet/drawer

PROCEDURE

- Stock concentration: 100 µM (that is 0.0392 mg/ml)
- Final concentration: 0.1 µM
- Dexamethasone MW: 392.46 g/mol
- Weight the proper amount of dexamethasone to prepare the stock solution (100 µM). For example, for 10 ml solution you will need 6.83 mg dexamethasone.
- Dissolve the powder in the proper volume of DMEM
- Filter the solution
- Aliquot in 1.5 ml sterile eppendorf tubes (1 ml/eppendorf) and store at -20°C.
- The working concentration is 0.1 µM (that is the stock solution has to be diluted 1000 times)

HUMAN BMSCs ISOLATION

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
αMEM (complete medium) (e.g.: GIBCO, Code: 22571-020)		2-8°C
PBS (e.g.: GIBCO, Code: 20012)		2-8°C
Crystal violet (e.g.: Sigma, Code: HT901-8FOZ)	Diluted 1:100 in PBS	

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Product example	Location
50 ml Falcon tubes	BD-352070	Cabinet/drawer
micropipettes		Cabinet/drawer
Sterile pipettes (50- 25-10 -5 and 2 ml)		Cabinet/drawer
Microcentrifuge tubes	COSTAR CELBIO-CORNING - 3621	Cabinet/drawer
Flask T150 – T75 – T25	T150 (CELBIO-CORNING CC430825), T75 (CELBIO-EUROCLONE SP70075), T25 (CELBIO-CORNING CC430639).	Cabinet/drawer

PROCEDURE

- Transfer the sample to a tube (maximum volume for each tube: 25 ml)
- Centrifuge at 510 g for 10 min
- Remove the supernatant
- Add 10 ml PBS in each tubes and collect all the samples in a single falcon.
- Centrifuge at 1800 rpm for 10 min
- Remove the supernatant and suspend in 10 ml PBS
- Centrifuge the sample at 1300 rpm for 10 min
- Remove the supernatant and suspend with complete medium (for hBMSCs)
- Mix well and count the sample diluting 1:10 v/v with Crystal Violet dye

NOTE

If it is impossible to count the sample, suspend in 5 ml hBMSC expansion medium and plate in a 25 cm^2 flasks

HUMAN BMSC EXPANSION

DESCRIPTION

This procedure describes the expansion of human BMSCs after isolation

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
αMEM (complete medium) (e.g.: GIBCO, Code: 22571-020)		+4 °C
PBS (e.g.: GIBCO, Code: 20012)		+ 4°C
Fibroblast growth factor 2 (FGF2) (e.g. Sigma, Code: HT901-8FOZ)	Aliquot 0,005 mg/ml	-20°C
Trypsin/EDTA 1X (e.g.: GIBCO, Code: 25300-054)		-20°C
Trypan blue dye (e.g.: Sigma, Code: T8154-20ML	Diluted falcon 1:100 LABCOLTCEL	RT

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Product example	Location
50 ml Falcon tubes	BD-352070	Cabinet/drawer
micropipettes		Cabinet/drawer
Sterile pipettes (50- 25-10 -5 and 2 ml)		Cabinet/drawer
Centrifuge		
Microcentrifuge tubes	COSTAR CELBIO-CORNING -3621	Cabinet/drawer
Flasks T150 – T75 – T25	T150 (CELBIO-CORNING CC430825), T75 (CELBIO-EUROCLONE SP70075), T25 (CELBIO-CORNING CC430639).	Cabinet/drawer

PROCEDURE

Expansion after isolation

- Warm complete medium, TGF-b1 and FGF2 in the water bath at 37°C
- Calculate the volume of cell suspension and fill a centrifuge tube or flask without vent cap with complete medium supplemented with the proper growth factor concentration (see the protocol for its preparation)
- Suspend the cells in medium containing the proper growth factor concentration (FGF-2 5 ng/ml)
- Plate the cell suspension in the proper dish/flask with the following density:
- Cells isolated (P0 cells): 100.000 cells/cm^2
- Cells after the first confluence (P1 cells), second confluence (P2 cells), ……..: 3.000 cells/cm^2
- Incubate the cells at 37°C and culture them until they are sub-confluence

BMSCS EXPANSION P_0 TO P_1 AND P_1 TO P_2 SPLIT:

PROCEDURE

- Upon reaching the confluence, wash the cells with PBS and detach with Trypsin-EDTA for 5 min at 37°C (for T150 flasks use 4-5 ml Trypsin-EDTA, for T75 flasks use 2.5-3 ml, for T25 flasks use 1.5-2 ml. The volume of Trypsin-EDTA can also be reduced, e.g. 0.5 ml can be used for T25 flasks)
- After checking cell detachment by an optical microscope, put the suspension in a tube containing 10 ml complete medium
- Wash the flask with complete medium to recover all cells and the centrifuge the tube for 5 min at 1400 rpm at room temperature
- Remove the supernatant and break the pellet
- According to the thickness of the pellet, suspend the cells with complete medium
- Count the cells using trypan blue dye (1:1 ratio) and plate the cells at 3000 cells/cm^2 in complete medium + FGF 20X

NOTE

The suitable volume for each flask is: 20 ml for a T150, 10 ml in a T75 and 5 ml in a T25

CULTURE OF RAT BONE MARROW STROMAL CELLS (rBMSCS)

DESCRIPTION

Rat bone marrow stromal cells are capable of differentiating into multiple lineages (osteogenic, chondrogenic, adipogenic, neurogenic and myogenic lineages). For this reason, they have attracted significant interest as useful somatic stem cells for use in tissue engineering and regenerative medicine. The following procedure regards the culture, passaging, freezing and thawing of rat BMSCs.

Rat bone marrow stromal cells at P0 (0h) in a T75 flask

CHEMICALS AND REAGENTS

Reagent	Characteristics	Storage
αMEM (Complete medium) (e.g.: GIBCO, Code: 22571-020)		2-8°C
Penicillin-Streptomycin 1%	Stock 100%	Stock -20°C 2-8°C
FBS (Fetal Bovine Serum) (e.g.: LONZA, Code: DE 14-801F)		Stock -20°C 2-8°C
PBS (e.g.: GIBCO, Code: 20012)	1X, sterile	2-8°C
Trypsin/EDTA (e.g.: GIBCO, Code: 25300-062)	1X, with Phenol Red	-20°C
DMSO (e.g.: GIBCO, Code: D2650)	100%	RT

PROCEDURE

CELL CULTURE

- After 24 h adhesion, transfer the supernatant in a new T75 flask
- After 72 h, wash the two flasks twice with PBS to remove non-adherent cells (24 h+72 h = BMSCs P0)

NOTE: In a T75 flask, P0 cells reach confluence in 8-10 days, for a total of about 15 million cells

PASSAGING

IN THIS STEP, CONFLUENT P0 CELLS ARE SPLIT TO MAKE A SUB-CULTURE:

- Remove the culture medium and wash twice with PBS (warmed in the water bath at 37°C)

- Add 2-3 ml (eventually only 1 ml) of 1X trypsin EDTA solution and wait for 3-4 minutes (try to facilitate cell detachment by gently tapping the flask);
- According to the the number of detached cells, split them into three (1:3), four (1:4) or five (1:5) new flasks. The removal of trypsin is not necessary.

PLEASE NOTE: After P0, passages reach confluence in 4-5 days

FREEZING

- Add 1X trypsin/EDTA solution to the flask to detach the cells
- Count the cells (see the specific procedure)
- Centrifuge in a 15 ml or 50 ml tube at 2000 rpm for 5 min (eventually, 800 rpm for 10 min)
- Discard the supernatant
- Suspend the cells in the freezing medium (αMEM, 30% FBS, 5% DMSO; see the specific protocol for its preparation). Consider 1.5 ml freezing mix for 2-3 million cells.
- Put 1.5 ml in each cryovial and move to Mr Frosty container and then to -80°C for at least 2 h
- Transfer the vials in liquid nitrogen

PLEASE NOTE: Freeze the cells at passage P1 to avoid ageing

THAWING

- Transfer the frozen from the liquid nitrogen to the water bath (37°C)
- Transfer the cell suspension in 10 ml of complete medium (pre-heated at 37°C)
- Wait for 5 min
- Centrifuge at 1000 rpm for 10 min at 22°C
- Suspend the cells in complete medium and plate in a 75 cm^2 flask

PLEASE NOTE: The frozen rat BMSCs should be used during the year following the freezing

HUMAN ADIPOSE STEM CELLS (hASCS) ISOLATION AND CULTURE

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
DMEM with 4500 mg/l D-Glucose, NEAA; without: L-Glutamine, Sodium Pyruvate. (e.g.: GIBCO, Code: 10938-025)		2-8°C
PBS (e.g.: SIGMA, Code: D8537)	1X, sterile	2-8°C
FBS (Fetal Bovine Serum) (e.g.: LONZA, Code: DE 14-801F)		-20°C
HEPES 1M (e.g.. GIBCO, Code 15630-056)	100X	2-8°C
Sodium Pyruvate 100 mM (e.g.. GIBCO, Code 11360-039)	100X	2-8°C
PSG (e.g. GIBCO. Code: 10378-016)	100X	-20 °C
Collagenase I (e.g.. WORTHINGTON, Code: LS004197)		2-8°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Location
Pipettes	Cabinet/drawer
Pipettor	Cabinet/drawer
Micropipettes and tips	Cabinet/drawer
Centrifuge tubes	Cabinet/drawer
Cell strainers	Cabinet/drawer
Syringes and syringe filters	Cabinet/drawer

PROCEDURE

- For a lipoaspirate sample: divide the sample in different 50 ml tubes (max 20-25 ml in each tube) and centrifuge at 1200 g for 2 min
- If the adipose tissue is a solid block: transfer to a Petri dish containing PBS and digest mechanically until obtaining very little pieces of fat, then move to one or more 50 ml tubes containing PBS and centrifuge at 1200 g for 2 min
- To remove all the blood contaminants, remove the PBS and the precipitate with a Pasteur pipette connected to a vacuum pump. Add new PBS, mix gently and centrifuge at 1200 g for 2 min
- Repeat these washing steps until the sample is clean from blood contaminants (at least 3 times).
- After the last centrifugation, remove the precipitate and PBS and quantify the volume of adipose tissue to be digested.

Collagenase preparation

To prepare the collagenase type-I solution, consider a volume of 0.6 ml of PBS (supplemented with PSG) for each ml of adipose tissue. For the enzymatic digestion use 0.075% (w/v) Collagenase type-I. The amount of collagenase type-I must be determined on the total volume (adipose tissue + PBS supplemented with PSG).

E.g. for 10 ml of adipose tissue, use 6 ml of PBS. The amount of collagenase must be determined on the total volume (16 ml):

0.075 g : 100 ml = x g : 16 ml

That is, x = 0.012 g

- Add Coll-I to PBS and filter through a sterile syringe filter, then add this solution to adipose tissue and incubate at 37°C on a shaker for 30 min. Every 5 min shake the sample very strongly
- Neutralize Coll-I by adding at least the same volume of complete medium and centrifuge at 1200 g for 10 min
- Remove the superrnatant and suspend the pellet in complete medium
- Filter through a cell strainer or a sterile lint to remove undigested tissue
- Centrifuge at 350 g for 4 min, suspend the pellet in a suitable volume of complete medium and count the cells by dilution with trypan blue dye
- Plate the cells at a density of $1 \cdot 10^5$ cells/cm^2

Note: hASCS are maintained in the complete medium composed by the same complete medium. This culture medium is prepared by supplementing DMEM with:

- 10% v/v fetal bovine serum (FBS)

- 0.01 M HEPES buffer (if the stock solution is 1 M, it means 1% v/v 1 M HEPES buffer)

- 2 mM L-glutamine (if the stock solution is 200 mM, it means 1% v/v 200 mM L-glutamine)

- 1% v/v penicillin and streptomycin 100X (that is 100 units/ml penicillin; 0.1 mg/ml streptomycin)

- 1 mM sodium pyruvate (if the stock solution is 100 mM, it means 1% v/v 100 mM sodium pyruvate).

PREPARATION OF COMPLETE MEDIUM WITH AUTOLOGOUS SERUM

CHEMICAL AND REAGENTS

Reagent	Characteristics	Storage
DMEM with 4500 mg/l D-Glucose, NEAA; without L-Glutamine, Sodium Pyruvate. (e.g.: GIBCO, Code: 10938-025)		+4 °C
FBS (Fetal Bovine Serum) (e.g.: LONZA, Code: DE 14-801F)	100X	+ 4°C
Sodium Pyruvate 100 mM (e.g.. GIBCO, Code 11360-039)	100X	+4 °C
PSG (e.g.. GIBCO, Code: 10378-016)	100X	-20 °C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Location
Sterile pipettes	Cabinet/drawer
Pipettor	Cabinet/drawer
Micropipettes and tips	Cabinet/drawer
Centrifuge tubes	Cabinet/drawer
0.22 µm syringe filters	Cabinet/drawer
Cryovials	Cabinet/drawer

PROCEDURE TO PREPARE AUTOLOGOUS SERUM

- Collect autologous blood in sterile tubes not containing any anticoagulants or preservatives. To allow clotting of the blood, do not shake the tubes and let them stand in an upright position for 1 h
- Centrifuge the tubes for 15 min at 1900 g
- Collect the supernatant (serum) with a 10 ml serological pipette and check visually if the serum is clear and/without coloration
- Pool the serum collected from all the tubes in a 15 ml sterile tube
- Filter by a 0.2 µm sterile syringe filter
- Aliquot in cryovials (1 ml/vial) and store at -20°C

SAMPLES PREPARATION FOR SCANNING ELECTRON MICROSCOPY (SEM)

DESCRIPTION

Scanning electron microscopy (SEM) allows capturing high-resolution images of objects. It exploits a focused high-energy electron beam to investigate the surface of solid specimens. The interaction between the electron beam and the surface of the sample produces different signals, such as secondary electrons (related to topographical contrasts), backscattered electrons (related to chemical contrasts) and X-rays. X-rays can be detected by EDS and they allow assessing the elemental chemical composition of the sample.

Fixation of the specimens has to be performed, followed by dehydration with ethanol. For observation by conventional SEM, an electrically conductive coating must also be applied to electrically insulating samples. It is not necessary for instruments (like environmental SEM, ESEM) able to operate in a low vacuum mode, but its presence improves the resolution of images.

CHEMICAL AND REAGENTS

Reagent	**Quantity**	**Storage**
Sodium cacodylate (Sigma)		+4°C
Glutaraldehyde 50% water		+4°C
Ethanol absolute		Cabinet
Anhydrous sodium sulfate		Cabinet
Hexamethyldisilazane		+4°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Quantity**	**Storage**
Micropipettes da 100-1000 µl	1	Bench
Sterile pipettes 5 ml, 10 ml		Cabinet
Manual pipettor	1	Bench
Multi channel pipette	1	Cabinet

Multi channel pipette tips		Cabinet
50 ml Falcon tubes	1	Cabinet
Cellulose acetate 0,2 µm filter	1	Cabinet
Becker or glass Petri dishes		Cabinet

PROCEDURE

- Remove culture medium and incubate the samples for 2 h at room temperature (eventually, overnight at 4°C) with a solution composed of 1.5% (v/v) glutaraldehyde 50% (v/v) in water and 0.1 M sodium cacodylate (pH=7.1-7.2).
- Prepare enough 0.1 M sodium cacodylate buffer also for sample rinsing after fixing and for sample storing.
- Basically, for 1 ml fixing solution, 30 µl glutaraldehyde 50% (v/v) has to be mixed to 970 µl 0.1 M sodium cacodylate (pH=7.1-7.2)
- Rinse the samples with 0.1 M sodium cacodylate buffer. If necessary, samples can be stored in this solution at 4°C for one week.
- Dip the samples for 5 min (twice) in increasing concentration of ethanol in deionized water. One possibility might be the use of these concentrations:
    - 20% v/v ethanol
    - 30% v/v ethanol
    - 40% v/v ethanol
    - 50% v/v ethanol
    - 60% v/v ethanol
    - 70% v/v ethanol (for overnight or storage, instead of 5 min twice)
    - 90% v/v ethanol
    - 96% v/v ethanol
    - 100% v/v ethanol (for storage, if required with anhydrous sodium sulphate)
- Book SEM observation by contacting Francesca Brunella (Tel: 02.2399.3157, francesca.brunella@polimi.it)

Only if strictly necessary, dip the samples for 5 min (twice) in graded series of hexamethyldisilazane in ethanol. One possibility might be the use of these concentrations:

25% v/v hexamethyldisilazane

50% v/v hexamethyldisilazane

75% v/v hexamethyldisilazane

100% v/v hexamethyldisilazane

To remove hexamethyldisilazane, move the samples to the dryer until SEM analysis

NOTE

- Fixation and dehydration have to be performed in a chemical hood
- Working in sterile conditions is not mandatory
- Fixing solution has to be prepared before using and cannot be stored for future fixing
- Sodium cacodylate and glutaraldehyde are highly toxic, especially for water animals. Pay attention to waste disposal
- Before using, adjust the pH of sodium cacodylate solution to 7.1-7.2
- Hexamethyldisilazane is used to gradually remove ethanol. However, it is very volatile (and so very toxic for the operator)! Try to avoid the use of hexamethyldisilazane: in particular, for observations with the SEMs available at CMIC, this step is not required

CELL BIOCHEMICAL ASSAYS

ALAMAR BLUE ASSAY FOR CELL METABOLIC ACTIVITY

DESCRIPTION

The assay, which is based on Alamar Blue, a viability redox indicator, responds to the chemical reduction of the added indicator in culture medium as an effect of cell proliferation, thus functioning as a cell health indicator. When cells are alive, they maintain a reducing environment within their cytosol. Resazurin, the active ingredient of Alamar Blue reagent, is a non-toxic, cell permeable compound that is blue in color and weakly fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is pink in color and highly red fluorescent. The results of the essay can be measured in both fluorescence and absorbance using a spectrophotometer. At the end of the procedure, Alamar Blue can be removed and substituted with fresh culture medium, allowing cells to remain in culture.

CHEMICALS AND REAGENTS

Reagent	**Quantity**	**Location**
Alamar Blue, solution (Serotec)	….	+4°C
PBS	….	Cabinet
Culture medium	….	+4°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Quantity**	**Location**
Micropipettes 20, 200, 1000 µl	1	Bench
Sterile pipettes: 1 ml, 5 ml, 10 ml	….	Cabinet
Multichannel pipette	1	Cabinet
Multichannel pipette tips	….	Cabinet
50 ml tubes	1	Cabinet
96-well Multiwell plates (transparent/white)	1	Cabinet

Cellulose acetate 0,2 µm filter	1	Cabinet

PROCEDURE

- Prepare 10% Alamar Blue solution, 10 ml: dilute Alamar Blue 1/10 in a 50 ml Centrifuge tube, adding to 1 ml of Alamar blue stock solution (bottle) to 9 ml of culture medium.
- Filter and sterilize
- Remove culture medium from the plates to be analyzed and substitute with an equal amount of 10% Alamar Blue solution.
- Incubate the plate for 1-4 hours.
- Transfer 100 µl of assay medium in a 96-multiwell plate (transparent for absorbance reading, white for fluorescence reading) and read absorbance at 570 nm and at 630 nm as reference. Fluorescence has to be analyzed with an excitation wavelength of 510 nm and an emission wavelength of 590 nm;
- Remove the excess of assay medium; rinse the samples in PBS twice, then add fresh culture medium and incubate again the multiwell plate.

NOTE

10% Alamar Blue solution has to be prepared just before using and can not be stored.

MTT CELL VIABILITY ASSAY

DESCRIPTION

The assay measures intracellular mitochondrial activity through the analysis of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction to formazan, which has a blue/violet color. Reduction is performed by intracellular dehydrogenase in living cells. This reaction only takes place if cells have a significant oxidative metabolism level. The resulting solution color is readable in absorbance at 570 nm and the value is proportional to the number of living cells.

CHEMICALS AND REAGENTS

Reagent	Quantity	Location
MTT, powder (Sigma)	50 mg	Cabinet
PBS	10 ml	Cabinet
Culture medium	9 ml	Freezer/Fridge
Dimetylsulfoxide (DMSO) (Sigma)	vial	Cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Location
Micropipettes 20, 200, 1000 µl	1	Bench
Sterile pipettes: 1 ml, 5 ml, 10 ml	….	Cabinet
Multichannel pipette	1	Cabinet
Multichannel pipette tips	….	Cabinet
50 ml tubes	1	Cabinet

96-well multiwell plates (transparent/white)	1	Cabinet
Cellulose acetate 0,2 μm filter	1	Cabinet

PROCEDURE

Preparation of MTT solution

Depending on the number of samples, prepare an adequate volume of MTT solution.

The MTT solution is composed by 1 part v/v of MTT solution (5 mg/mL in PBS) and 9 parts v/v of pre-warmed CM without phenol red.

The MTT solution must be filtered in a centrifuge tube with 0.22 syringe filter and covered by light with a silver foil.

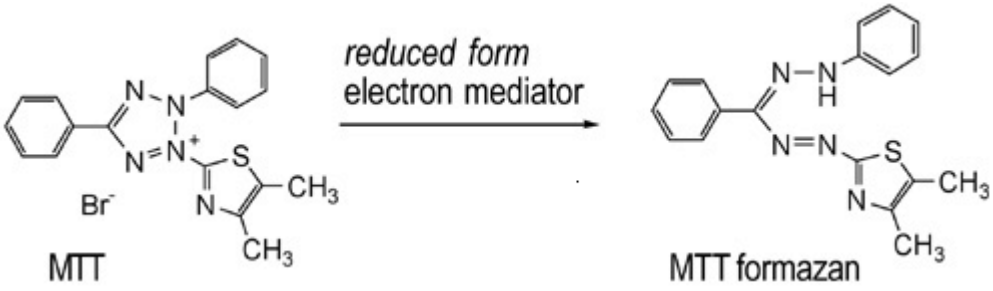
It can be stored for one week at 4°C.

Preparation of MTT extraction solution

Use 2- propanol with 10% v/v DMSO or pure DMSO.

Incubation of adherent cells with MTT

- Remove the culture medium and wash the cells with PBS.
- Put an adequate volume of MTT solution to cover the cells.
- Cover the plate with a silver foil and incubate for 1-4 hours in incubator at 37°C.
- After the incubation, remove MTT solution (e.g. by inversion of the plate) and add an equal amount of extractant solution.
- Put the plate in agitation for 40-60 minutes until complete dissolution of crystals (check using a microscope).
- Put the plate in incubator at 37°C for 2h.
- Plate the samples in a 96-well plate and read the absorbance at 570 nm (with a reference wavelength of 630 nm).



Schematic representation of MTT reduction to formazan

PREPARATION PROCEDURE FOR ASSAYS ON CELL LYSATE

DESCRIPTION

Cell lysis corresponds to the breakage of cell wraps so to have the leakage of cellular components. The solution containing lysed cells is called "lysate". For cell lysis, cells are exposed to stressful stressful conditions. There are different methods to produce cell lysate. Subsequent cell freezing and defrosting is an example of a physical method.

CHEMICALS AND REAGENTS

Reagent	**Quantity**	**Location**
Distilled water	...	Cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Quantity**	**Location**
Pasteur pipettes 3 ml	...	Cabinet
Multichannel pipette	1	Cabinet
Multichannel pipette tips	….	Cabinet
50 ml tubes	1	Cabinet
24-well multiwell plates	1	Cabinet
Cellulose acetate 0,2 µm filter	1	Cabinet

PROCEDURE

- Remove culture medium from the wells and substitute with an equal amount of distilled sterile water.
- Incubate at -80° C for 20 minutes and then at 37° C for 20 minutes, repeating the procedure three times.
- Freeze at -20°C (or -80°C) until the day of the assay.
- Defrost the plate at room temperature and check by an optical microscope that cellular lysis has completed occurred.
- Place the plate on a shaker for at least 20 minutes before using.

NOTE

Store the removed supernatants at -20°C in sterile conditions, so that they can be used for further analysis.

HOECHST 33258 ASSAY: MEASUREMENT OF TOTAL DNA CONTENT

CHEMICALS AND REAGENTS

Reagents	Amount	Stored
DNA standards (SIGMA)		Freezer
Sodium chloride (SIGMA)		Cabinet
Trisodium citrate (SIGMA)		Cabinet
HOECHST 33258 (dissolved in DMSO) (Sigma)	Bottle	Freezer
Sodium hydroxide or hydrochloric acid (for pH adjustment)	Drops/a few mL	Aspirate cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	Amount	Stored
20, 200, 1000 µl micropipettes		Bench
Sterile pipettes: 1 ml, 5 ml, 10 ml	….	Cabinet
Pipette tips	….	Cabinet
Pipetboy	1	Bench
50 ml and 15 ml centrifuge tubes	….	Cabinet
Microcentrifuge tubes	….	Cabinet
White 96-multiwell culture plates (for fluorescence measurements)	….	Cabinet
Spectrophotometer		Bench

Principles

The assay measures the total intracellular DNA content with a DNA specific dye, HOECHST 33258, which binds to A-T base pairs emitting fluorescence at 460 nm. The assay must be performed on cell lysate (which must be kept sterile for any further further tests).

Solutions

DNA stock solution (1 mg/ml): Prepare the DNA solution (1 mg/ml) in sterile demineralized water. Vortex until complete solubilization. Aliquot into microcentrifuge tubes and store at -20 ° C (freezer).

HOECHST 33258 stock solution (1 mg/ml): Prepare the HOECHST 33258 solution (1 mg/ml) in sterile demineralized water. Vortex until complete solubilization. Aliquot into microcentrifuge tubes and store at -20 ° C (freezer).

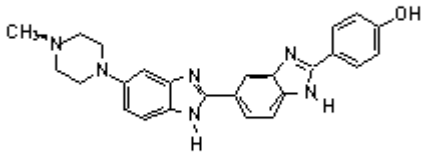
SSC stock solution (20X): Prepare 500 ml of 20X SSC solution (with respect to the working concentrations) in demineralized water. Weight 87.65 g sodium chloride and 44.10 g trisodium citrate. Dissolve in 480 ml demineralized water and adjust the pH to 7.0 with 1 M hydrochloric acid or 1 M sodium hydroxide, if required. Make up to 500 ml in a Pirex bottle and sterilize by autoclaving, if required.

Before starting the assay, dilute the solution 1 to 20 (e.g. 1 ml solution + 19 ml demineralized water), to get the working concentration (indicated as 1X).

Method

- To prepare the calibration line, label a series of microcentrifuge tubes with the final standard DNA concentrations (20; 10; 5; 2.5; 1.25; 0.625; 0.3125; 0 µg/ml).
- Thaw a quantity of stock DNA solution suitable to the number of plates to be analyzed (each plate requires 0.5 ml of standards).
- Prepare 1 ml of 20 µg/ml standard DNA solution for each 96-well plate to be tested by diluting the 1 mg/ml DNA stock solution 1:50 in 1X solution of SSC (replace 20 µl of SSC per ml with 20 µl of stock DNA solution) e mix thoroughly with the vortex.
- Measure a volume of 1X SSC solution equal to the final volume of the required standard (0.5 ml for each 96-well plate) in each of the tubes prepared for the calibration line, starting from 10 µg/ml (included) onwards.
- Sterile transfer an equal volume of 20 µg/ml standard DNA solution in the 10 µg/ml standard DNA tube, vortex and repeat the operation from the tube with the 10 µg/ml standard DNA tube into the 5 µg/ml tube and so on until the final dilution (0.3125 µg/ml), always mixing on vortex after each dilution. Use the 1X SSC solution in the 0 µg/ml tube.
- Sterile transfer 100 µl of each standard and sample to the wells of a white 96-multiwell plate.
- Prepare the volume of 1 µg/ml HOECHST 33258 solution required for the assay by transferring in a tube the volume of 1X SSC solution suitable to the number of plates to be analyzed (each 96-well plate requires 10 ml of solution).
- Remove a volume of SSC solution equal to the volume of HOECHST 33258 stock solution to be added (in the case of 10 ml, remove 10 µl) and replace with an equal volume of HOECHST 33258 stock solution (note: the solution obtained can be stored for a maximum of 2 hours without losing its activity, in the case that you need to repeat the assay).

- Add 100 µl of 1 µg/ml HOECHST 33258 solution to each well and mix by placing the plate on a plate shaker for up to 1 minute.
- Measure the fluorescence with a multimethod plate reader (excitation wavelenght: 360 nm; emission wavelenght: 460 nm).



Representation of the HOECHST 33258 molecule

QUANT-IT PICOGREEN dsDNA ASSAY

CHEMICALS AND REAGENTS

Reagents	Composition	Stored
PBE Buffer	For 500 ml: 7.1 g Na_2HPO_4, 1.86 g NaEDTA, HCl; pH 6,8	+4 °C
L-Cysteine 5 mM (es. Fluka, code: 30129)		RT
Papain (e.g.. Worthington)		+4 °C
PicoGreen reagent kit (ThermoFisher)		+4 °C/RT
water free of DNase and RNase		RT

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	Stored
1.5 ml and 2 ml microcentrifuge tubes	Cabinet/drawer
20, 200, 1000 µl micropipettes and tips	Cabinet/drawer
50 ml and 15 ml centrifuge tubes	Cabinet/drawer
Spectrophotometer (Victor)	Bench
96-multiwell black flat-bottomed plate (CELBIO-NUNC)	Cabinet/drawer

Principle

Using the PicoGreen® dsDNA quantitation assay, you can selectively detect as little as 25 pg/ml of dsDNA in the presence of ssDNA, RNA, and free nucleotides. The assay is linear over three orders of magnitude and has little sequence dependence, allowing you to accurately measure DNA from many sources, including genomic DNA, viral DNA, miniprep DNA, or PCR amplification products.

PROCEDURE:

Sample Digestion

- Wash the sample with PBS.
- Prepare the digestion solution (in the case of digestion of a pellet, consider 500 uL per sample) by diluting papain 1:100 in PBE buffer and cysteine [e.g.: 20 mL PBE buffer* + 0.035 g Cysteine + 100 ul papain].
  (*) PBE buffer: for 500 mL solution use 7.1g Na_2HPO4 (dibasic sodium phosphate) + 1.86 g NaEDTA + 500 ml distilled water then adjust the pH 6.8
- Digest the pellet in 500 uL of digestion solution using a mechanical mixer for 16 h at 60°C without agitation. Store at -20°C.

PicoGreen Assay

- Prepare COMPONENT B 1X:
- Dilute Component B from 20X to 1X in water free of DNase and RNase.
- Prepare the COMPONENT A 1X: dilute component A from 200X to 1X using component B 1X as diluent.
- Prepare DNA standard (or COMPONENT C): dilute component C from 100 µg/ml to 2 µg/ml using component B 1X as diluent.
- To prepare the calibration curve (standard curve) it is necessary to prepare the standards as follows:

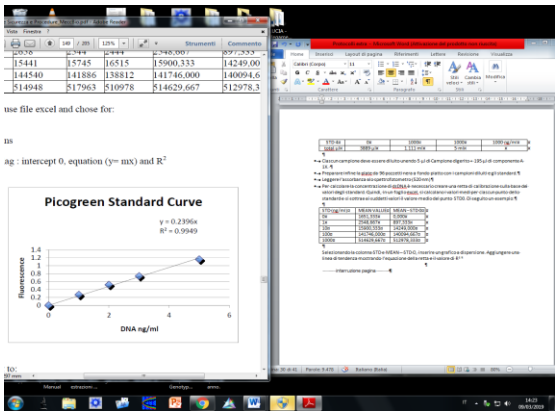
Dilution Table:

	COMPONENT B 1X (µl)	COMPONENT C (µl)	COMPONENT A 1X (µl)	DNA FINAL CONC. (µl)
STD 0	1000	0	1000	0 ng/ml
STD 1	999	1	1000	1 ng/ml
STD 2	990	10	1000	10 ng/ml
STD 3	900	100	1000	100 ng/ml
STD 4	0	1000	1000	1000 ng/ml
Total µl	3889 µl	1.111 ml	5 ml	

- Each sample must be diluted by combining 5 µl of digested sample + 195 µl of component A 1X.
- Finally, prepare the 96-multiwell black flat-bottomed plate with diluted samples and standards.
- Read the absorbance at 520 nm by the spectrophotometer.
- To calculate the dsDNA concentration it is necessary to create a calibration line based on the values of the standards. Then, in an Excel spreadsheet, the average values for each point of the standard are calculated and the average value of the STD 0 point is subtracted from the aforementioned values. Here is an example:

STD (ng/ml)	MEAN VALUE	MEAN - STD 0
0	1651.333	0.000
1	2548.667	897.333
10	15900.333	14249.000
100	141746.000	140094.667
1000	514629.667	512978.333

By selecting the STD and MEAN - STD 0 column, insert a scatter plot. Add one trend line showing the equation of the line and the value of R^2.



Calculate the average value of each sample (if loaded in duplicate or tripled) and subtract the value STD 0 at the average value. To obtain the concentration in ng/ml, enter the values obtained in the equation:

y = mx and obtain the x.

To calculate the total amount of ng: concentration (ng/ml) x (loaded volume$_{200\ \mu l}$/1000 µl) x (digestion volume$_{500\ \mu l}$/sample loaded$_{5\ \mu l}$).

From the total amount of DNA it is possible to derive the total number of cells by dividing the total ng for the amount of DNA contained in a single cell (e.g. chondrocytes contain 7 pg DNA).

Normalization using DNA results

DNA Picogreen results are used to normalize both GAG Blyscan results (see protocol) and MTT viability assay.

ALKALINE PHOSPHATASE: MEASUREMENTS OF ENZYMATIC ACTIVITY

CHEMICALS AND REAGENTS

Reagents	Amount	Stored
Randox KIT (AP 542; AP 501; AP 502; AP 307).		Fridge

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Amount	Stored
20, 200, 1000 µl micropipettes	...	Bench
Sterile pipettes: 1 ml, 5 ml, 10 ml	….	Cabinet
Pipette tips	….	Cabinet
50 ml and 15 ml centrifuge tubes	...	Cabinet
White 48-multiwell culture plates (for fluorescence measurements)	...	Cabinet
Pipetboy	1	Bench
Spectrophotometer		Bench

Principle

The assay is based on the use of p-nitrophenol phosphate in diethanolamine buffer, since the enzyme alkaline phosphatase (ALP) cuts the phosphate groups of the substrate producing P-nitrophenol, yellow at pH alkaline, absorbing at 405 nm. The assay should be performed on supernatants and/or cell lysate (which should be kept sterile for further tests).

Note: to allow the enzyme recovering its activity, it is necessary to thaw the samples the night before, place in the fridge and then, in the morning, leave at room temperature under stirring for at least 2-3 h.

Solutions

Substrate solution: reconstitute the substrate solution by adding to the KIT vial indicated as "Substrate 2" the appropriate volume of buffer solution indicated as "Buffer 1": 3 ml for the KIT AP 542; 10 ml for the KIT AP 307; 20 ml for the AP 501 KIT; 30 ml for the AP 502 KIT. The solution is stable for 30 days at 2-8 ° C.

Method

Follow the next steps:

1. Transfer 500 µl substrate solution into the wells of a transparent 48-well multiwell plate;
2. Withdraw 10 µl sample in sterile conditions, add to each well and mix by placing the plate on a plate shaker for two minutes;
3. Measure the absorbance with the microplate multimethod reader at a wavelength of 405 nm after 1, 2 and 3 minutes;
4. Calculate the ALP activity using the following formula: $U/I = A_{405} \text{ X } 2760$ [nm/min]

REFERENCES

[1] Bessey, Otto A., Oliver H. Lowry, and M. J. Brook, **A method for the rapid determination of Alkaline Phosphatase with five cubic millimeters of serum**. *Landmark Papers in Clinical Chemistry* 135 (2005).

PAPAIN DIGESTION

CHEMICALS AND REAGENTS

Reagents	Amount	Stored
Papain suspension		Fridge
Cysteine hydrochloride (Cys-HCl)	0.788 g	RT
EDTA (Ethylenediaminetetraacetic acid)	0.403 g	RT
Phosphate buffered saline solution		RT
Sodium hydroxide		Aspirated cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Amount	Stored
20, 200, 1000 µl micropipettes	...	Bench
Sterile pipettes: 1 ml, 5 ml, 10 ml	….	Cabinet
Pipette tips	….	Cabinet
Centrifuge tubes (50 ml and 15 ml)	...	Cabinet
Pipetboy	1	Bench

Principle

Papain is a proteolytic enzyme that is extracted from the immature fruit of papaya (*Carica papaya*). It has a digestive action superior to pepsin and pancreatin, it acts like pineapple bromelain. Taken during the digestive process of proteins, it dissolves the albuminoids and converts into peptones (that are easily

soluble and diffusible, capable of being absorbed and assimilated) and it will act as a catalytic agent acting as acidic, alkaline or neutral medium.

Solutions

1) **PAPAIN DIGESTION BUFFER** (Preparation for 1l of stock buffer)

- Add 0.788g cysteine hydrochloride and 0.403g EDTA to 480 ml PBS.
- Adjust the pH with NaOH to 6.00.
- Add PBS until a final volume of 500 ml. Dilute the solution 1:2 with distilled water (final concentrations: cysteine hydrochloride 0.005 M; EDTA 0.0015 M 1l).
- Make 20 ml aliquots and store in the freezer.

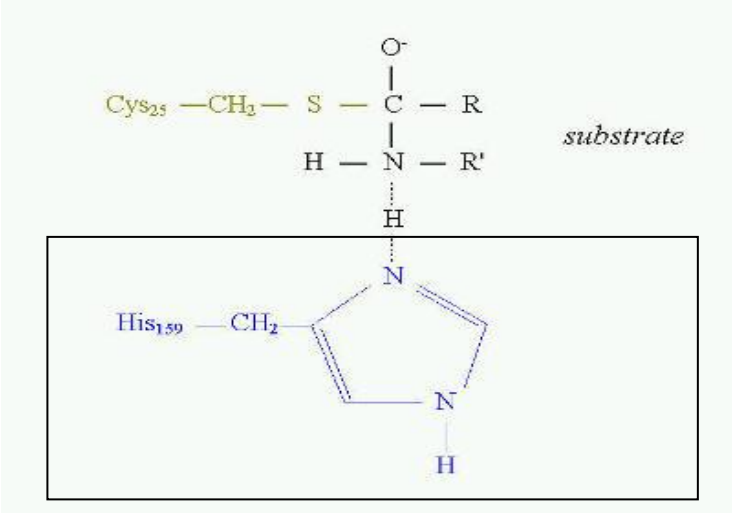
2) **PREPARATION OF PAPAIN SOLUTION**

In the required volume of digestion buffer, add 1 µl of papain suspension per ml of buffer.

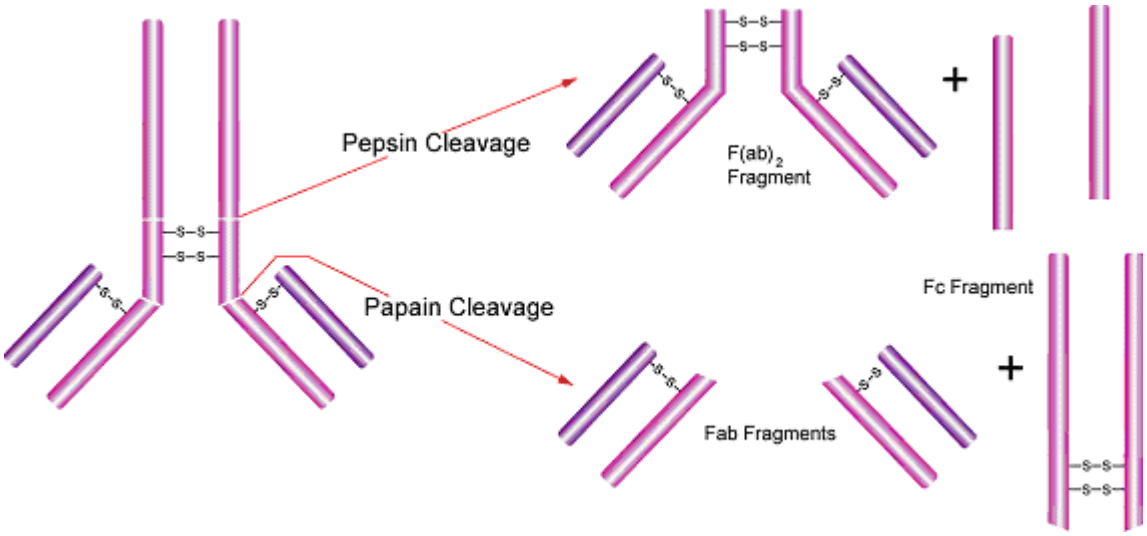
Method

- Prepare the digestion mixture in papain: 1 µl papain solution for 1 ml digestion buffer
- Add papain digestion mixture to the samples.
- Seal carefully and then keep at 65 °C overnight in the stove or bath.

Scheme of papain protein (surrounded in the box)



Papain protease cutting action on antibody substrate



HYDROXYPROLINE COLORIMETRIC ASSAY

CHEMICALS AND REAGENTS

Reagents	Amount	Stored
Citric acid monohydrate	25 g	Cabinet
Sodium Acetate	41 g	Cabinet
Glacial Acetic Acid	6 ml	Aspirated Cabinet
Perchloric Acid 70%	2.74 ml	Aspirated Cabinet
Sodium Hydroxide	17 g	Aspirated Cabinet
Hydroxyproline		Cabinet
Chloramine-T	0.14 g	Cabinet
Methoxyethanol	13 ml	Aspirated Cabinet
Dimethylaminobenzaldehyde (DMAB)	1 g	Aspirated Cabinet
Concentrated HCl		Aspirated Cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	Amount	Stored
Scale	1	Bench
pHmetre	1	Bench
Vortex	1	Bench
Drier or Lyophilizer	1	Bench
Multimethod plate reader	1	Bench

20, 200, 1000 µl micropipettes		Bench
Sterile pipettes: 1 ml, 5 ml, 10 ml		Cabinet
Micropipette tips		Cabinet
Transparent 96-well multiplate		Cabinet
Centrifuge tubes (50 ml and 15 ml)		Cabinet
Pipetboy		Bench

Principle

The amino acid hydroxyproline is oxidized by chloramine-T (sodium N-chloro-p-toluenesulphonamide) in neutral buffer. The chromogen formed combines with paramethylaminobenzaldehyde (DMAB) in high concentration perchloric acid, producing a chromophore whose absorbance is detected at 570 nm.

1. Prepare the stock buffer solution (10X) (to be diluted 1:10 before use, 1 ml of 10X stock buffer + 9 ml of distilled water)

Quantity per 500 ml of solution:

- citric acid monohydrate, 25 g
- glacial acetic acid, 6 ml
- sodium acetate, 41 g
- sodium hydroxide, 17 g

Add distilled water up to 450 ml and adjust the pH to 6.0. Make up to a final volume of 500 ml.

Store at 4 ° C.

2. Hydroxyproline stock solution 200 mg/ml (1000X), aliquoted at -20C °

Note: In this assay we use a hydroxyproline solution at a concentration of 20 µg/ml. For prepare 1 ml solution, proceed with serial dilutions, in this way:

- 10 µl of hydroxyproline stock solution 1000x + 990 µl of buffer solution (the final solution has concentration 2 mg/ml), then mix the solution with a vortex;
- 10 µl of hydroxyproline 2mg/ml + 990 µl of buffer solution (the final solution has concentration 20 µg/ml), then mix the solution with a vortex.

Preparation of 70% perchloric acid solution (10 ml): put 2.74 ml distilled water and add perchloric acid up to 10 ml (sufficient for two microplates).

Prepare the chloramine-T solution: dissolve 0.14 g of chloramine-T in 2 ml of distilled water. Add 3 ml of methoxyethanol and 5 ml of 10X stock buffer (10 ml will be sufficient for 2 microplates).

This solution is stable for 2-3h after preparation, it is best to prepare it after the samples, just before starting the essay.

Prepare the Elrich's reagent: dissolve 1 g of DMAB in 10 ml of methoxyethanol, mixing carefully (enough for 2 microplates).

Also this solution is stable for 2-3h after the preparation, it is better to prepare it after the samples, just before starting the test.

NOTE: Perchloric acid is VERY dangerous: handle with the TECHNICIAN or LAB MANAGER.

Method

Sample preparation

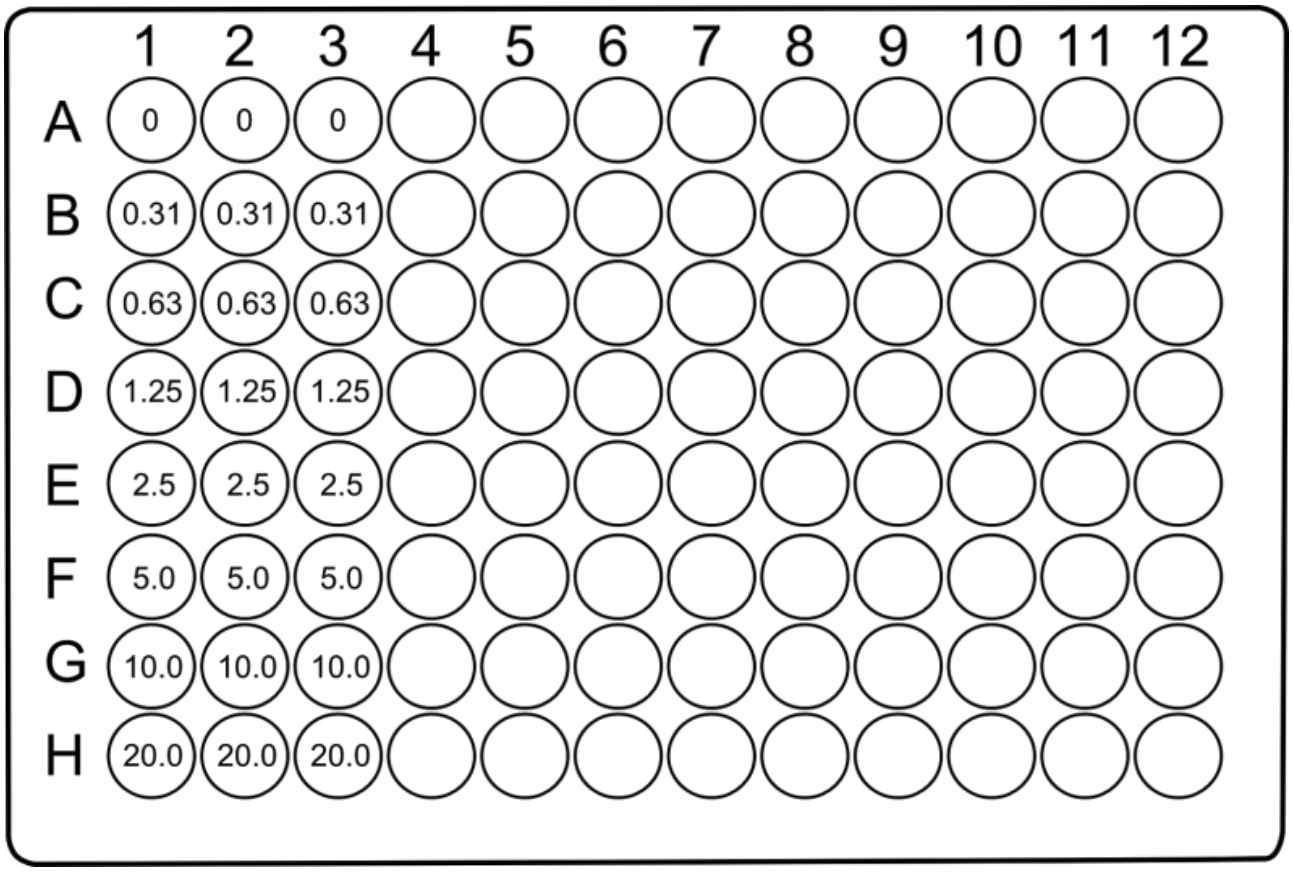
Tissue samples must be digested in papain (approximately 10 µg of tissue in 250 µl digestion buffer)

- Place 40 µl of papain digest in glass containers. Add 0.25 ml concentrated HCl and 0.25 ml distilled water until the neck of the ampoule is filled before sealing with parafilm. Seal and leave overnight (minimum 16 hours) in an oven at 110 ° C.
- Dry the sample in the lyophilizer (or dryer) overnight.
- Resuspend in 0.5 ml of 1X stock buffer.

ASSAY

Follow the steps below:

- Add 1 ml of 20 µg/ml hydroxyproline solution to H1 (see figure below)
- Pipette 500 ml of 1x buffer into B1, C1, D1, E1, F1 and G1
- Transfer 500 µl from H1 to G1 to obtain 1 ml of 10 µg/ml of diluted solution. Mix the diluted solution with the vortex. Repeat the last two operations to obtain the concentrations of F1, E1, D1, C1 and B1.
- Pipette 100 µl of sample into standard wells and into the control
- Add 50 µl of chloramine-T solution to each well and stir the multiwell plate for 5 minutes. The solution may become cloudy.
- Add 50 µl of 70% perchloric acid solution to each well and stir the plate for 5 minutes. The solution may become cloudy.
- Add 50 µl of DMAB solution to each well and stir the plate for 10 minutes.
- Incubate at 60 ° C for about 30-40 minutes, until the solution turns pink. The solution may become clear and yellowish.
- Insert the plate into the spectrophotometer and read the absorbance at 570 nm.



Scheme of the 96-well microplate with all the relative concentration (expressed in µg/ml). Each well will contain 500 µl of solution.

GLYCOSAMINOGLYCANS (GAGS) WITH DIMETHYLMETHYLENE BLUE (DMMB) ASSAY

CHEMICALS AND REAGENTS

Reagents	Amount	Stored
DMMB (e.g.. Sigma, 341088)	16 mg for 1L solution	Cabinet
Ethanol	5 mL for 1L DMMB solution	Aspirated Cabinet
Sodium formate	2 g for 1L DMMB solution	Cabinet
Formic acid	2 mL for 1L DMMB solution	AspiratedCabinet
Chondroitin sulfate (e.g Sigma, C8529)		
Distilled water		

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	Stored
Micropipettes and tips	Bench and Cabinet
Pasteur pipette (3 mL)	Cabinet
Pipetboy	Bench
96-well microplates	Cabinet
Vortex	Bench
Multimethod plate reader	Bench
Microcentrifuge tubes (1.5 mL)	Cabinet

GAGs are long unbranched chains formed by disaccharide units; one of the two saccharides contains an amino group instead of a simple -OH group (this is called aminosaccharide). Given the high heterogeneity of the type and number of sulfate and carboxyl groups that make up the GAGs, various types of glycosaminoglycans can be distinguished. GAGs mainly support and protect the tissues (especially in the cartilaginous tissue).

- They create and keep constant the extracellular turgor pressure. Being hydrophilic, GAGs can very easily bind with water molecules, creating hydrated molecules. Hydration leads to a sort of "swelling" of the GAG molecule, which therefore takes on the appearance and function of a cushioning pad. The tissue is thus protected from the traumas that can be caused by external compression forces;
- They transport water-soluble molecule,s that can rapidly spread inside the porous structure of the GAG;
- They can bind with other molecules, giving rise to proteoglycans and glycoproteins.

Procedure

The dimethylmethylene blue dye (DMMB) combines with sulfated GAGs causing a metachromatic variation of the absorbance that presents a maximum peak from 530 nm to 600 nm.

- For the DMMB solution, there are two options:
  1) add 5 ml ethanol, 2.0 g sodium formate and 2 ml formic acid to 990 ml distilled water, adjust the pH to 3.00 using formic acid. Dissolve 16 mg DMMB, then make up to 1 L and store it in a dark closed bottle at room temperature

  2) add 3.04 g glycine (Sigma, code G7126-100g), 2.36 g NaCl, 800 µl concentrated HCl in 1L of ddH_2O. While stirring, dissolve 16 mg DMMB.
- For the standard solutions: solubilize chondroitin sulfate A (1 mg/ml), prepare 500 µl aliquots and store in the freezer. To prepare the standard curve it is necessary to dilute 1:10 the stock solution 1 mg/ml.
- Using the first and second row of the multiwell plate, prepare the standard solution (using stock solution 1:10)
- Prepare GAG standard solution by diluting 1:10 the stock solution and calculating the total volume from the table. Add distilled water in microcentrifuge tubes as indicated in the table. Add the complement to 200 µl with respect to the volume of standard GAG solution (previously diluted 1:10 with respect to the stock).
- Transfer into the 96 multiwell plate the first two lines with the calibration line and the samples and controls, as shown in the table. Vortex each sample and each standard before pipetting. Transfer 40 µl to all the wells (both for samples and standard).
- Add 250 microliters of DMMB solution to each well.
- Read the absorbance at 535 nm.

Standard GAG curve

Standard no	**GAG conc (µg/ml)**	**Vol standard (µl)**	**ddH_2O (µl)**
1	0	0	200
2	5	10	190
3	10	20	180
4	15	30	170
5	20	40	160
6	25	50	150
7	30	60	140

8	35	70	130
9	40	80	120
10	50	90	110
11	60	100	100

Multiwell GAG plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0**	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
B	**0**	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
C												
D												
E												
F												
G												
H												

DIMETHYLMETHYLENE BLUE ASSAY (DMMB)

CHEMICALS AND REAGENTS

Reagents	**Amount**	**Stored**
Na_2HPO_4 (e.g. Sigma, S7907)	7.1 g for 500 mL PBE buffer	Cabinet
Na_2EDTA (e.g. Sigma, E1644)	1.86 g for 500 mL PBE buffer	Cabinet
Glycine (e.gs. Sigma, G7126)	1.52 g for 500 mL DMMB solution	Cabinet
NaCl	1.18 g for 500 mL DMMB solution	Cabinet
Concentrated HCl	0.4 mL for 500 mL DMMB solution	Aspirated Cabinet
DMMB (e.g. Sigma, 341088)	8 mg for 500 mL DMMB solution	Cabinet
Chondroitin sulfate (es. Blyscan Assay Kit)		Cabinet
Distilled water		

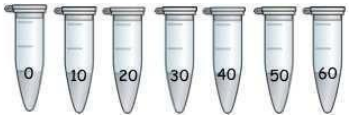
EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	**Stored**
Micropipettes	Bench
Serological pipettes	Cabinet
Pipetboy	Bench
96-well microplate	Cabinet
Microcentrifuge tubes (1,5 mL)	Cabinet
Multimethod plate reader	Bench

Procedure:

Solution preparation

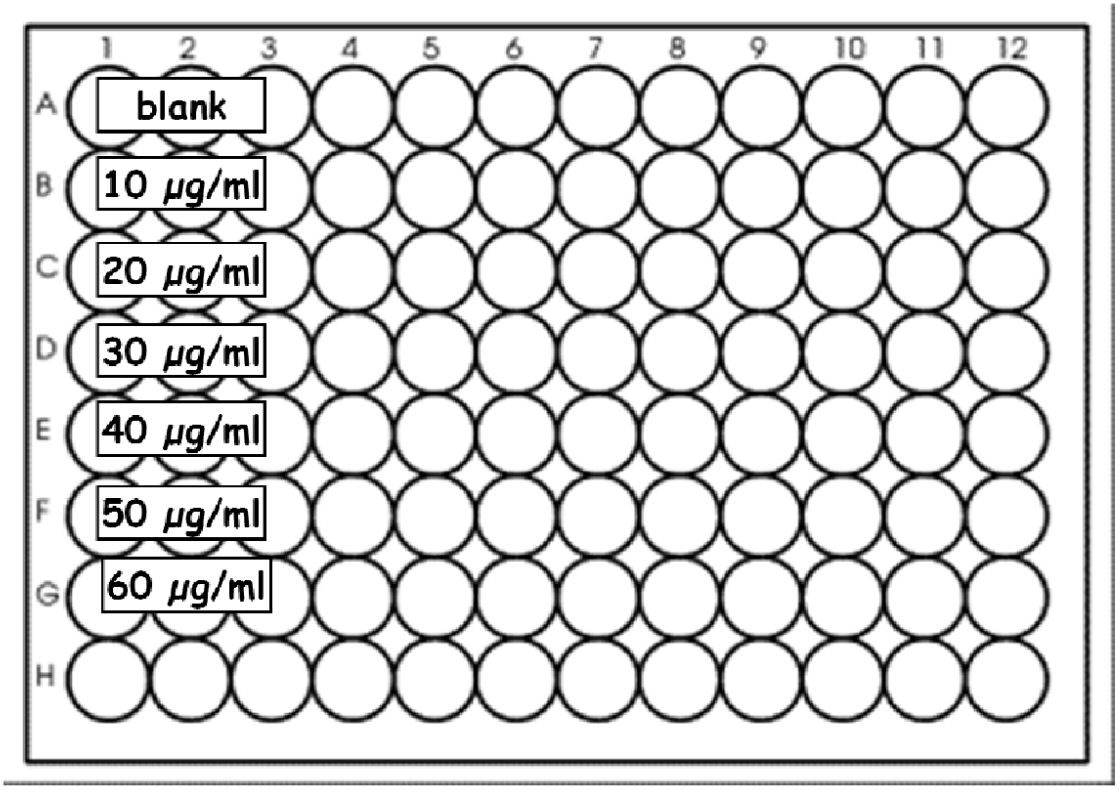
- For PBE buffer (100 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 6,5): dissolve 7.1 g Na_2HPO_4 and 1.86 g Na_2EDTA in 500 mL distilled water. Store PBE buffer at 2-8°C
- For DMMB solution (16 g/mL DMMB in glycine/NaCl, pH 3): add 1.52 g glycine; 1.18 g NaCl and 400 µl HCl concentrated to 500 mL distilled water. In agitation, dissolve 8 mg DMMB
- Chondroitin sulfate (CS) standard solutions: 100 µg/mL.
  Prepare CS standard solutions: take 7 clean microcentrifuge tubes and prepare the samples for the calibration line in a final volume of 50 µL (0, 10, 20, 30, 40, 50 and 60 µL/mL). Use Blyscan kit standards.



- Use the PBE buffer to dilute CS stock solutions

Standard No.	**CS Conc. (µg/mL)**	**CS a 100 µg/ml (µL)**	**PBE buffer (µL)**
1	0	0	50
2	10	5	45
3	20	10	40
4	30	15	35
5	40	20	30
6	50	25	25
7	60	30	20

- Pipette 12 µl of each standard (in triplicate) in a 96-well multiwell plate and add 38 µL PBE buffer.
- Sample preparation: place 25-35 µl (depending on sample concentration) in a 96-well microplate and add PBE buffer up to 50 µL
- Measurements: add 100 µl of DMMB solution to all the wells and read the absorbance at 500 nm



Note: The absorbance values obtained for the samples must be in the range measured for the standards, otherwise sample readings may be affected by saturation. If sample readings are higher than the last point of the standard curve, repeat the analysis with a smaller amount of sample.

REFERENCES

Coulson-Thomas, V. and Gesteira, T. F. (2014). Dimethylmethylene Blue Assay (DMMB). *Bio-protocol* 4(18): e1236. http://www.bio-protocol.org/e1236

Warren, S. (2000). A critical analysis of the 1, 9-dimethylmethylene blue assay for sulfated glycosaminoglycans in *Synovial fluid*. University of Guelph.

BLYSCAN-SULFATED GLYCOSAMINOGLYCAN (GAG) ASSAY

CHEMICALS AND REAGENTS

Reagents	Amount	Storage
PBE Buffer pH 6.8 (1M NaCl, 1mM EDTA, HCl)	500 mL	2-8°C
L-Cysteine 5 mM (e.g. Fluka, Sigma, 30129)		Cabinet
Papain (Worthington)		2-8°C
Standard reagent (Blyscan Assay kit-Biocolor)	120 mL	2-8°C
Blyscan Dye Reagent (Blyscan Assay kit-Biocolor)	120 mL	Cabinet
Blyscan Dissociation Reagent (Blyscan Assay Kit)	120 mL	Cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	Storage
Micropipettes and tips	Bench and cabinet
Serological pipettes	Cabinet
Pipetboy	Bench
Mixer (e.g Eppendorf, Thermomixer Comfort)	Bench
Microcentrifuge tubes (1.5 mL)	Bancone
96-well microplate	Cabinet
Multimethod plate reader	Bench

Principle

The **Blyscan™ Glycosaminoglycan Assay** è un metodo quantitativo per l'analisi di proteoglicani solfati e glicosaminoglicani, a cui si lega un colorante.

PROCEDURE

Sample Digestion:

- Wash the sample with PBS.
- Prepare the digestion mix (for pellet digestion, use 500 uL per sample) = 1:100 papain in filtered PBE buffer and cysteine [e.g. 10 mL PBE buffer* + 0.035 g cysteine + 100 ul papain].

(*) PBE buffer: for 500mL solution, use 7.1 g Na_2HPO4 (dibasic sodium phosphate) + 1.86 g NaEDTA + 500 ml distilled water then adjust pH to 6.8.

- Digest the pellet in 0.5 mL digestion mix in a mechanical mixer for 16 h at 60°C, without agitation.
- After incubation use the sample directly or store at –20°C.

BlyScan Assay

Process fresh samples or thawed samples.

- Samples dilution: take 50 uL digested samples and put in a microcentrifuge tube with 50 uL filtered PBE buffer and Cysteine.
- Calibration curve: prepare 5 microcentrifuge tubes containing:
- Blank = 100 uL (PBE+ Cys) + 0 uL GAG Standard (0 ug)
- STD1= 90 uL (PBE+ Cys) + 10 uL GAG Standard (1 ug)
- STD2= 80 uL (PBE+ Cys) + 20 uL GAG Standard (2 ug)
- STD3= 70 uL (PBE+ Cys) + 30 uL GAG Standard (3 ug)
- STD4= 50 uL (PBE+ Cys) + 50 uL GAG Standard (5 ug)
- Add 1 mL of BLYSCAN DYE REAGENT to all microcentrifuge tubes (for both samples and standards).
- Shake the microcentrifuge tubes in a mechanical mixer at 24°C, 950 rpm, 30 min.
- Centrifuge the microcentrifuge tubes at 10000xg,10 min.
- Discard the supernatant and add 1 mL of DYE DISSOCIATION REAGENT.
- Keep the microcentrifuge tubes resting for 10 min at room temperature then vortex to dissolve the pellet totally.
- Load a 96-well microplate with 200 uL/well dissolved samples and standard in triplicate.
- Read the absorbance at 656 nm within 2h.

RESULTS AND CALCULATIONS

Measure the absorbance values for samples and standards

Draw the standard curve

Use an Excel format to:

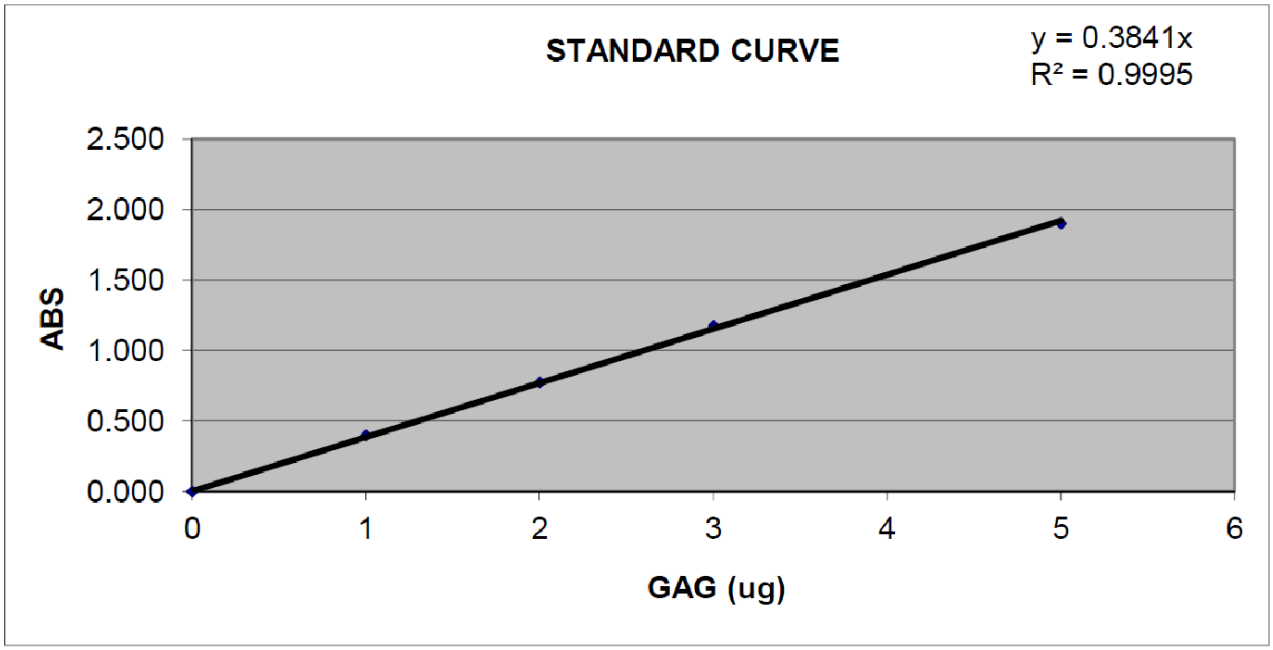
- Calculate the mean values of each triplicate
- Subtract blank to means
- To obtain ug_{tot} multiply values by 2.5 (papain volume/loaded volume, i.e. 500ul/200ul)
- To obtain ug/ml, multiply values by 5 [ug_{tot} * 1000 (ml)/papain volume (500)]
- Use the obtained ug values to create the linear standard curve.

EXAMPLE

sample	Absorbance @ 656 (1.0s) (A)	Mean	Calculation formulae			

BLANK	0.051							
BLANK	0.051	0.051		Blank (0ug)	STD1 (1ug)	STD2 (2ug)	STD3 (3ug)	STD4 (5ug)
BLANK	0.052		Mean-blank	0.000	0.159	0.310	0.470	0.761
STD1	0.207		ug(*2.5)	0.000	0.397	0.775	1.175	1.902
STD1	0.213	0.210	ug/ml(*5)	0.000	0.794	1.550	2.349	3.803
STD1	0.211							
STD2	0.354			ug GAG	Abs			
STD2	0.360	0.361		0	0			
STD2	0.371			1	0.397			
STD3	0.502			2	0.775			
STD3	0.530	0.521		3	1.175			
STD3	0.532			5	1.902			
STD4	0.784							
STD4	0.817	0.812						
STD4	0.835							

- To create the graph in the Excel file chose:
- Insert graph
- Dispersion
- interval data: columns
- save the graph
- add trend line and flag : intercept 0, equation (y= mx) and R^2



SAMPLE RESULTS

Use an Excel format to:

- Calculate the mean values of each triplicate
- Subtract blank to means.
- To correct the mean values with the standard curve, use the formula y = mx as follow: mean value/m
- To obtain ug_{tot} values, multiply the previous result by 2.5
- To obtain ug/ml values, multiply the previous result by 5

Sample results (ABS)	sample means	mean - blank	y = mx	ug (*2.5)	ug/ml (*5)
0.203					
0.212	0.,208	0.156	0.407	1.017	2.034
0.208					
0.203					
0.205	0.205	0.153	0.399	0.997	1.995
0.205					
0.186					
0.200	0.194	0.143	0.373	0.931	1.863

0.198					

GAG (Blyscan)/DNA (Picogreen) Normalization

Convert DNA Picogreen results from ng/ml to ug/ml (multiply by 10^3)

Divide as follow = GAG Blyscan ug/ml : DNA Picogreen ug/ml

Obtain an adimensional result (ADIM)

Create an histogram graph: x = samples; y = ADIM

QUANTIFICATION OF PROTEINS ON CELLULARIZED SCAFFOLDS: BICINCHONINIC ACID ASSAY

CHEMICALS AND REAGENTS

Reagents	Composition	Stored
Distilled buffer		
Extraction buffer		Cabinet
BCA Kit (Pierce, Code 23227)	Reagent A (2 bottles of 500 ml) Reagent B (1 bottle of 25 ml) Albumin Standard-BSA (10 aliquots of 1 ml. The aliquot in use is stored at -20°C, the new aliquots are stored at room temperature)	Shelves
Isopentane		Aspirated Cabinet
Liquid nitrogen		

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	Stored
Microcentrifuge tubes 1.5 mL	Cabinet
Micropipettes and tips	Bench
Ice container	
Multimethod plate reader	Bench
Flat bottom 96-well microplate	Cabinet
A pair of scissors (or a Potter homogenizer)	
Blotting paper	Drawer

Principle

The bicinchoninic acid (BCA) assay is a biochemical assay to determine the total concentration of proteins in a solution. The presence of proteins is highlighted by a color change of the solution from green to purple in proportion to protein concentration, which can be measured using colorimetric techniques.

PROCEDURE

The following procedure refers to 1 scaffold-seeded sample.

The samples have to be soaked in a becker containing isopentane (partially submerged in liquid nitrogen) and stored at -80°C: Fill up the ice container to prevent samples thawing and dry possible isopentane drops by placing the sample on blotting paper

Fragment the sample with a pair of scissors or an appropriate Potter homogenizer directly inside the microcentrifuge tube;

Add x µl of Extraction Buffer and incubate for 45 minutes at 4°C;

Repeat the same procedure on a blank scaffold sample (not cell-seeded. This is useful to build a calibration line);

Centrifuge at 2000 rpm for 4 minutes at 4°C;

Transfer (x- 20) µl in a new microcentrifuge tube;

Store at -80°C.

The second part of the procedure regards the preparation of the 96 well microplate.

The samples and the preparations for the calibration line will be placed into the microplate (a second calibration line could be necessary to consider the presence of the scaffold).

Prepare the Working Reagent (WR) by mixing 50 parts of reagent A and 1 part of reagent B (reagents are supplied in the BCA Protein Assay Kit). The WR volume is computable as: (9 + number of samples for examination)·2·200 µl, where 9 is the number of calibration points for the calibration line (18 calibration points are needed if a second calibration line is required to take into account the presence of the scaffold) and 2 is the number of replicates;

Pipette 200 µl into the wells;

Calibration line without scaffold: take 25 µl from each preparation in the kit (tagged with letters from "A" to "H") and transfer into 8 wells (corresponding to the 8 points of the calibration line);

Calibration line considering the scaffold: take 25 µl from each preparation in the kit (tagged with letters from "A" to "H") plus x µl from the extract from the blank scaffold and transfer in 8 wells;

Sample: mix the extract using the micropipette and put x µl in the well, then add (25 - x) µl of dH_2O to have the same volume level in all the wells.

Prepare the two calibration lines and the samples in duplicate

Stir the microplate for 2 minutes at 40 rpm

Incubate at 37°C for 30 minutes

Measure the absorbance at 562 nm by the multimethod plate reader.

NOTE: This procedure is highly dangerous and requires an accurate waste disposal: isopentane must be thrown into the specific laboratory jerry can. In any case, always remember to contact the laboratory Safety Officer.

BCA PROTEIN ASSAY (PIERCE)

CHEMICALS AND REAGENTS

Reagents	Composition	Stored
Lysis buffer	0.1% Triton X-100 in ddH_2O	Fridge
Triton X-100 (e.g. SIGMA, Code T8787)		Cabinet
BCA Kit (Pierce, Code 23225)	Reagent A (2 bottles of 500 ml) Reagent B (1 bottle of 25 ml) Albumin Standard-BSA (10 aliquots of 1 ml. The aliquot in use is stored at -20°C, the new aliquots are stored at room temperature)	Shelves

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	Stored
Microcentrifuge tubes 1.5 mL	Cabinet
Micropipettes and tips	Bench
Centrifuge tubes	Cabinet
Multimethod plate reader	Bench
Flat bottom 96-well microplate	Cabinet

Principle

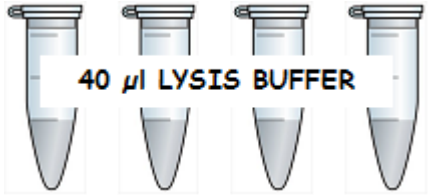
The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The BCA reagent is approximately 100 times more sensitive (lower limit of detection) than the pale blue color of the first reaction.

The reaction that leads to BCA color formation is strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in the amino acid sequence of the protein.

PROCEDURE

1. **Preparation of BSA STANDARDS**



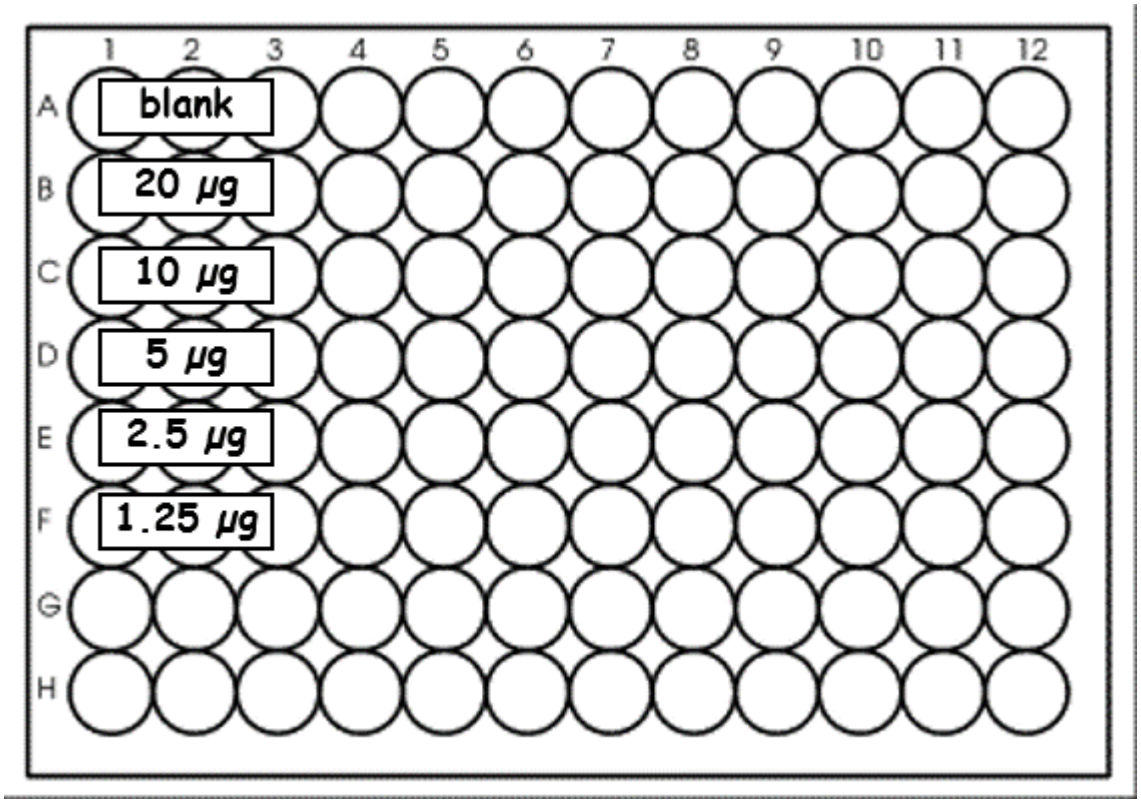
- Take 4 eppendorf tubes and add 40 µl of Lysis Buffer each.
- Add 40 µl BSA 2 mg/ml (stock solution) to the first eppendorf tube to obtain a final concentration
- of 1 mg/ml.
- Add 40 µl BSA 1 mg/ml to the second eppendorf tube to obtain a final concentration of 0.5 mg/ml.
- Add 40 µl BSA 0.5 mg/ml to the third eppendorf tube to obtain a final concentration of 0.25 mg/ml.
- Add 40 µl BSA 0.5 mg/ml to the fourth eppendorf to obtain a final concentration of 0.125 mg/ml.
- From each BSA solution, take 10 µl and pipette in triplicate in a 96 microplate.
- As a blank, put 10 µl of Lysis Buffer in 3 wells.

2. **SAMPLE PREPARATION**

- Based on the sample concentration, transfer 96 wells from 1 to 10 µl of sample (in triplicate) to the plate. Add the diluent to have a total volume of 10 µl.
- To prepare the AB reaction solution, consider a solution volume of 200 µl/well. Reagent A and reagent B must be mixed in a 50:1 ratio (e.g. 24.5 ml of Reagent A and 0.5 ml of Reagent B).
- Add 200 µl / well of AB reaction solution and incubate for 30 minutes at 37 ° C.

3. **MEASUREMENT**

- Read absorbance at 562 nm.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
blank
20 µg
10 µg
5 µg
2.5 µg
1.25 µg

HISTOLOGY ON PELLETS AND TISSUES

HEMATOXYLIN MAYER AND EOSIN STAINING, SAFRANIN O

CHEMICALS AND REAGENTS

Reagents	**Stored**
Safranin 1% w/v (e.g.. Bio-Optica, 05-07008/L)	Cabinet
Safranina O (e.g. Fluka, Sigma, 84120)	2-8°C
Killik glue (e.g. Bio-Optica, 05-9801)	Cabinet
Formalin 10% w/v (e.g. Olcelli Farmaceutici, 605-001-00-5)	Aspirated cabinet
PBS	2-8°C
Hematoxylin Mayer (e.g. Bio-Optica, 05-06002/L)	Cabinet
Eosin Y 1% w/v (e.g. Bio-Optica, 05-10002/L)	Cabinet
Fast green FLF (e.g. Sigma, F7252)	Cabinet
Ethanol (e.g. Fluka, Sigma, 02860)	Aspirated cabinet
Isopentane (e.g. Sigma, 494437)	Aspirated cabinet
Xylene (e.g. Sigma, 13444)	Aspirated cabinet
Acetic acid (e.g. Sigma, A6283)	Aspirated cabinet
Liquid nitrogen	Dewar
Dry Ice	-80°C
MOUNT quick aqueous (e.g. Bio-Optica, 05-1740)	Aspirated cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	**Stored**
Moulds depending on the sample size	Cabinet/Drawer
Microtome blade (*carbon steel C35*)	Cabinet/Drawer
Cryostat ed equipment	Cabinet/Drawer
Superfrost plus slides	Cabinet/Drawer
Coverslip (e.g. Bio-Optica)	Cabinet/Drawer
Plastic trays	Cabinet/Drawer
Brush	Cabinet/Drawer

Tweezer	Drawer
Mortar	Drawer

Principle

Hematoxylin is a dark blue or violet stain that is basic/positive. It binds to basophilic substances (such DNA/RNA - which are acidic and negatively charged). DNA/RNA in the nucleus, and RNA in ribosomes in the rough endoplasmic reticulum are both acidic because the nucleic acid building blocks that come off the phosphate backbone are negatively charged. These form salts with basic dyes containing positive charges. Therefore, dyes like hematoxylin will bind to them and stain them violet.

Eosin is a red or pink stain that is Acidic/Negative. It binds to acidophilic substances (such as proteins - which are basic and positively charged).

Most proteins in the cytoplasm are basic because they are positively charged due to the arginine and lysine amino acid residues. These form salts with acid dyes containing negative charges, like eosin. Therefore, eosin binds to these amino acids/proteins and stains them pink. This includes cytoplasmic filaments in muscle cells, intracellular membranes, and extracellular fibers.

Safranin (also Safranin O or basic red 2) is a biological stain used in histology and cytology. Safranin is used as a counterstain in some staining protocols, coloring all cell nuclei red. It can also be used for the detection of cartilage[1], mucin and mast cell granules.

Procedure

- Before starting staining, the sample must be fixed in 4% formalin

1. Fix the pellet in 4% formalin. After 2 weeks of expansion:
- Wash the pellet with 0.5 mL of PBS
- Fix the pellets in 0.5 mL of 4% formalin for 4 hours at 4 ° C

2. Fix other samples in 4% formalin
- Native cartilage: wash the sample with PBS and fix it overnight in 4% formalin in just enough volume to cover the sample

- In order to cut the sample for histological sections, it must be frozen

1. Freezing in liquid nitrogen
- Put the Killik glue in the mold and insert the pellet
- Use liquid nitrogen carefully and pour it into a mortar
- Transfer the isopentane to a beaker and place the beaker in liquid nitrogen
- Quickly put the mold in the beaker and wait for a moment to freeze the pellet
- Take the pellet from the isopentane and prepare to be processed in the cryostat or store it at -80 ° C
2. Freezing in dry ice
- Prepare dry ice in a polystyrene box
- Put Killik glue in a mould, insert the pellet and cover with other Killik glue, being careful not to form bubbles
- Put the molds with the samples on dry ice until the sample is completely frozen (about 1h)

- One hour before cutting, place the sample in the cryostat.

- Insert the blade lock.
- Mount the sample on the cryostat support with a some killik glue.
- Include the sample with killik glue and wait for freezing.
- Mount the cryostat support on the cryostat slide.
- Insert the blade into the support.
- Approach the sample with progress command.
- Set the section size to 6 um.
- Turn the crank to trim the sample.
- Spread the section with the brush.
- Collect the section on the Superfrost plus slides.

If not immediately stained, store the slides in the fridge (covered, in a box).

Staining protocol:

Hematoxylin Mayer and Eosine (nuclear and cytoplasmic stain)

- Hematoxylin Mayer (2 min)
- Running tap water (5 min)
- Eosine 1% (2 min)
- Tap water (1 min)
- Ethanol 50 % (30 sec)
- Ethanol 70 % (30 sec)
- Ethanol 95 % (30 sec)
- Ethanol 100 % (30 sec)
- Xylene (30 sec)

Staining protocol: Safranin O (Safranin O will stain the sulfated glycosaminoglycans, that are a component of the cartilaginous extracellular matrix)

- Hematoxylin Mayer (3 min)
- Running tap water (5 min)
- 0.02% Fast green (0.1 g Fast Green FCF/500 mL) (3 min)
- 1% acetic acid (30 sec)
- 0.1% Safranin O (0,5 g Safranina O/500 mL) (12 min)
- Ethanol 50% (30 sec)
- Ethanol 70% (30 sec)
- Ethanol 95% (30 sec)
- Ethanol 100% (30 sec)
- Xylene (3 min)

COLORING WITH ALIZARIN RED-S (AR-S) AND EXTRACTION WITH CPC (QUANTIFICATION OF THE CALCIFIED MATRIX)

CHEMICALS AND REAGENTS

Reagents	**Amount**	**Storage**
PBS (e.g. Sigma D8537)		RT
Frozen Ethanol 70% v/v		-20°C
Alizarin Red-S (e.g. Sigma, A5533)	1.37 g	2-8°C
Sodium hydroxide (e.g. Sigma, S8045)	Drops/a few mL	Aspirated cabinet
Na_2HPO_4 (e.g. Sigma, S7907)	100 mL	Cabinet
NaH_2PO_4 (e.g. Sigma, S8282)	100 mL	Cabinet
CPC (e.g. Sigma, C0732)	1 g	Cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	**Stored**
Micropipettes	Bench
Serological Pipettes	Cabinet
Piperboy	Bench
96-well microplate	Cabinet
Multimethod plate reader	Bench

Procedure:

- Preparation of solutions:
  - Alizarin Red-S solution (40 mM): Dissolve 1.37 g of Alizarin-S in 100 mL distilled water, adjust the pH to 4.1-4.2 with 10% NaOH m/v, store in a refrigerator away from light;

  - Phosphate buffer (0.1 M): Prepare 100 mL of Na_2HPO_4 1 M and 100 ml of NaH_2PO_4 1 M. Mix 57.7 mL of Na_2HPO_4 1 M and 42.3 mL of NaH_2PO_4 1 M, check that the pH of the final solution is 7.0 and keep at room temperature. Do not store in the refrigerator (the solution precipitates!);

  - Cetylpiridium chloride 10% m/v (CPC 10%): Take 1 mL of 0.1 M phosphate buffer and add distilled water up to a volume of 10 mL (obtaining 0.01 M phosphate buffer). Dissolve 1 g of CPC in 10 mL of 0.01 M phosphate buffer.

- Wash each well twice with PBS;
- Fix the cells with 70% v/v frozen ethanol for 1h;
- After incubation, remove the ethanol and allow to dry;
- Wash with distilled water:

- Add the Alizarin Red-S 40 mM solution (see the following table for volumes) and incubate for 15 minutes while stirring;
- Remove the Alizarin Red-S solution and add PBS. Incubate for 5 minutes to reduce non-specific staining;
- Remove the PBS and allow the wells to dry completely;
- Take some photos and proceed with the destaining;
- Incubate the wells for 15-30 minutes (until the destaining is complete) with 10% CPC in agitation to extract the Alizarin Red-S (see the following table for CPC volumes);
- Transfer the extracted solution to a 96-well microplate and measure the absorbance at 550 nm (1 sec; read at least 100 µl of sample and maintain the same reading volume between different experiments to compare the data).

	6-well microplate	**12-well microplate**	**24-well microplate**
Surface (cm^2)	10	4	2
Alizarin Red S (mL)	1	0.4	0.4
CPC 10% v/v (mL)	1,5	0.6	0.3

COLORING WITH OIL RED O AND EXTRACTION

CHEMICALS AND REAGENTS

Reagents	Amount	Stored
PBS (e.g. Sigma D8537)		RT
Formalin 4% m/v		Aspirated cabinet
Oil Red O (e.g. Sigma, O0625)	0.7 g	2-8°C
Isopropanol 60% and 100% v/v	50 mL (60% v/v) 230 mL + mL for extraction (100% v/v)	Aspirated cabinet
Distilled water		

EQUIPMENT, GLASSWARE AND CONSUMABLES

Equipment	Stored
Micropipettes	Bench
Serological Pipettes	Cabinet
Piperboy	Bench
96-well microplate	Cabinet
Multimethod plate reader	Bench

Procedure:

- PREPARATION OF SOLUTIONS:
  - 8.5 mM Oil Red O stock solution: dissolve 0.7 g of Oil red O in 200 ml of isopropanol. Stir overnight, filter with a 0.22 mm filter and keep refrigerated away from light.

  - Oil Red O working solution (to be prepared just before use): for 10 mL, mix 6 mL Oil Red O stock solution (8.5 mM) with 4 mL distilled water. Leave at room temperature for 20 minutes and filter with a 0.22 μm filter;

  - Isopropanol 60% v/v: for 50 mL, take 30 mL of isopropanol and add 20 mL distilled water.
- Wash each well twice with PBS;
- Fix the cells with 4% m/ v formalin for 30 minutes at room temperature;
- Wash the wells with 60% v/v isopropanol and allow to dry;
- Add the Oil Red O working solution (without touching the wells) and incubate for 15 minutes;
- Remove the Oil Red O solution and immediately add distilled water;
- Wash the wells with distilled water to remove excess dye;
- Take pictures (keep distilled water in the wells while taking pictures);
- Remove the distilled water and let it dry before proceeding with the extraction of the Oil Red O;

- Elute the Red O Oil by adding 100% v/v isopropanol (see the following table for volumes) and incubate about 10 min (but the time may be longer);
- Pipette isopropanol with Oil Red O up and down several times to be sure that all the Oil Red O is in solution;
- Transfer the extracted solution to a 96-well multiwell plate and measure the absorbance at 490 nm (1 sec; read at least 100 µl of sample and maintain the same reading volume between different experiments to compare the data).

	6-well microplate	**12-well microplate**	**24-well microplate**
Surface (cm^2)	10	4	2
Oil Red O (mL)	1	0.4	0.4
Isopropanol (mL)	1,5	0.6	0.3

COLONY FORMING UNITS-FIBROBLAST ASSAY (CRYSTAL VIOLET)

DESCRIPTION

The colony forming cell (CFC) assay, also referred to as the methylcellulose assay, is an *in vitro* assay used in the study of (hematopoietic) stem cells. The assay is based on the ability of (hematopoietic) progenitors to proliferate and differentiate into colonies. The colonies formed can be enumerated and characterized according to their unique morphology.

CHEMICALS AND REAGENTS

Reagents	**Quantity**	**Location**
PBS (es. Sigma, D8537)		Biological cabinet
Complete Medium with 20% FBS (e.g. DMEM supplemented with 20% FBS, 1% HEPES, 1% Sodium Pyruvate and 1% PSG)	2 mL/sample + 990 µL	2-8°C
Formalina 4% m/v		Fumet hood
Crystal violet solution in dH_2O	1 mL/campione	Fumet hood
Distilled water		

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Location**
Micropipette	Chemical hood
Falcon for microcentrifuge	Cabinet
Single use pipette	Cabinet
Pipettor	Biological cabinet
6 well plate	Cabinet

PROCEDURE

CELL SEEDING PROCEDURE:

Prepare 1 ml of a cellular suspension (in complete medium) containing $1x10^5$ cells/ml (suspension A).

Take 10 µl from suspension A and add to 990 µl complete medium to obtain 1 ml of a cellular suspension containing $1x10^3$ cells/ml (suspension B). Add 2 ml complete medium with 20% FBS in each well of a 6-wells microplate. Plate cells from suspension B ($1x10^3$ cells/ml) according to the following table:

1 cell/cm^2 (9.6 cells) 9.6 µl	3 cell/cm^2 (28.8 cells) 28.8 µl	6 cell/cm^2 (57.6 cells) 57.6 µl
12 cell/cm^2 (115.2 cells) 115.2 µl	24 cell/cm^2 (230.4 cells) 230.4 µl	48 cell/cm^2 (460.8 cells) 460.8 µl

Culture the cells for 10 days at 37°C (5% CO_2)

CFU-F STAINING PROCEDURE

After 10 days of culture, remove medium and wash the wells with PBS.

Fix the cells by incubating for 10 minutes with formalin 4%.

Filter Crystal Violet solution to remove precipitates.

After incubation, remove formalin and add about 1 ml Crystal Violet solution to each well (check that the wells are completely covered with the staining solution).

Incubate for 10 minutes at room temperature.

After incubation remove Crystal Violet and wash at least 5-6 times with ddH_2O. Let dry.

Once the wells are completely dried, take some photos and count the Colony Forming Units (a group of cells must contain at least 20 cells to be considered as a colony).

MINERALISED MATRIX AND CALCIUM DEPOSITION (VON KOSSA STAINING)

DESCRIPTION

The population of bone progenitor cells, osteoblasts, are able to differentiate and proliferate *in vitro*, contributing to the formation of mineralized nodules. The matrix has an organic component and a mineral calcified component, becoming a suitable site for mineral deposition.

Von Kossa staining remains the gold standard for the identification, visualization and quantification of mineralization - that is the quantity of deposited calcium salts - in cell cultures and in tissue sections. The method is based on the fixation of metallic silver, obtained by the reduction of nitrate by exposure to sunlight or ultraviolet, by calcium salts. This staining is usually characterized by a fast red staining to display cultured cells nuclei.

CHEMICAL PRODUCTS AND REAGENTS

Reagents	**Quantity**	**Location**
Silver nitrate (es. Sigma, 5193)	4 mL (per soluzioni 2,5% m/v)	2-8°C
Sodium thiosulfate (es. Sigma, 72049)	5 g	Chemical fumet hood
Aluminum sulfate (es. Sigma, 202614)	5 g	Chemical fumet hood
Nuclear fast red (es. Sigma, 60700)	0,1 g	Chemical fumet hood
Paraformaldeide 2% m/v in PBS		Freezer -20°C
PBS (es. Sigma, D8537)		Biological cabinet
Acqua distillata		
Etanolo (per disidratare, varie concentrazioni)	Suitable for filling the trays in which to dip the slides	Chemical fumet hood
Xilene	Suitable for filling the trays in which to dip the slides	Chemical fumet hood
Bio Mount HM (es. Bio-Optica, 05-BMHM100)	Few drops per slides	Chemical fumet hood

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Location**
Micropipette P1000 and tips	Chemical hood
Pyrex Petri Dish	Cupboard

Centrifuge tubes	Cupboard
Histology trays	Chemical hood
Slides for the assembling	Cupboard

PROCEDURE

Solution preparation

1% silver nitrate: i.e. for 10 ml of ddH_2O, dilute 4 ml of 2.5% silver nitrate by adding 6 ml of distilled water;

5% sodium thiosulfate: 5 g in 100 ml of distilled H_2O;

0.1% Nuclear fast red: dissolve 5 g of aluminum sulphate in distilled water, add 0.1 g of nuclear fast red powder, heat slowly until boiling, let it cool and filter.

Staining procedure

Fix the cells with paraformaldehyde 2% for 10 minutes and then wash with PBS 1X.

Incubate with 1% silver nitrate solution in Pirex Petri dish placed under the ultraviolet lamp of the biological hood, for at least 20 minutes (up to several hours, depending on the type of UV lamp).

Wash 2-3 times with distilled water.

Remove the unreacted silver nitrate solution by using 5% sodium thiosulfate for 5 minutes.

Wash in distilled water.

Counterstain with nuclear fast red for 5 minutes.

Wash in distilled water.

Dehydrate with solutions at increasing ethanol concentration, up to xylene.

Assemble the coverslips using the synthetic upright for histology (Biomount).

NOTE

Calcium deposits are stained brown/black, nuclei are stained red and cell cytoplasm is stained pink.

A standard protocol is not available, refer to these procedures:

http://www.abcam.com/ps/products/150/ab150687/documents/ab150687-Calcium%20Stain%20Kit%20(website).pdf

http://www.ihcworld.com/_protocols/special_stains/von_kossa.htm

CONNECTIVE TISSUE STAINING (MASSON TRICHROMIC STAINING)

DESCRIPTION

The connective tissue provides structural and metabolic support to the surrounding tissues. It consists mainly of extracellular matrix interposed between the cells: fibroblasts, osteoblasts, chondrocytes, mast cells, adipocytes, white blood cells and macrophages. By varying the composition of the extracellular matrix, the features of the connective tissue change and the resulting tissue can be: bone, cartilage, tendons, ligaments, adipose tissue, blood or lymph.

Masson trichrome staining is the most suitable staining method for the connective tissue and allows identifying its specific components. It involves the combination of a nuclear and a basophilic structures staining with Weigert ferric hematoxylin, a red blood cell staining with picric acid and a connective tissue staining with two possible different acid dyes (Light Green or aniline blue).

CHEMICALS AND REAGENTS

Reagents	**Quantity**	**Location**
Kit Masson Tricromica (Bio-Optica, 04-010802) Reagent A: Weigert's Iron hematoxylin solution A (hematoxylin 1 g + Ethanol 100 ml) Reagent B: Weigert's Iron hematoxylin, solution B (Iron (III) Chloride 30% aqueous solution, 4 ml, Reagent C: Picric acid alcoholic solution Reagent D: Biebrich Scarlet solution Reagent E: Phosphomolybdic acid solution Reactive F: Blue aniline solution	Few drops per slide	Chemical fumet hood
Paraformaldehyde 2% m/v in PBS	Few drops per slide	Freezer -20°C
PBS (es. Sigma, D8537)	Few drops per slide	Chemical fumet hood
Distilled water		
Ethanol	Suitable for filling the trays in which dip the slides	Chemical fumet hood
Xilene	Suitable for filling the trays in which dip the slides	Chemical fumet hood

Bio Mount HM (es. Bio-Optica, 05-BMHM100)	Suitable for filling the trays in which dip the slides	Chemical fumet hood

Materials	**Location**
Micropipette P1000 and tips	Chemical hood
	Cupboard
Histology trays	Chemical hood
Slides for assembling	Cupboard

PROCEDURE

Take the special kit (reagents A-F).

Fix the cells with paraformaldehyde 2% for 10 minutes.

Wash the cells with PBS 1X, for 5 minutes.

Apply 6 drops of Reagent A, add 6 drops of Reagent B (equal vol.) for 10 minutes.

Do not wash, drain and add 10 drops of Reactive C for 4 minutes

Quickly wash for 3-4 seconds in distilled water and add 10 drops of Reagent D for 4 minutes.

Wash in distilled water and add 10 drops of Reagent E for 10 minutes.

Do not wash, drain and then add 10 drops of Reagent F for 5 minutes.

Wash in distilled water and dehydrated with ethanol-scale while pausing for 1 minute in absolute ethanol before switching to Xylene (operate under a chemical hood).

Mount the slide with a synthetic upright (Biomount).

NOTE

Nuclei and gametes appear black

Cytoplasm, keratin, muscle fibers and granules acidophilus appear red

Collagen, mucus, basophilic granulations pituitary appear blue

Pituitary delta cells granules appear blue

Red blood cells appear yellow

REFERENCES

Due to the absence of a standard protocol, two similar procedures are reported below:

http://www.bio-optica.it/pdf/010802.pdf

https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/1/ht15.pdf

GLYCOSAMINOGLYCANS (SAFRANIN STAINING)

DESCRIPTION

Safranin O is a cationic dye that stains acidic proteoglycan present in cartilage tissues, which are indicators cell chondrogenesis. Safranin O binds to glycosaminoglycan and shows an orange-red color. **It should be noted that it is considered a cationic dye, and as such can also bind nucleic acids.**

Reagents	**Quantity**	**Location**
Hematoxylin (Masson Trichromic Kit) (es.kit Masson Tricromica, Bio-Optica, 04-010802)	Few drops per slides	Chemical fumet hood
Safranin O (es. Sigma-Aldrich, 84120)	0,5 g	Chemical fumet hood
Ethanol 50%, 70%, 95% v/v		Chemical fumet hood
Xilene	Suitable for filling trays in which incubate slides	Chemical fumet hood
Bio Mount HM (es. Bio-Optica, 05-BMHM100)	Suitable for filling trays in which incubate slides	Chemical fumet hood
Distilled water	500 mL	

Materials	**Quantity**	**Location**
Quantitative filter papers: circle grade 40 (es. Fisher, 09-845)	1	Cupboard
Becher	1	Cupboard
Micropipette P1000 and tips		Chemical fumet hood
Histology trays		Chemical fumet hood
Centrifuge tubes		Cassetti/Armadi
Slides for assembling		

PROCEDURE

Hematoxyline (Masson Trichromic Kit)

- Add 6 drops of reagent A

- Add 6 drops of reagent B (e.g., same volume of reagent A)
- After 10 minutes, dry and do not wash
- Add 6 drops of reagent C for 4 minutes

- Wash the samples with distilled water for 5 seconds

- Add **0.1% Safranin O** for 5 minutes (to be prepared **in advance** - 0.5 g/500 mL of distilled water and filtered).
    - Ethanol 50%, 30 sec.
    - Ethanol 70%, 30 sec.
    - Ethanol 95%, 30 sec.
    - Ethanol 100%, 30 sec.
    - Xylene, 3 min (chemical hood!)
- Mount the samples (Biomount)

ADIPOGENIC CULTURE (OIL RED O STAINING)

DESCRIPTION

Oil Red O staining is an assay performed to stain induced adipogenic cultures to detect mature adipocytes.

CHEMICALS AND REAGENTS (Thermo Scientific SC 00011)

Reagents	Quantity	Location
PBS (es. HyClone™ AdvanceSTEM™ Dulbecco's Phosphate Buffered Saline, ES Cell Qualified, Fisher, SH3085002)	2 mL per sample	Biological cabinet
Formaline 10% m/v, pH neutral (es. Fisher, SF98)	2 mL per sample	Chemical fumet hood
Oil Red O (es. Fisher, M312512)	300 mg	Chemical fumet hood
Hematoxylin (es. Fisher, SH30-500D)	2 mL per sample	Chemical fumet hood
Isopropilic alcohol 60% e 99% v/v	2 mL/sample (60% v/v) 100 mL (99% v/v)	Chemical fumet hood
Acqua distillata		
Acqua del rubinetto		

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Quantity	Location
Quantitative filter papers: circle grade 40 (es. Fisher, 09-845)	1	Cupboard
Micropipette P1000		Chemical cabinet
Becher	1	Cupboard
Centrifuge tubes		Cupboard

PROCEDURE

GENERAL CONSIDERATIONS

- All the procedures involving formalin must be done in a fume hood.
- Take care to not leave the cells dry for more than 30 seconds during the assay.

- Gently add and remove all reagents indirectly to the monolayer to avoid cell detachment. For example, drip the reagent down the side of the culture plate.

FIXING ADIPOGENIC CULTURES

- Remove cell cultures from incubator and place in a fume hood
- Remove culture medium from the wells, starting from the control
- Gently rinse the plate with 2 mL of sterile HyClone ES-Qualified DPBS
- Remove the PBS and add 2 mL 10% formalin
- Incubate for 30-60 minutes at room temperature

OIL RED O STOCK SOLUTION

- Prepare a stock solution by dissolving 300 mg of Oil Red O powder in 100 mL of 99% isopropanol. This stock solution is stable for one year.
- Mix the solution until the powder is dissolved (the Oil Red O powder solubility is quite low!)

OIL RED O WORKING SOLUTION

- In the fume hood, mix 3 parts of Oil Red O stock solution with 2 parts of distilled water.
- Incubate for 10 minutes at room temperature. This working solution is only stable for 2 hours.
- Place a piece of Whatman filter paper in a funnel above a vessel.
- Filter the Oil Red O working solution through the filter funnel.

OIL RED O STAINING OF ADIPOGENIC CULTURE (1)

- Remove formalin from each well and discard according to procedures for chemical waste disposal.
- **Add 2 mL 60% isopropanol to each well and wait for 5 minutes.**
- **Remove isopropanol** and add the appropriate volume of Oil Red O working solution to each well, be sure to cover the entire monolayer (3 mL/well for 6-well plate; 1 mL/well for 12-well plate; 3 mL for 10 cm Petri dish).
- Incubate 5 minutes at room temperature.
- Remove Oil Red O and rinse cultures with room temp tap water until the water rinses off clear.
- Add 2 mL Hematoxylin stain into each well, be sure to cover the entire monolayer. Incubate 1 minute at room temperature.
- Remove the hematoxylin and rinse the cultures with tap water at room temperature, until the water rinses off clear.
- Add 2 mL tap water to each well and observe the samples with a phase contrast microscope.

FLUORESCENCE MARKING METHODS

LIVE/DEAD ® VIABILITY/CYTOTOXICITY KIT

Reagente	Caratteristiche	Conservazione
D-PBS		+4 °C
LIVE/DEAD® viability/cytotoxicity Kit *for mammalian cells* (Thermo Fisher, Cod prodotto L3224)	It contains calcein AM 4 mM e EthD-1 2 mM	-20 °C

Example Dilution Protocol (Invitrogen Kit)

This example protocol use a concentration of 2 µM calcein AM and 4 µM EthD-1 diluted in culture medium (eventually serum free).

We found these dye concentrations to be suitable for NIH 3T3, PtK2 and MDCK, MSC cells when incubated at room temperature for 5-40 minutes. Cultured mouse leukocytes (J774A.1), which have higher esterase activity, require 5-10 times less calcein AM than that required for the three other cell types, but the same amount of EthD-1. This is an example protocol only; the optimal dye concentrations for any experiment will vary.

Remove the LIVE/DEAD® reagent stock solutions from the freezer and allow them to warm to room temperature.

Add 20 µL of the supplied 2 mM EthD-1 stock solution (Component B) to 10 mL of sterile, tissue culture–grade D-PBS, vortexing to ensure thorough mixing. This gives an approximately 4 µM EthD-1 solution.

Combine the reagents by transferring 5 µL of the supplied 4 mM calcein AM stock solution (Component A) to the 10 mL EthD-1 solution.

Vortex the resulting solution to ensure mixing.

Incubate the cells with this solution at room temperature (or in incubator if it takes more than 5 minutes).

The final concentration of DMSO is ≤ 0.1%, a level generally innocuous to most cells.

Note that aqueous solutions of calcein AM are susceptible to hydrolysis (see Storage and Handling of Reagents). Aqueous working solutions should therefore be used within one day.

PHALLOIDIN STAINING FOR CYTOSKELETAL ACTIN

DESCRIPTION

Phalloidin is one of a group of toxins from the death cap mushroom (*Amanita phalloides*) known as phallotoxins. Its toxicity is due to its stabilizing of actin filaments within cells. Since the discovery that fluorescently labelled phalloidin still retained its actin binding properties [1], it has been widely used in biomedical research to visualize filamentous actin.

Reagents	**Characteristics**	**Conservazione**
D-PBS		+4 °C
Triton X-100 (es. SIGMA, Cod prodotto T8787)		RT
Phalloidin (SIGMA, Cod prodotto P5282)	The aliquots of phalloidin stock solution are prepared by diluting the powder in DMSO at a concentration of 500 ug/ml	-20 °C

PROCEDURE

Prepare stock solutions of phalloidin conjugates in methanol or DMSO at 0.1-5 mg/ml. Final staining solutions in aqueous physiological buffers are in the concentration range of 0.1-100 mM with corresponding incubation times from 15 minutes to 72 hours.

The following procedure may be used as a guideline for staining the cells:

Wash the cells with phosphate buffered saline (PBS).

Fix the cells for 10 minutes in 3.7% formaldehyde solution in PBS, then washed extensively in PBS.

Permeabilize the cells with 0.1% TRITON X-100 in PBS and washed again in PBS.

Stain the cells with 50 mg/ml fluorescent phalloidin conjugate solution in PBS (containing 1% DMSO from the original stock solution) for 40 minutes at room temperature.

Wash several times with PBS to remove unbound phalloidin conjugate.

QUANTUM DOTS STAINING FOR CYTOPLASM IN LIVING CELLS

DESCRIPTION

Long-term tracking of cell movement and location requires long-lasting probes that are highly resistant to photobleaching and are retained through many cycles of cell division.

Semiconductor fluorescent quantum dots are nanometre-sized functionalized particles that are readily excitable and display high photo-stability. When conjugated with natural ligands they are quickly internalized into cells, do not interfere with intracellular signalling, and are nontoxic.

These properties make them particularly well suited for visualizing and tracking molecular processes in cells in long-term culture using standard fluorescence microscopy.

Structure of a semiconductor fluorescent quantum dot nanocrystal. The heavy metal core is responsible for the fluorescence properties of the quantum dot. The non-emissive shell stabilizes the core, whereas the coating layer provides anchor sites to organ.

CHEMICAL AND REAGENTS

Reagents	Conservation
Qtracker kit (Thermo Fisher)	+4 °C

Q-TRACKER, Invitrogen: it is a commercial kit for long-term studies of living cells including migration, proliferation, motility and morphology. It delivers fluorescent Quantum dot nanocrystals into living cells using a custom targeting peptide

Q-tracker cell labelling kit is supplied with 100 µL of the Q-tracker Reagent A and 100 µL of the Q-tracker Carrier Reagent B

Complete Medium. Aliquots in use are stored at +4°C, new aliquots are stored at -20°C

PBS (Phosphate Buffered Saline), stored at room temperature

PROCEDURE

Pick up the kit from the refrigerator (+ 4°C)

Prepare a 10 nM solution of quantum dots in sterile conditions. E.g. to prepare 200 µL of solution, mix 1 µL of reagent A to 1 µL of reagent B in a centrifuge tube. Wait for 5 minutes at room temperature. Add 200 µL of complete medium and swirl for 30 seconds. Incubate the cells with the prepared solution for 40-60 minutes at 37°C. Wash the cells twice with complete medium.

NOTES

The marked cells can be observed in living conditions or after fixing with formaldehyde (4% in PBS for 15 minutes). It is possible to counterstain the nucleus with DAPI (a fluorescent stain that binds strongly to A-T rich regions in DNA).

REFERENCES

X. Michalet, F. F. Pinaud, L. A. Bentolila, “Quantum Dots for Live Cells, in Vivo Imaging, and Diagnostics”, *Science 2005*, January 28, 307(5709): 538–544.

5-DTAF STAINING FOR COLLAGEN IN LIVING CELLS

DESCRIPTION

Visualization of the formation and orientation of collagen fibres in tissue engineering experiments is crucial for understanding the factors that determine the mechanical properties of tissues. 5-(4,6-dichlorotriazinyl)aminofluorescein (5-DTAF), the product of the reaction of aminofluorescein with cyanuric chloride, is a nonspecific amine-reactive fluorescent dye for visualizing collagen fibres in live tissue, both for in vivo healing experiments and for in situ mechanical studies. Chemical structure of 5-DTAF.

CHEMICAL AND REAGENTS

Reagents	Characteristics	Conservation
PBS	1X	Fridge +4 °C
5(46dichlorotriazinyl) (Thermo Fisher)		Freezer -20 °C

PROCEDURE

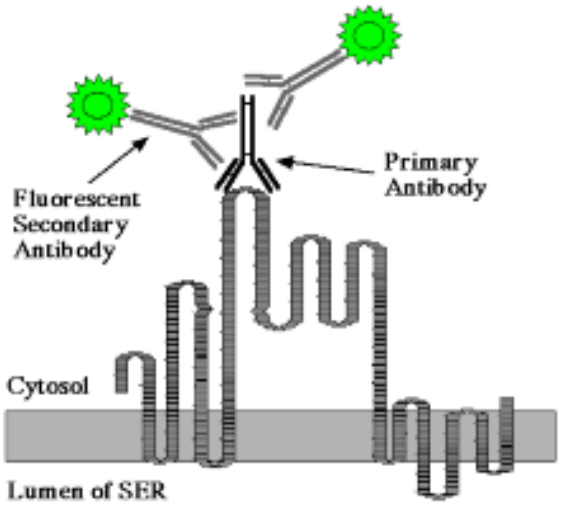
Prepare a 7.5-micromolar solution of 5-DTAF (0.0037 g/L) [STOCK SOLUTION]: 0.0037 g of 5-DTAF powder + 1 mL PBS. The stocking solution can be stored at -20°C. Prepare the WORKING SOLUTION: dilute the stock solution 1:1000. Filter the working solution at 0.22 micron and incubate the cells for 90 minutes in the incubator. Wash the cells with PBS 1x and then with complete medium

NOTE

The working solution has to be made just before using.

IMMUNOFLUORESCENCE PROTOCOLS

Immunofluorescence is a powerful technique that utilizes fluorescent-labelled antibodies to detect specific target antigens. As it is not possible to see an antibody directly, it is necessary to use a fluorescent dye that is covalently attached to the antibody. When a light illuminates the fluorescent dye, it absorbs the light and emits a different colour light, which is visible to the investigator and can be photographed. In most immunofluorescence experiments, two antibodies are employed (indirect immunofluorescence). The first one, called the primary antibody, binds to the protein that has to be detected. The secondary antibody is generally purchased from a company that sells antibodies and has a fluorescent dye covalently attached to it. As illustrated in the following figure, the secondary antibodies can bind to multiple sites on the primary antibody and thus produce a brighter signal since more dyes are brought to a single location.



Immunofluorescence principles

Applications include the evaluation of cells in suspension, cultured cells, tissue, beads and microarrays for the detection of specific proteins.

REFERENCES

J. Paul Robinson PhD, Jennifer Sturgis BS and George L. Kumar PhD, "Immunofluorescence", *IHC Staining Methods, Fifth Edition*, Chapter 10.

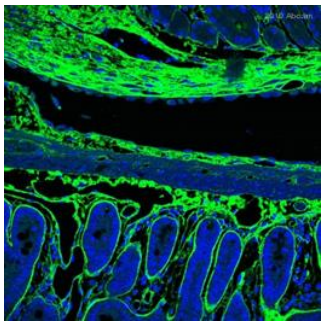
Ernst H. Beutner, "Immunofluorescent staining: the fluorescent antibody method", Bacteriological Revisions, 1961, 25(1): 49-76.

http://www.scbt.com/protocol_immunofluorescence_cell_staining.html

IMMUNOFLUORESCENCE STAINING FOR COLLAGEN TYPE I, II, AND OSTEOCALCIN

DESCRIPTION

Specific antibodies against type I and II collagen and osteocalcin can be used to localize these proteins in cultured human cells. These studies can indicate if the same cells produces the above-mentioned proteins.



Antibody 21286 staining collagen I in Mouse small intestine tissue sections

Reagents	**Characteristics**	**Conservation**
Triton X-100		RT
PBS	1X	RT
Acetone		+4 °C
BSA		+4 °C
Rabbit anti-collagene II	1:80	-20 °C
Mouse anti-collagene I	1:2000	-20 °C
Mouse anti-osteocalcina	10 µg/ml	-20 °C
Fluorescin (FITC)-conjugated Goat Anti-Rabbit igG (H+L) (Jackson ImmunoResearch Cod prodotto 111-095-144	1:25	-20 °C
Cy3-conjugated F(ab')2 Fragment Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Cod prodotto 715-166-151)	1:100	-20 °C
Goat anti-mouse IgG (H+L) coniugato TRITC (BioFX Cod prodotto 110-121-04)		-20 °C

Goat anti Rabbit IgG-H+L coniugato FITC (Bethyl Cod prodotto No. A120-201F)		-20 °C

GENERAL PROCEDURE

- Fix the cells with acetone (stored at 4°C) for 5 minutes
- Wash the cells twice with PBS for 5 minutes
- Cover the cells with a solution of Triton at a concentration of 0.1% for 5-10 minutes
- Wash the cells twice with PBS for 5 minutes
- Block the cells with a solution of BSA 3% for 30 minutes
- Wash the cells twice with PBS for 5 minutes
- Add the primary antibodies overnight at 4°C
- Wash the cells twice with PBS for 5 minutes
- Add the secondary antibodies for 1h at room temperature
- Wash the cells twice with PBS for 5 minutes
- Add DAPI (1 µg/Ml) for 15 minutes
- Wash the cells twice with PBS for 5 minutes
- Remove PBS and mount with aqueous mounting medium

Experiments conducted by Irene Cattaneo at Mario Negri (6/7-03-2012)

PROCEDURE

- MG-63 cells (human osteosarcoma) were plated on coverslips placed in a multi well plate (20x20 mm^2 , 160 µm thick, autoclavable or disinfectable with ethanol) at a density of 104 cells/cm^2 and brought to confluence
- Cells were then fixed with cold acetone taken from the fridge at 4°C
- After fixing operations, the samples were conserved for some days in the fridge in a solution of PBS + Sodium Azide (0.02% v/v).
- Secondary antibodies used:

Goat anti mouse IgG (H+L) conjugated TRITC (BioFX, code 110-121-04); dilution 1:100

- Goat anti Rabbit IgG (H+L) conjugated FITC (Bethyl, code No. A120-201F); dilution 1:100
- Construction of the moist chamber: an opaque plastic box for histology slides was lined with towel paper soaked with PBS and lightly squeezed. A layer of parafilm was overlapped. With a permanent marker the parafilm was divided in slots that were then numbered
- A drop (80 µl) solution of antibodies was added on the parafilm and the slide was leaned on the cells side; the solution wetted the entire slide by capillarity, without wasting the reagent

- To facilitate the extraction of the slide from the multiwell plate a fine tip pincer and an hypodermic needle were used; with the same needle they lightly scratched the glass: only the cellularised side remained scratched so they could recognize it
- To continue the operations, the slide with the cells was put in PBS (not in the moist chamber) and some towel paper was used on the parafilm in order to dry the drops of the solution used in the previous phase
- Diluted antibodies were shaked with the vortex just before deposition on the parafilm
- The negative control was made by incubating the samples only with the secondary antibody
- To improve the fluorescence, the secondary antibodies were centrifuged before the dilution at 15000 rpm for a few seconds (to precipitate impurities)
- The slides were mounted with aqueous mounting medium just after the last wash, being careful not to introduce air bubbles

RESULTS

The examination was made in a dark room with an inverted confocal microscope; the magnification used was 40X with oil immersion.

The secondary antibodies marking done by the Mario Negri professionals was successful: the marked sites appeared as fluorescent dots within the cell while the background appeared as a widespread fluorescence. The secondary antibodies marking done by Irene Cattaneo was not successful: the background prevailed, making samples very similar to their negative controls.

CONCLUSIONS

While primary antibodies used by Irene Cattaneo worked well (they were the same used with the secondary ones of Mario Negri professionals) with the secondary antibodies there were some problems, so it may be necessary to repeat the experiment with different dilutions, to find the most effective.

The observation of the samples with the fluorescence microscope (Olympus IX 70, 40X objective in the air) was difficult because the marking was faintly visible (it was possible to observe the samples only conveying with the knob the entire light signal to the oculars). It was possible to acquire some images with the camera provided with the microscope only by using a long exposure time.

PLEASE NOTE: All operations must be conducted at room temperature and the antibodies must be kept in ice.

Sodium Azide solution is highly toxic (classified T+), therefore its preparation and use have to be performed under chemical hood. If the powder has to be weighed, it is necessary to wear gloves, glasses and a mask with a P2 filter (EN-143).

REFERENCES

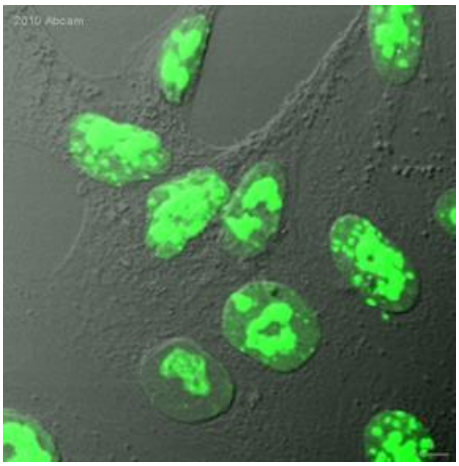
Helga von der Mark, Klaus von der Mark, Steffen Gay, “Study of differential collagen synthesis during development of the chick embryo by immunofluorescence: I. Preparation of collagen type I and type II

specific antibodies and their application to early stages of the chick embryo", *Developmental Biology*, 1976, Volume 48, Issue 2, Pages 237-249.

CELL PROLIFERATION MARKER (KI67)

DESCRIPTION

The expression of the human Ki67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki67 protein is present during all active phases of the cell cycle but is absent from resting cells makes it an excellent marker for determining the so-called growth fraction of a given cell population. The fraction of Ki67-positive tumor cells (the Ki67 labelling index) is often correlated with the clinical course of cancer.



Antibody 166667 staining Ki67 - Proliferation Marker in Human HEp-2 cells

CHEMICAL AND REAGENTS

Reagents	Characteristics	Conservation
Triton X-100		RT
PBS	1X	RT
FBS		-20 °C
BSA		+4 °C
anti-KI67	1:100	-20 °C
Secondary antibody	1:100	-20 °C
Fluoromount Aqueous (es. SIGMA-ALDRICH)	10 µg/ml	-20 °C

PROCEDURE

Fix the cells in paraformaldehyde 2% for 10 minutes under the chemical hood

Wash the cells two or three times with PBS for 5 minutes

Cover the cells with a solution of Triton at a concentration of 0.1% (0,2% if the cells are highly sticky) in PBS for 3 minutes to permeabilize

Wash the cells two or three times with PBS for 5 minutes

Block the cells with a solution of BSA 3% and FBS 10% in PBS for 30 minutes (filter the solution with a 0.22 μm filter before using)

Add the primary antibody for KI67 (diluted 1:1000). Incubate for 2 hours at room temperature

Wash the cells two or three times with PBS for 5 minutes

Add the secondary antibody (diluted 1:100). Incubate for an hour at room temperature

Wash the cells two or three times with PBS for 5 minutes

Add DAPI (1 μg/ml) and incubate for 15 minutes

Wash the cells two or three times with PBS for 5 minutes

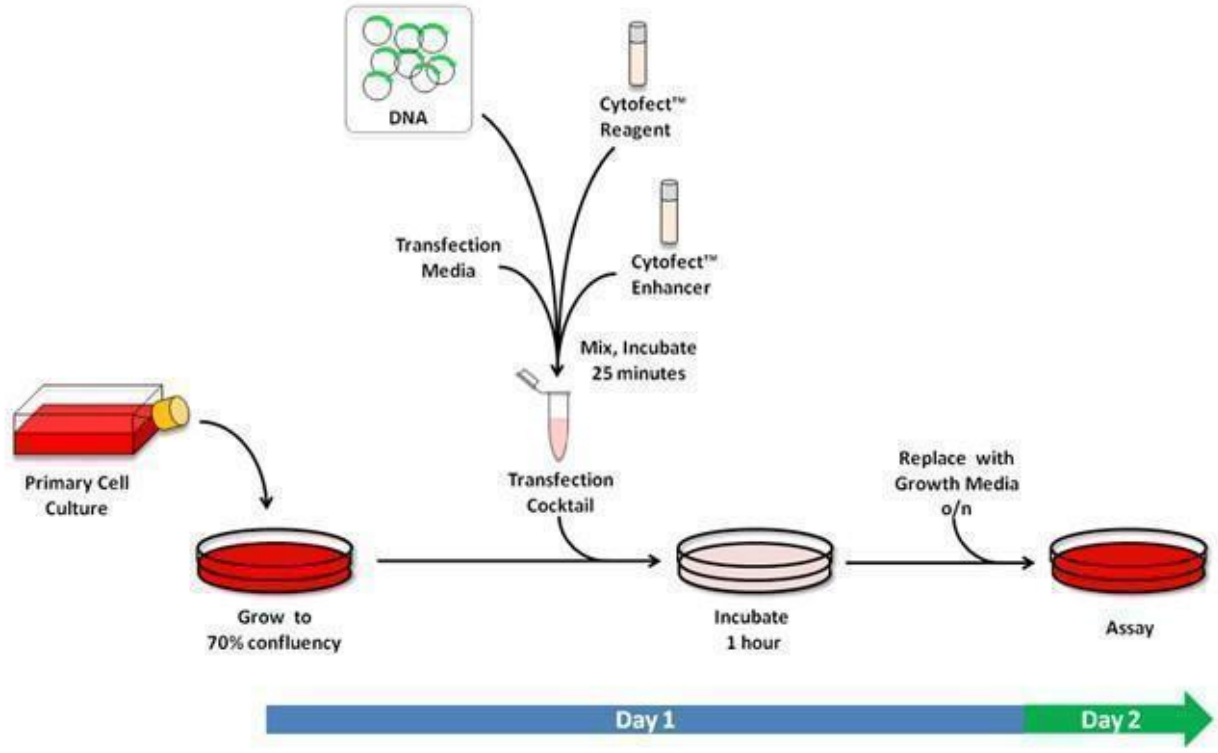
Remove PBS and mount with aqueous mounting medium (e.g. SIGMA fluoromount)

NOTE: All operations must be conducted at room temperature and the antibodies must be kept in ice.

TRANSFECTIONS AND TRANSDUCTIONS

DESCRIPTION

Transfection is the process that introduces exogenous biological material in eukaryotic cells (mainly mammalian cells). Insertion of genetic material, such as DNA and siRNA is most habitual but also proteins (antibodies for example) can be transfected. Transfection can be carried out using calcium phosphate, by electroporation, by cell squeezing or by mixing a cationic lipid with the material to produce liposomes, which fuse with the cell membrane and deposit their cargo inside (Fig.1).



Conceptual scheme to perform the transfection process of DNA

The following protocol describes the procedure for performing transfection, by mixing a cationic lipid with DNA, on cells cultured in 25 cm^2 flasks. The protocol continues describing the process for splitting transfected cells.

TRANSFECTION WITH DNA ON MAMMALIAN CELLS

CHEMICAL AND REAGENTS

Reagents	Characteristics	Location
Cationic lipid, stock solution	1 ml	+4 °C
Plasmid		-20°C
Culture medium without serum		+4 °C
Complete culture medium		+4 °C
PBS 1x		-20 °C
Sterile water		RT
Cationic lipid, stock solution		RT

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Location
Micropipette P20, P200, P1000	Bench
Pipettor	Bench
Pipette 10 mL	Bench
Sterile Eppendorf	Bench
Sterile tips	Bench

PROCEDURE

Preparation of the cationic lipid stock solution

Dissolve the transfectant film with 1 ml sterile water.

Collect an aliquot of the solution (store the remaining solution at +4°C).

Dilute with sterile water up to obtain a concentration of 1 mg/ml.

Mix carefully with a pipette.

The day before transfection, plate the cells on the flask. This step allows reaching 50% of cell confluence at the time of transfection.

Preparation of the cationic lipid/DNA complex

· Prepare the DNA solution in a sterile eppendorf tube: 2 µg plasmid (mix long and carefully with a pipette before sampling) in 50 µl sterile water. Mix carefully with a pipette.

· Prepare the cationic lipid solution in a sterile Eppendorf: x* µl of work solution diluted in 50 µl of sterile water. *The amount of working solution depends on the charge ratio we want to obtain in the cationic lipid/DNA complex. The charge ratio is the ratio between positive charges (due to cationic lipid amino group) and negative charges (due to DNA phosphate group. 1 µg DNA contains 3.03 nmol of phosphate group). Mix carefully with a pipette.

· Transfer carefully the cationic lipid solution in the eppendorf tube containing the DNA solution. The final volume is 100 µl. Mix long and carefully with a pipette. NOT use a vortex.

· Incubate the mixture at room temperature for 20-30 minutes to enable the creation of the lipid cationic/DNA complex.

· Add the culture medium without serum to the mixture up to reach a total volume of 1 ml.

· Remove the culture medium from the cells seeded the day before the experiment.

· Wash with 2 ml of PBS to remove the remaining serum.

· Add 4 ml culture medium without serum to the seeded cells.

· Add the medium containing the cationic lipid/DNA complex (step 3). Final volume is 5 ml.

· Incubate at 37°C in the incubator for 4 hours.

· Remove the culture medium and add 5 ml complete culture medium.

· Incubate at 37°C in incubator for several hours (usually 48 hours).

NOTE

Reagent amounts and solution volumes have been evaluated for one 25 cm^2 flask.

FIXING FOR TRANSFECTION

DESCRIPTION

The fixing process is necessary to preserve chemical and physical properties of tissues, after that they were excised from an organism. Depending on the nature of samples and the type of analyses we wish to perform, we can use chemical or physical fixing methods.

The following protocol describes the procedure for chemically fix transfected cells cultured in 25 cm^2 flasks. We use paraformaldehyde, depolymerised in solution to formaldehyde, as fixative agent.

CHEMICALS AND REAGENTS

Reagents	**Characteristics**	**Location**
PBS	1x	Cupboard
p-formaldehyde solution (PFA)		-20°C
4% (w/v) di PFA in PBS heated at 60°C		-20°C
Culture medium with serum		-20°C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Location**
Micropipette P200 and P1000	Bench
Pipette 10 ml	Cupboard
Pipettor	Bench
Tips	Cupboard

PROCEDURE

Remove culture medium from the flask and wash the cells with 2.5 ml PBS

Carefully, add 300 µl Trypsin-EDTA and incubate at 37°C in the incubator for 2 minutes

Detach the cells by tapping the bottom of the flask

Add 700 µl of culture medium with serum to block trypsin effects

Suspend the cells with a pipette and transfer in an eppendorf tube. If interested in carry out a cell count test, collect an aliquot now

Centrifuge at 1600 rpm at 4°C for 5 minutes. Remove the supernatant

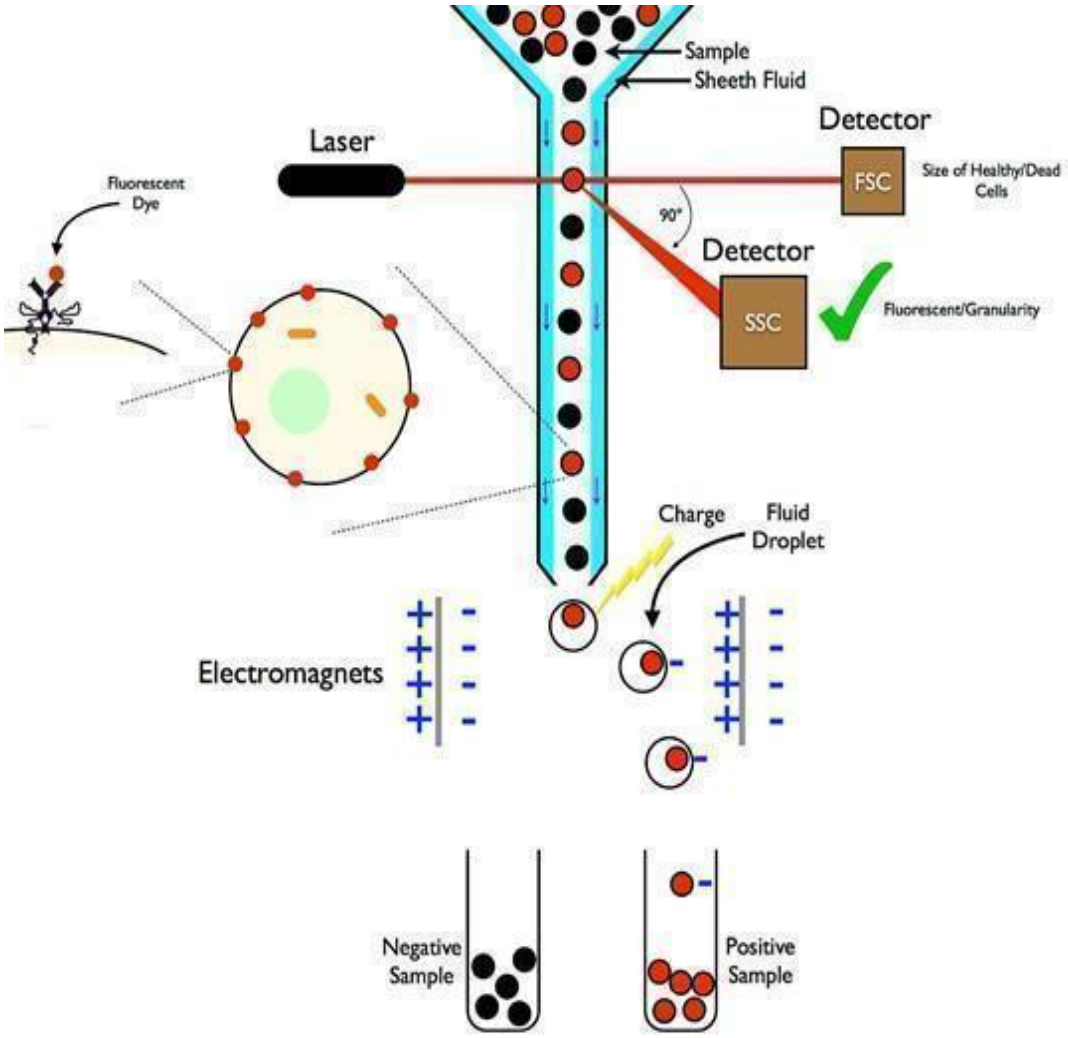
Wash the pellet with 1ml of PBS, without re-suspending the pellet. Remove the supernatant
Add 300 µl PAF solution and re-suspend the pellet with a 200 µl micropipette
Store at 4°C in a dark box until use.

NOTE: Reagent amounts and solution volumes have been evaluated for one 25 cm^2 flask.
Use cells at 50-70% of confluence.

PREPARATION OF TRANSFECTED SAMPLES FOR FLOW CYTOMETRY (FACS)

DESCRIPTION

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry that provides a method for sorting a heterogeneous mixture of biological cells into two or more containers.



Scheme of Fluorescence-Activated Cell Sorting (FACS)

The following protocol describes the procedure to prepare transfected cells for flow cytometry, and in particular for FACS.

CHEMICAL AND REAGENTS

Reagents	Characteristics	Location

FBS (Sigma, F7524)	5%	-20°C
PBS (Sigma)	Sterile	RT

PROCEDURE

Fix the cells (Refer to the previously described procedure “Fixing for transfection”).

Prepare a 5% FBS solution in PBS buffer solution at 4°C.

Add 700 µl of the obtained solution (step 2) to fixed samples containing 300 µl of buffer solution for the fixing procedure.

Remove the supernatant and re-suspend the pellet in 500 µl PBS.

Transfer the cell suspension in a 15 ml Centrifuge tube for FACS analysis.

REFERENCES

R.F. Kalejta, T. Shenk, A.J.Beavis. Use of membrane-localized green fluorescent protein allows simultaneous identification of transfected cells and cell cycle analysis by flow cytometry. Citometry. 1977 Dec 1; 29(4):289-91

ELECTROPHORESIS - WESTERN BLOTTING

DESCRIPTION

Western blotting is an important technique used in cell and molecular biology to separate and identify proteins. Firstly, the protein of a mixture are separated from each other based on molecular weight through gel electrophoresis. The different protein are then transferred to a membrane maintaining the position gained during electrophoresis and the membrane is incubated with labels antibodies specific for the proteins analysed. After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a specific portion of the primary antibody, which emit a signal(e.g. cleaving a chemiluminescent agent) corresponding to the position of the target protein. This signal is captured on a film which is usually developed in a dark room.

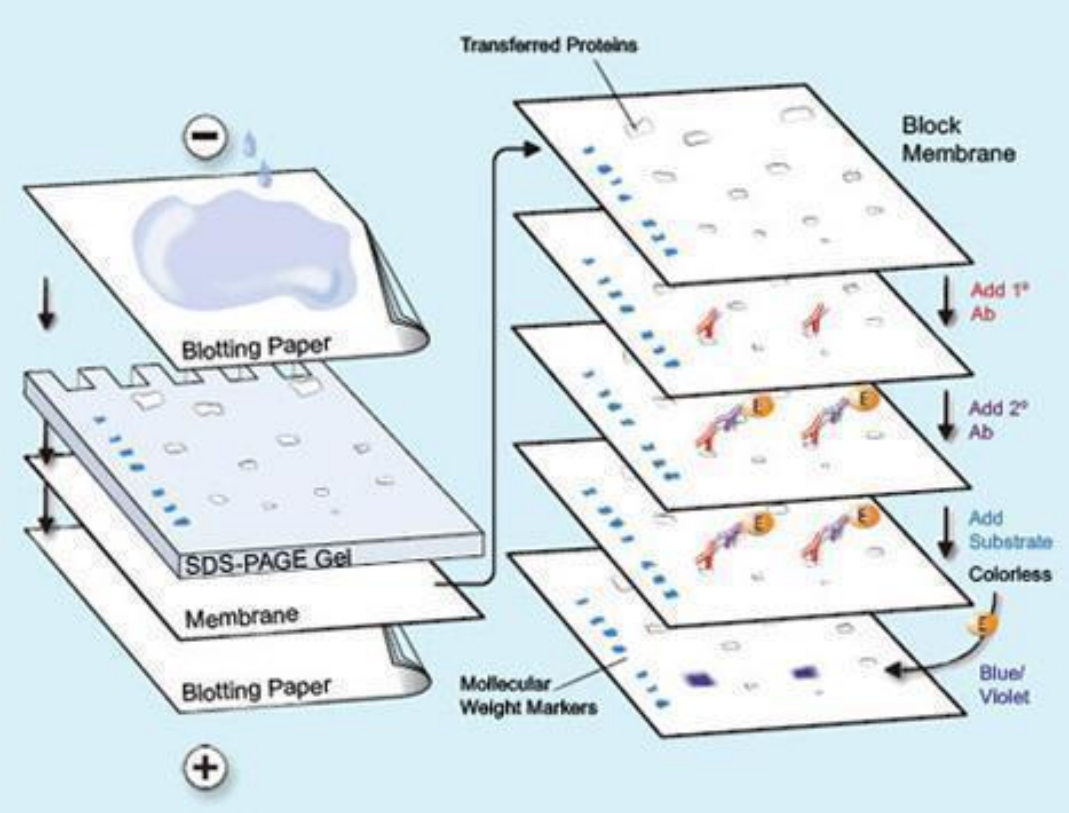


Figure 1 Western blotting technique

The following protocol is meant for the Western Blot of gel using an electrophoresis apparatus and the Western Blot Biorad.

CHEMICAL AND REAGENTS

Reagents	Characteristics	Location
MeOH	400 ml	Safety hood

H_2O		RT
Tris	~12 g	RT
Glicine	~6 g	RT
SDS 10 %	3.75 ml	RT
PBS	500 ml	RT
Tween 20	0,250 ml	RT
BSA		+4 °C
Primary antibody		-20°C
Secondary antibody		-20°C
Lite Ablot solution (fornita da Cell Bio)		+4 °C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Location**
Falcon 50 ml	Bench
Micropipette P20, P200, P1000	Cupboard
Pipettor	Bench
Tips	Cupboard
Pipette 10 ml	Bench
Parafilm	Bench
Absorbing paper	Bench
Acrylonitrile paper	Cupboard

PREPARATION OF SOLUTIONS, GEL AND BUFFER:

TRANSFER BUFFER 1 (1000 mL)

Reagents	**Characteristics**	**Location**
MeOH	200 ml	Safety hood

H_2O	To 1000 ml	RT
Tris	5.82 g	RT
Glycine	2.93 g	RT

TRANSFER BUFFER 2 (1000 mL)

Reagents	**Characteristics**	**Location**
10% SDS	3.75 ml	RT
H_2O	To 1000 ml	RT
Tris	5.82 g	RT
Glycine	2.93 g	RT

WASHING BUFFER (500 mL)

Reagents	**Characteristics**	**Location**
PBS o H_2O	500 ml	RT
Tween 20	0.250 ml	RT

BLOCKING SOLUTION (300 mL)

Reagents	**Characteristics**	**Location**
BSA	3*x g (fraction x % m/v)	RT
Washing Buffer	To 300 mL	RT

WORKING SOLUTION LITE ABLOT

Reagents	**Characteristics**	**Location**
Solution A	1 µl	+4°C
Solution B	1 mL	+4°C
Solution C	3 µl	+4°C

PROCEDURE

- Prepare four containers suitable for containing the gel, filled with H_2O, MeOH, Transfer Buffer 1 and Transfer Buffer 2.
- Pour the content of the electrophoresis apparatus inside a container bigger enough and insert the two plates.
- Cut one of the corner of the acrylonitrile membranes to recognize the right disposal.

FROM HERE, THE PROCEDURE CONCERNS ONLY ONE GEL.

IT IS REALLY IMPORTANT NOT TO TOUCH THE MEMBRANE WITHOUT PERFECTLY CLEAN TWEEZERS.

- Dip the membrane in MeOH for almost one minute.
- Wash the membrane in H_2O.
- Moisten the blotting paper in the Buffer 1 and place it on the plate of the Western Blotting apparatus.
- Place the acrylonitrile membrane on the blotting paper (avoid the formation of air bubbles).
- Gently remove the Running Gel from the chamber and place it on the membrane (avoid the formation of air bubbles).
- Moisten a second blotting paper in the Trasfert Buffer 2 and place it on the gel (avoid the formation of air bubbles).
- Repeat the procedure with the second gel, as quickly as possible.
- Turn on the Western blotting apparatus at a controlled voltage of 12V for 22 minutes.
- Prepare two bath of Blocking Solution, dip the two membrane and incubate in the shaker at 40 rpm for 1 hour.
- Pure the Blocking Solution, replace it with the Washing Solution and incubate in the shaker for 5 minutes.
- Prepare 35 ml of Blocking Solution and 35 ml of primary antibody solution in two Centrifuge tubes and warm up at room-temperature.
- Replace the Washing Buffer with the primary antibody solution and incubate in the shaker at 40 rpm for 1 hour.
- Wash three times with the Washing Buffer (5 minutes +5 minutes + at least 15 minutes in the shaker at 40 rpm).
- Prepare 35 ml of Blocking Solution and 35 ml of secondary antibody solution in two centrifuge tubes and warm up at room-temperature.
- Replace the Washing Buffer with the secondary antibody solution and incubate in the shaker at 40 rpm for 1 hour.
- Wash three times with the Washing Buffer (5 minutes +5 minutes + at least 15 minutes in the shaker at 40 rpm).
- Prepare the Working Solution Lite Ablot for the Scanning Densitometry.
- Pure the Washing Buffer and dry the membrane using a corner of the blotting paper.
- Cover the membrane with the Working Solution and leave it act for at least 1,5 minutes.
- Flip the membrane on the analysis surface of the Densitometry machine (avoid the formation of air bubbles).
- Place a contact paper on the membrane.
- Scan with at least 4 minutes of exposition.

NOTE

The Western blotting procedure is meant to be used after an electrophoretic gel preparation and electrophoresis analysis of the samples.

A procedure to prepare polyacrylamide gel and perform the electrophoresis analysis is provided below

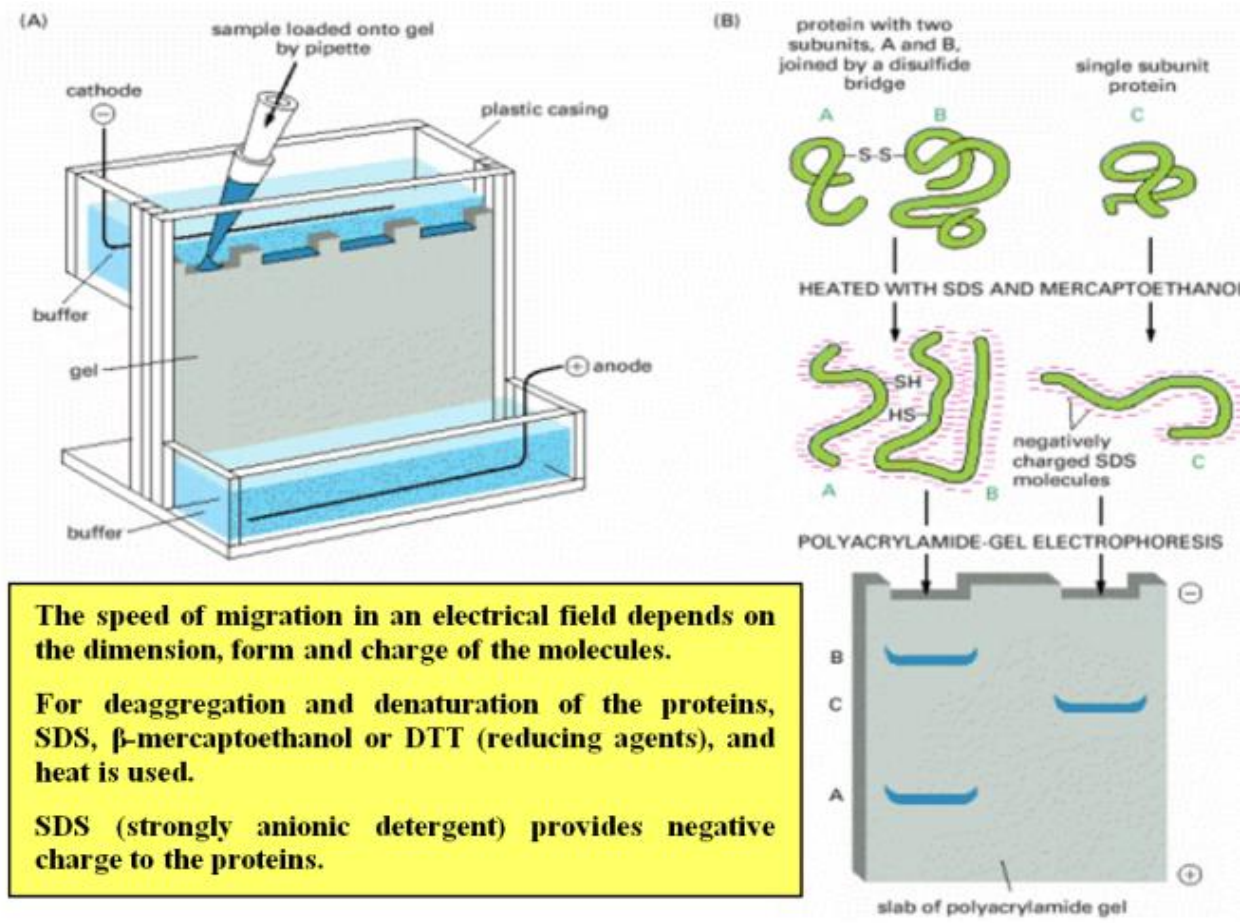
REFERENCE

Tahrin Mahmood and Ping-Chang Yang *"Western Blot: Technique, Theory, and Trouble Shooting"* N Am J Med Sci. Sep 2012; 4(9): 429–434

SDS-ELECTROPHORESIS ON POLYACRYLAMIDE GEL

DESCRIPTION

SDS polyacrylamide gel electrophoresis is a widely used technique for analyzing mixtures of proteins. In this technique, proteins are reacted with the anionic detergent, sodium dodecylsulfate (SDS, or sodium lauryl sulfate) to form negatively charged complexes. The amount of SDS bound by a protein, and so the charge on the complex, is roughly proportional to its size. The mixture is then inserted in the polyacrylamide gel, across which an electric field is applied, and each protein migrates accordingly to its size to a different position in the gel. The use of a Polyacrylamide gel is due to its ability to restrain larger molecules from migrating as fast as smaller molecules.



The following procedure is meant for the electrophoretic separation of proteins through an electrophoretic Biorad cell equipped with two running chambers.

CHEMICALS AND REAGENTS

Reagents	Characteristics	Location
Isobutanol	2 ml	safety cabinet

TEMED	9 µl	+4°C
40% APS	17 µl	-20°C
37.5-1 Acrilamide-Bis		+4°C
Tris HCl		RT
10% SDS	8 ml	RT
SDS	1 g	RT
Glycerol		RT
H_2O		RT
Bromofenol blue	500 µl	RT
β-mercaptoetanol	2.5 ml	RT
Tris	30 g	RT
Glycine	143.6 g	RT

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Location**
Parafilm	Bench
Blotting paper	Drawer
Micropipettes (20, 200, 1000 µl) and tips	Bench
Serological pipettes (10 ml)	Drawer
Pipette controller	Drawer
Eppendorf tubes	Drawer

PREPARATION OF SOLUTIONS, GEL AND BUFFERS

RUNNING GEL SOLUTION X% (100 ml)

Reagents	Caratteristiche	Collocazione
37.5-1 Acrilamide-Bis	X ml (at 100%)	+4°C
Tris HCl 1.5 M pH 8,8	25 ml	RT
10% SDS	1 ml	RT
Glycerol at 60%	12.5 ml	RT
H_2O	to 100 ml	RT

RUNNING GEL X% (~7 ml)

Reagents	Characteristics	Location
Running gel solution X%	7 ml	RT
TEMED	4.7 µl	+4°C
APS at 40%	11.7 µl	-20°C

STACKING GEL SOLUTION X% (50 ml)

Reagents	Characteristics	Location
37.5-1 Acrilamide-Bis	X/2 ml (at 100%)	+4°C
Tris HCl 0.5 M pH 6.8	12.5 ml	RT
SDS at 10%	0.5 ml	RT
Glycerol at 60%	12.5 ml	RT
H_2O	to 50 ml	RT

STACKING GEL (~3.5 ml)

Reagents	Characteristics	Location
Stacking gel solution X%	3.5 ml	RT
TEMED	3.5 µl	+4°C
APS at 40%	4.8 µl	-20°C

LOADING BUFFER x5 (~10 ml)

Reagents	Characteristics	Location
Tris HCl 1 M pH 6.8	2.75 ml	RT
SDS	1 g	RT
Glycerol	4 ml	RT
Bromophenol blue	500 µl	RT
β-mercaptoetanol	2.5 ml	Safety cabinet

PRE RUNNING BUFFER x10 (1000 ml)

Reagents	Characteristics	Location
H_2O	To 1000 ml	RT
Tris	30 g	RT
Glycine	143.6 g	RT

RUNNING BUFFER (500 ml)

Reagents	Characteristics	Location
Pre-running buffer 10X	50 ml	RT
10% SDS	5 ml	RT
H_2O	To 500 ml	RT

PREPARATION

- Prepare almost 7 ml of Running Gel.

- Load 3 ml of Running Gel into each electrophoretic chamber using the pipette controller.
- Load 1 ml of isobutanol into each electrophoretic chamber to level off the gel.
- Cover the superior extremity of the chamber with the parafilm and leave it polymerize for 30-60 minutes.
- Remove the isobutanol and dry with the blotting paper.
- Prepare almost 3.5 ml of Stacking Gel.
- Load the Stacking Gel into the two plates up to the upper extremity (try to avoid the formation of bubbles).
- Insert the combs and leave it polymerize.
- Dilute the samples in the eppendorf tubes with the Loading Buffer (the final concentration of the Loading Buffer has to be 1x).
- Place the samples in boiling water for 5 minutes.
- Centrifuge for 5 minutes at 2000 rpm and remove the drops of condensed water.
- Remove the combs and load the samples in the wells created.
- For each chamber, load into two wells a colorant to monitor the evolution of the running and a molecular weight marker.
- Insert the two plates into the specific lodging of the electrophoretic cell and fill it with the cold Running Buffer (4°C).
- Begin the electrophoresis in controlled current (0.03-0.05 A).
- Stop the electrophoresis when the colorant reaches the lower extremity of the chambers or when the separation is believed to be happened.

DNA ELECTROPHORESIS ON AGAROSE GEL

DESCRIPTION

Nucleic acid electrophoresis is an analytical technique used to separate DNA or RNA fragments by size and charge. Nucleic acid molecules to be analyzed are inserted into the gel across which an electric field is applied. Due to the net negative charge of the sugar-phosphate backbone of the nucleic acid chain, the different nucleic acids migrate toward the anode. The separation of the fragments is accomplished by exploiting the motilities with which different sized molecules are able to pass through the gel (smaller fragments end up nearer to the anode than longer ones). After a given period, each nucleic acids gained a specific position accordingly to its size and charge.

The following procedure refers to the DNA separation with a BioRad electrophoretic cell.

CHEMICALS AND REAGENTS

Reagents	**Characteristics**	**Location**
Agar (powder)		RT
TAE 1X		RT
H_2O		RT

PROCEDURE

- Prepare TAE 1X solution (1L)
- Weight agarose powder to reach the desired concentration in a fixed volume of TAE 1X.
- Put agarose powder into a 250 ml beaker and mix with the TAE 1X.
- Put the beaker into the microwave and warm up until boiling (the solution has to be homogenous and transparent).
- Once the solution is ready, pour (avoiding the formation of many air bubbles) into the electrophoretic running cell, containing the combs, which will constitute the wells for DNA loading.

TISSUE ENGINEERING

DESCRIPTION

Cell microencapsulation allows immobilization of cells within a polymeric semi-permeable membrane that permits the bidirectional diffusion of molecules: nutrients inward, waste and therapeutic proteins outward. The encapsulated cells would provide a source of sustained continuous release of therapeutic products for longer durations at the site of implantation. Alginates are regarded as the most suitable biomaterials for cell microencapsulation due to their abundance, excellent biocompatibility and biodegradability properties.

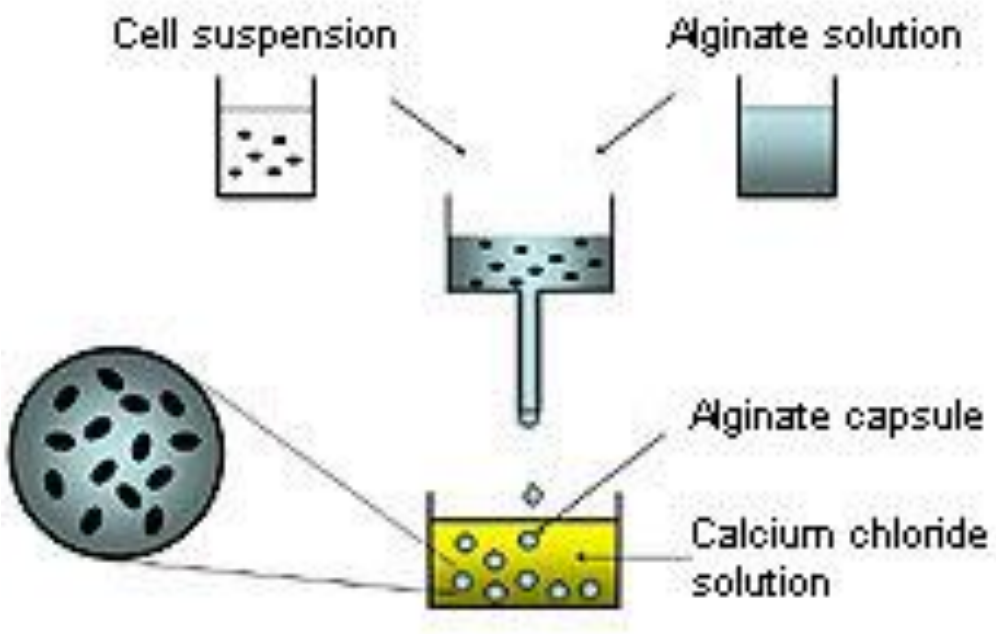


Figure: Conceptual scheme for alginate cell encapsulation

ALGINATE ENCAPSULATION OF PANCREATIC ISLETS

The following protocol describes the procedure to perform encapsulation of pancreatic cells with alginate gel.

CHEMICAL AND REAGENTS (the solutions have to be prepared in sterile conditions)

Reagents	**Characteristics**	**Location**
$CaCl_2$ x2H_2O	7350 mg + 184 mg + 0,37 mg	+4°C
KCI	74,6 mg + 175 mg + 350,4 mg + 0,35 mg	-80°C, aliquots at -20°C
$MgCl_2$	122 mg	RT
Hepes	1190 mg + 2975 mg + 5958 mg 5,96 mg	RT
NaCl	3857 mg + 7889,4 mg + 6,90 mg	RT
KH_2PO_4	163,3 mg + 0,16 mg	RT
$MgSO_4 \cdot 7H_2O$	295,8 mg + 0,29 mg	RT
Alginate Manugel DMB (Monsanto plc) 1.7%		

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Location
Syringe with a cut and rounded needle tip	Drawer
Centrifuge tubes	Drawer
UV lamps	Drawer

PROCEDURE

Prepare the sterile solutions:

- **CALCIUM CHLORIDE DEHYDRATE 100 mM. (I)**

Add to 500ml of distilled water these reagents following this order:

  - 7350 mg $CaCl_2 \cdot 2H_2O$ (100 mM)
  - 74.6 mg KCl (2 mM)
  - 1190 mg Hepes (10 mM)

- **SPOLHEPES (II)**

Add to 500ml of distilled water these reagents following this order:

  - 3857 mg NaCl (132 mM)
  - 175 mg KCl (4.7 mM)
  - 122 mg $MgCl_2$ (1.2 mM)
  - 2975 mg Hepes (25 mM)
  - 184 mg $CaCl_2 \cdot 2H_2O$ (2.5 mM)

- **KREBS WITHOUT CALCIUM (III)**

Add to 1L of distilled water these reagents following this order:

  - 7889.4 mg NaCl (135 mM)
  - 350.4 mg KCl (4.7 mM)
  - 5958 mg Hepes (25 mM)
  - 163.3 mg KH_2PO_4 (1.2 mM)
  - 295.8 mg $Mg_2SO_4 \cdot 7H_2O$ (1.2 mM)

- **KREBS RINGER HEPES 25mM (IV)**

Add to 1L of distilled water these reagents following this order:

  - 0.35 g KCl (4.69mM)
  - 0.16 g KH_2PO_4 (1.18mM)
  - 0.29 g $Mg_2SO_4 \cdot 7H_2O$ (1.18mM)
  - 5.96 g Hepes (25mM)
  - 0.37 g $CaCl_2 \cdot 2H_2O$ (2.52mM)

- For each solution adjust pH to 7.4 and sterilize
- Prepare 1.7% alginate solution
- Sterilize alginate powder for 30 minutes under UV lamps

- Dissolve the alginate powder in Krebs without calcium (solution III).
- Leave the solution at 4°C for several hours (usually overnight).
- Prepare the encapsulated cells.
- Collect the cells in a Centrifuge tube.
- Wash the cells with Hanks medium in order to remove all the calcium particles that were present in the medium.
- Remove the supernatant.
- Suspend the cells in the 1.7% alginate solution. We use a concentration of 3 islets/alginate µl
- Collect the cells with a syringe.
- Pour forth carefully the alginate and cell drops in the calcium chloride solution (solution I).
- Wait for 5 minutes from the last capsule fall in order to obtain the alginate gel.
- Remove the calcium chloride.
- Add Spoelhepes (solution II) and wait for EXACTLY 1 minute to replace calcium with sodium.
- Remove the supernatant
- Wash capsules twice (1 minute for each washing) with Krebs without calcium (solution III). Remove the supernatant.
- Add Krebs Ringer Hepes (solution IV).
- Culture the cells.

NOTE

Drop carefully in order to obtain capsules with 2-3mm dimension. If interested in obtain smaller capsules, use a capsule generator with an air flow that regulates capsule dimensions

REFERENCES

M. Figliuzzi, T. Plati, R. Cornolti, F. Adobati, A. Fagiani, L. Rossi, G. Remuzzi, A. Remuzzi. Biocompatibility and function of microencapsulated pancreatic cells. Acta Biomater 2006 Mar; 2(2):221-7

R. Cornolti, M. Figliuzzi, A. Remuzzi. Effect of micro- and macroencapsulation on oxygen consumption by pancreatic islets. Cell Transplant 2009; 18(2):195-201

ALGINATE ENCAPSULATION OF CELLS + ALGINATE DISSOLUTION FOR CELL HARVESTING

DESCRIPTION

Alginates are natural hetero-polysaccharides isolated from brown algae and are of particular interest due to their unique properties, including injectability and biodegrability. Due to their biocompatibility they can be used as support for cells proliferation or to produce scaffolds.

CHEMICAL AND REAGENTS

Reagents	**Characteristics**	**Location**
Saline solution	NaCl 0.154 M	RT
$CaCl_2$ Solution	2 mM	RT
Alginate		RT

PROCEDURE

- Alginate solution must be prepared at 2% in saline solution (NaCl 0.154 M).
- Suitable quantity of alginate has to be melted in a little beaker shaking it overnight (preparing more than 20 mL of solution is not advised as it might result in a very dense solution).
- Filter the solution (use a filter with 0.22 micron pores and a syringe).
- The filtration is a difficult procedure, please do it for little volumes with 5mL syringes.
- The solution will be used for cell encapsulation.
- To cross-link the solution, immerse the sample holder in a sterile solution of NaCl 0.154 M.
- Gelation occurs at room temperature in about 30 minutes.
- After gelation, rinse quickly the support for the cells in PBS with a low concentration of $CaCl_2$ (2 mM)
- Low concentration of $CaCl_2$ is necessary only to remove the surplus of Ca.
- The protocols for assembling the bioreactor must include this stop phase, under hood, in order to allow the housing of the samples preparation in the support.
- Dissolve the cellularised gel at 37° shaking mildly the test tube until the alginate is completely dissolved.
- When the experiments are finished, the dissolved solution is composed by: NaCl 0.154 M + citrate sodium 55 mM. Typically, 200 microliters of solution are used for each small alginate disc with cells (each small disc had a volume of 50 microliters).
- By maintaining this proportion, no problems should emerge.

REFERENCES

Wong, M. et al.; Development of mechanically stable stable alginate/chondrocyte constructs: effects of glucuronic acid content and matrix synthesis. J Orthop Res, 19: 493-499; 2001.

Giannoni, P., et al.; The mechanosensitivity of cartilage oligomeric matrix protein (COMP); Biorheology, 40:101-10; 2003.

PREPARATION OF AGAROSE-ALGINATE CULTURE SUBSTRATES WITH VARIABLE STIFFNESS

CHEMICALS AND REAGENTS

Reagents	Characteristics	Location
Alginate powder	to prepare a 0.2% w/v solution	RT
Agarose powder	to prepare a 1.8% w/v solution	RT
$CaCl_2$	100 mM	RT
Saline solution NaCl 0.9%		RT
PBS		RT

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Location
autoclavable bottles and cap	Armadietto
0.2 micron syringe filter and syringe	Scaffale
24-well plate	Armadietto
FluoSpheres Ø 0.5µm	

PROCEDURE

- Solutions to be prepared separately in autoclavable bottles and cap hermetical
- Solution 1 - soft matrix: dissolve agarose powder (1.8% w/v) and alginate powder (0.2% w/v) in physiological solution
- Solution 2 - rigid matrix: dissolve agarose powder (4.5% w/v) and alginate powder (0.2% w/v) in physiological solution
- Maintain the solutions 1 and 2 under stirring for 30 minutes at 80 ° C with the cap tightly closed
- Autoclave solutions 1 and 2, and keep stirring under the hood at 80 ° C with the cap closed. (If not using right away, go to "Before use")
- Prepare a solution of 100 mM $CaCl_2$ (1.1% w/v) in physiological solution
- Filter the solution of $CaCl_2$ with 0.2 micron filter
- Ready to use
- Heat solutions 1 and 2 at 80 ° C under stirring with the cap tightly closed
- Dispense the solutions 1 and 2, 0.3 ml/well in 24-well plate pre-heated
- Dilute the solution containing the FluoSpheres Ø 0.5µm to have a quantity of microspheres (in 100 L of solution/well) corresponding to 10 microspheres/cell that will be plated
- Let polymerize the agarose under a hood for a time varying between 10 and 15 minutes

- Dispense the solution containing the FluoSpheres, 100 L of solution / well, after it is heated to 40 ° C
- Film the plate and store at 4 ° C for 30 minutes to complete agarose polymerization including the microspheres below the surface
- Remove the solution from the wells of Fluorospheres
- Dispense 0.5 ml/well of $CaCl_2$ solution
- Store at 4 ° C for 30 minutes to polymerize the alginate
- Vacuum the $CaCl_2$ solution
- Wash with PBS
- Plate cells > 150000/well (remember to respect the ratio of 1 cell/10 microspheres)

DYNAMIC CELL SEEDING OF CUSTOM-MADE POLYSTYRENE SCAFFOLDS

DESCRIPTION

In this section, the procedure for dynamic cell seeding of 3D Biotek polystyrene scaffolds is discussed. We observed that using the seeding technique suggested by the producer, which consisted in capillary diffusion starting from a cellular suspension drop at the center of the scaffold, there was evidence of inhomogeneity and cell aggregates. The following protocol is the result of several experiments, which aimed at improving the seeding procedure to obtain a homogeneous cell distribution without requiring dynamic seeding in perfusion. The technique consists in seeding the scaffold with a cellular suspension and keeping the construct in a shaking state (dynamic seeding) with an orbital shaker.

CHEMICALS AND REAGENTS

Reagents	Characteristics	Location
MG63 cell line (adherent cells)		Liquid nitrogen
Culture medium		+4 °C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	Location
PS-M3D multifunction orbital shaker	Drawer
24-well plate	Cupboard
micropipettes (1000 µl)	Bench
Scaffold 3D biotek (w=3 mm; l=6 mm; h=0,4 mm; fibre diameter 0,1 mm; grid light = 300 µm; grid layers displacement = 150 µm)	

PROCEDURE

- Obtain a cell suspension with a density of 2.5 million cells/mL culture medium;
- Place a scaffold inside a well of the MW24;
- Inject 400 µl of cell suspension (corresponding to 1 million cells) in each well;
- Place the MW24 on the shaker plate, inside the incubator (37°C, 5% CO_2, vapor saturation), and set:
- Orbital: OFF
- Reciprocal: mode=270°; time=01
- Vibro: mode=5°; time=2
- Cycle: mode=00; time=stop
- Keep the scaffolds in a shaking state for 5 hours;
- Stop the shaker and gently put the multiwell plate on the incubator shelf;

- The following day, transfer the scaffolds into clean wells and add 400 µl culture medium;
- Change culture medium daily.

NOTE

Transferring the scaffolds into clean wells is necessary because many cells adhere to the bottom of the wells;

if using a different culture environment (not a 24-well microplate), add a suitable volume of medium;

if not using a 24-well microplate, turnover times could be different.

REFERENCES

3D BIOTEK LLC, Ilene Court, Hillsborough, New Jersey 08844; *3D Insert Cell Seeding Protocol*, Version: 3.2, Updated: 2013; http://www.3dbiotek.com/documents/3DCellSeedingProtocol.pdf

Griffon DJ, Abulencia JP, Ragetly GR, Fredericks LP, Chaieb S; A comparative study of seeding techniques and three-dimensional matrices for mesenchymal cell attachment; J Tissue Eng Regen Med. 2011 Mar;5(3):169-79. doi: 10.1002/term.302

BIOREACTOR FOR 3D TISSUE CULTURE UNDER INTESTINAL PERFUSION

MATERIALS

The culture system consists of two parts, each housing two independent perfusion circuits, featuring a total of four independent perfusion circuits.

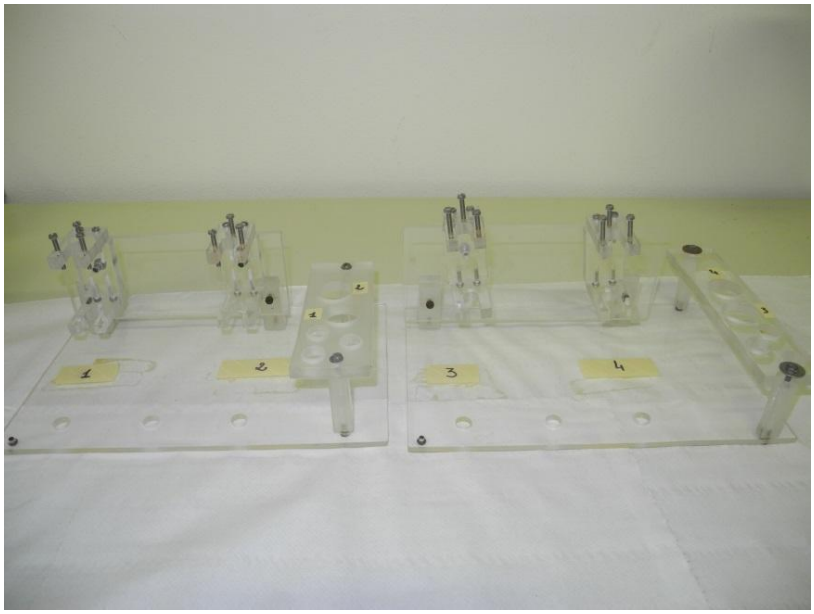


Figure 1- Housings of the culture system

For each circuit, the components to be assembled must be sterilized with a low-temperature method, such us Ethylene Oxide or Hydrogen Peroxide Plasma.

Never use steam, which causes damage to the polycarbonate components.

The components of each circuit must be grouped into the following sterilization envelopes.

- Envelope 1: 1 reservoir + 1 cap with 3 holes (with passing tubes for inlet, outlet and air flow)
- Envelope 2: 1 air filter (commercially available - already sterilized)
- Envelope 3: scaffold holder, 2 parts and 1 screw
- Envelope 4: culture chamber, 2 parts and one internal gasket
- Envelope 5: 1 de-bubbler with cap
- Envelope 6: 2 oxygenator tubes, 1 connecting the reservoir to the chamber inlet and 1 connecting the chamber outlet to the de-bubbler
- Envelope 7: 2 oxygenator tubes with anti-squeeze sleeve, 1 connecting the pump tubing to the reservoir and 1 connecting the pump tubing to the de-bubbler
- Envelope 8: 1 pump tubing.

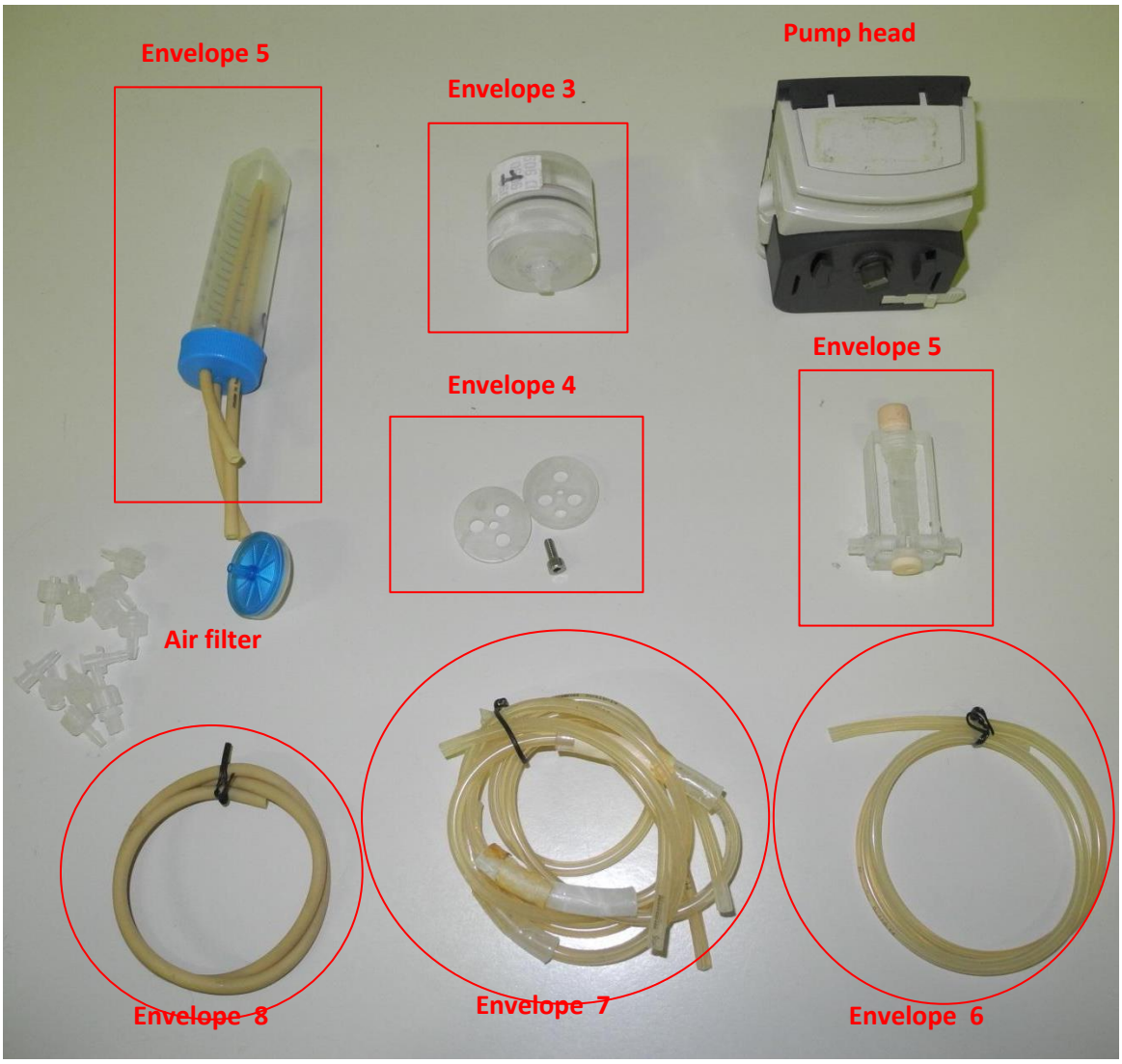


Figure 2- Materials to be grouped into sterile envelopes

The other materials to be used to mount one circuit are:

Materials	**Location**
Sterile gloves	Bench
Sterile hexagonal screw-driver (brugola) to operate the scaffold holder screw	Drawer
Complete culture medium	+4 °C
sterile forceps	Drawer
Pipettes	Drawer

Figure 3 - Other materials

PROCEDURE

Assembly must take place in a biological safety cabinet, by two operators: one wearing sterile gloves (main operator) and one wearing normal gloves (support operator), who opens sterile packets for the main operator.

Before starting the assembly, the main operator wears sterile gloves and leaves the sterile paper package open on the cabinet ground.

ASSEMBLY OF THE CULTURE CHAMBER

- Insert the cell-seeded scaffolds in the first half of the scaffold holders.
- Assemble the second half of the scaffold holder to close the holder by fitting the holder peg as shown in Figure 4.

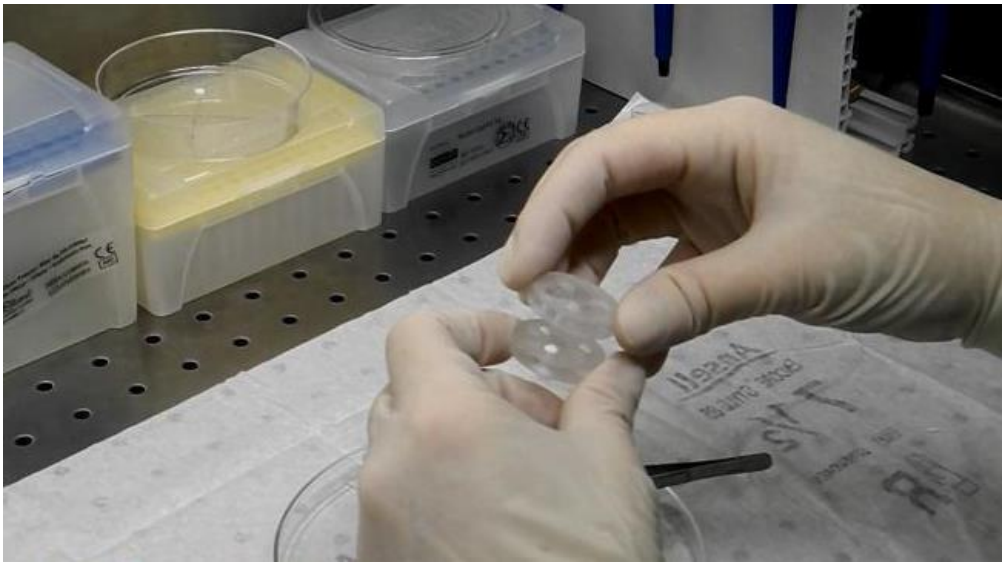


Figure 4

- Lock the holder with its screw using the hexagonal screw driver (Figure 5)
- Insert the gasket in the culture chamber and insert the scaffold holder firmly in one of the two chamber parts (Figure 5).

Figure 5

- Assembly the culture chamber (Figure 6) by rotating one half on the other until the external gasket is completely covered.

Figure 6

ASSEMBLY OF THE CULTURE CIRCUIT

- Fill the reservoir with 40 mL of complete culture medium. Lock the reservoir. Connect a sterile filter to the shorter tube of the reservoir (Figure 7).

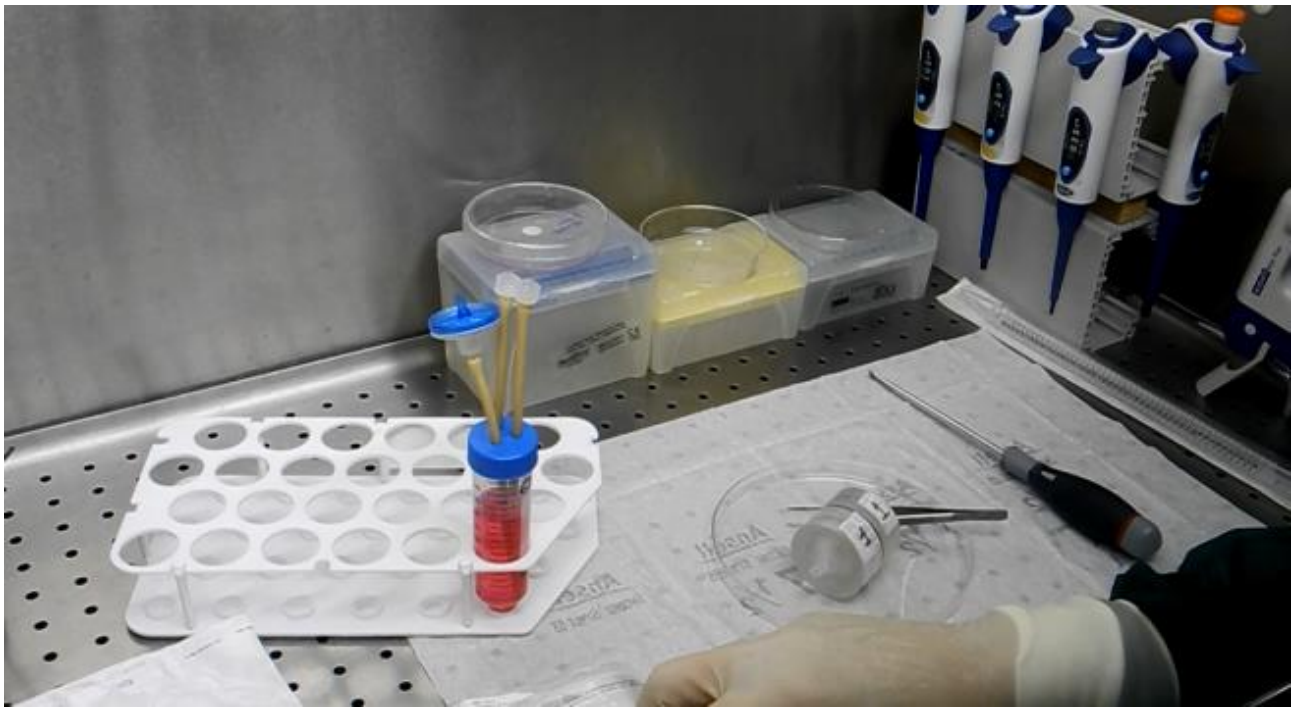


Figure 7

- Connect one oxygenator tube to the reservoir outlet on one side, and to the culture chamber inlet on the other side (Figure 8).

Figure 8

- Lock firmly the de-bubbler cap.
- Connect the other oxygenator tube to the culture chamber outlet on one side and to the de-bubbler on the other side (Fig. 9).

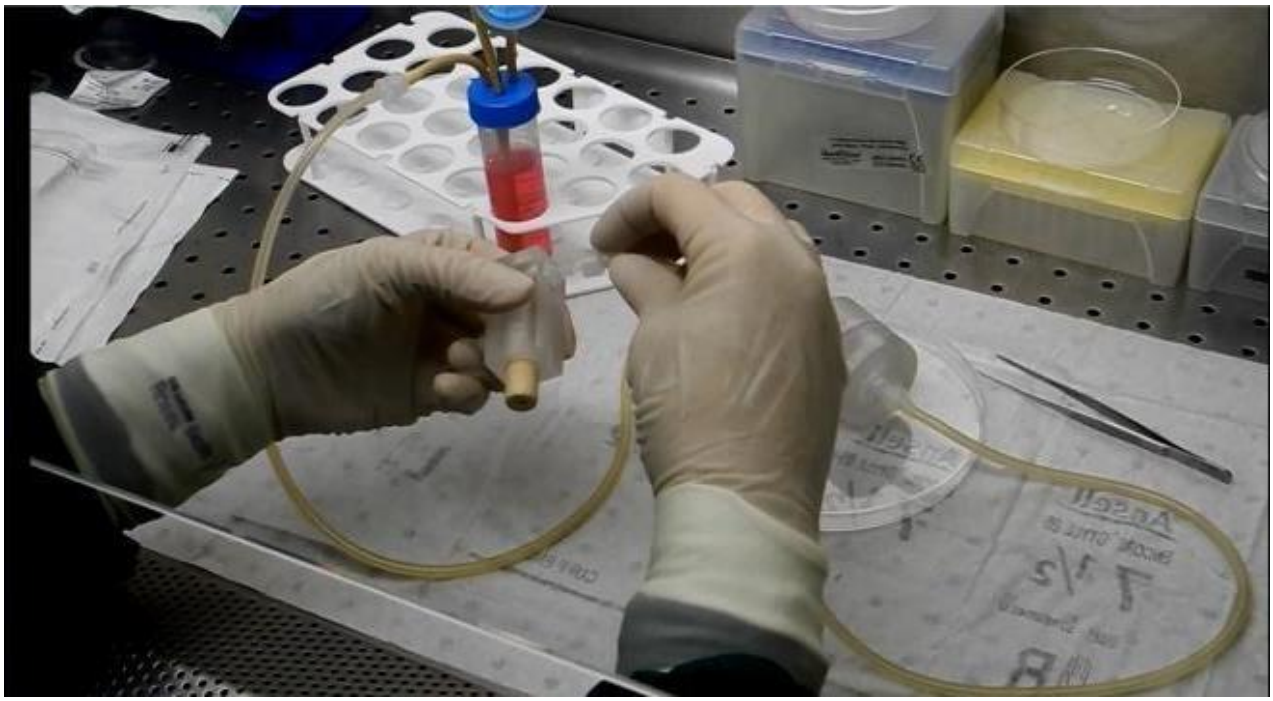


Figure 9

- Connect the two oxygenator tubes with anti-squeeze sleeve to the pump tubing. One oxygenator must connect the de-bubbler to the pump tubing, the other oxygenator must connect the pump tubing to the reservoir. At this stage, the first perfusion circuit is ready.
- Place the culture chamber ,vertically, on its holder support and lock it with the four support screws (Figure 10).

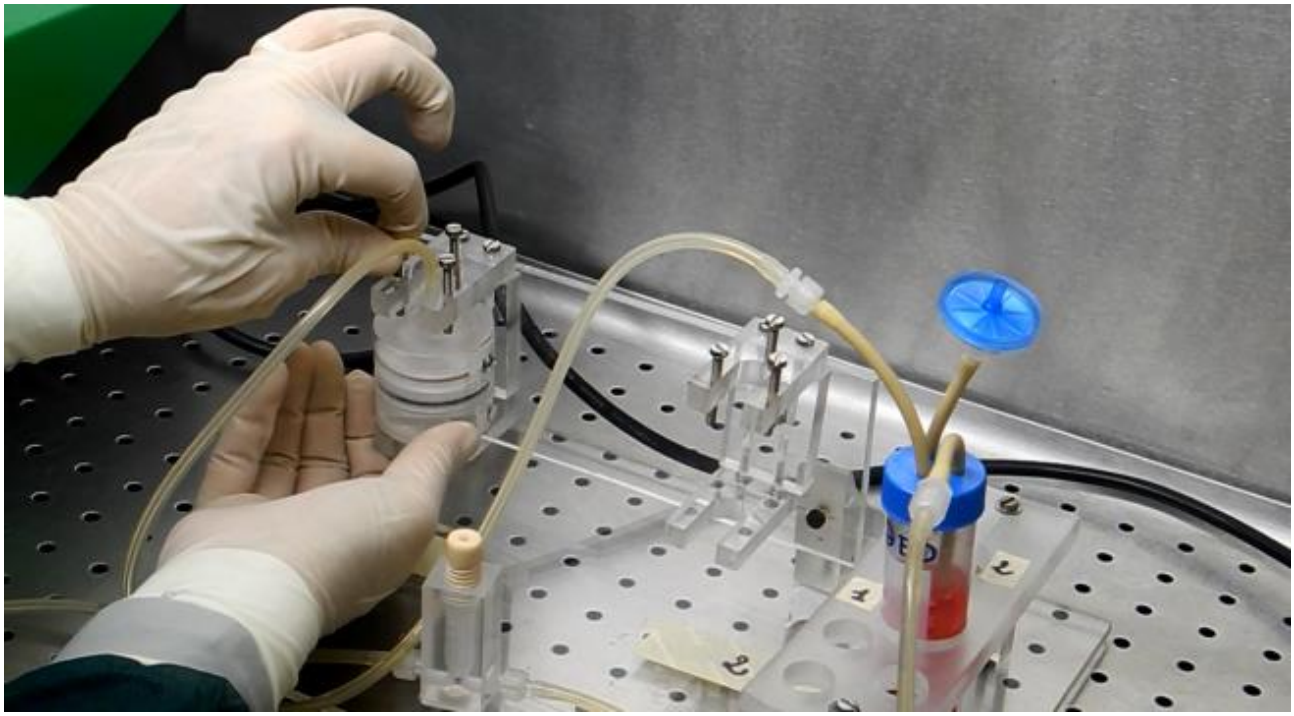


Figure 10

- If needed, assemble the other three circuits using the same procedure detailed at points 1-12.
- Insert for each circuit one pump head on the roller pump. Insert each pump tubing in an individual pump head (Figure 11), lock the pump heads on the pump tubing.

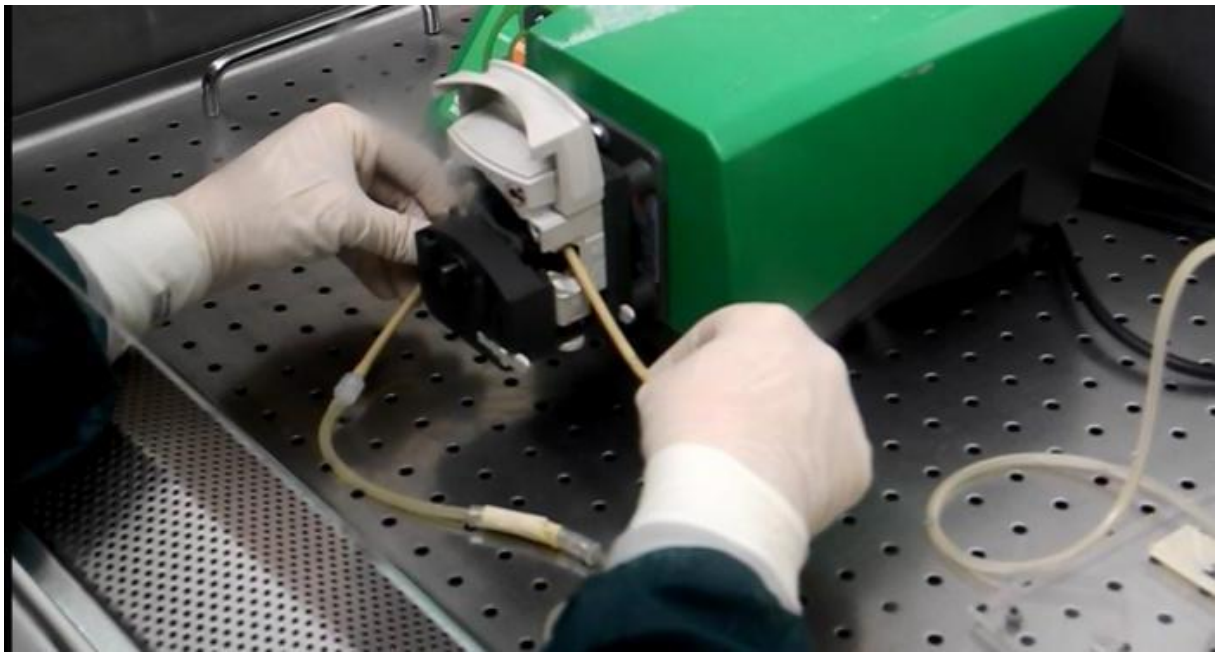


Figure 11

- Operate the pump to fill the four circuits. The culture chambers must be rotated in vertical position to obtain filling of the culture chamber and simultaneous de-bubbling (Figure 12a,b). Use a very low flow rate to fill the circuits, not exceeding 0.1 mL/min, to avoid damage and/or washout of the seeded cells.

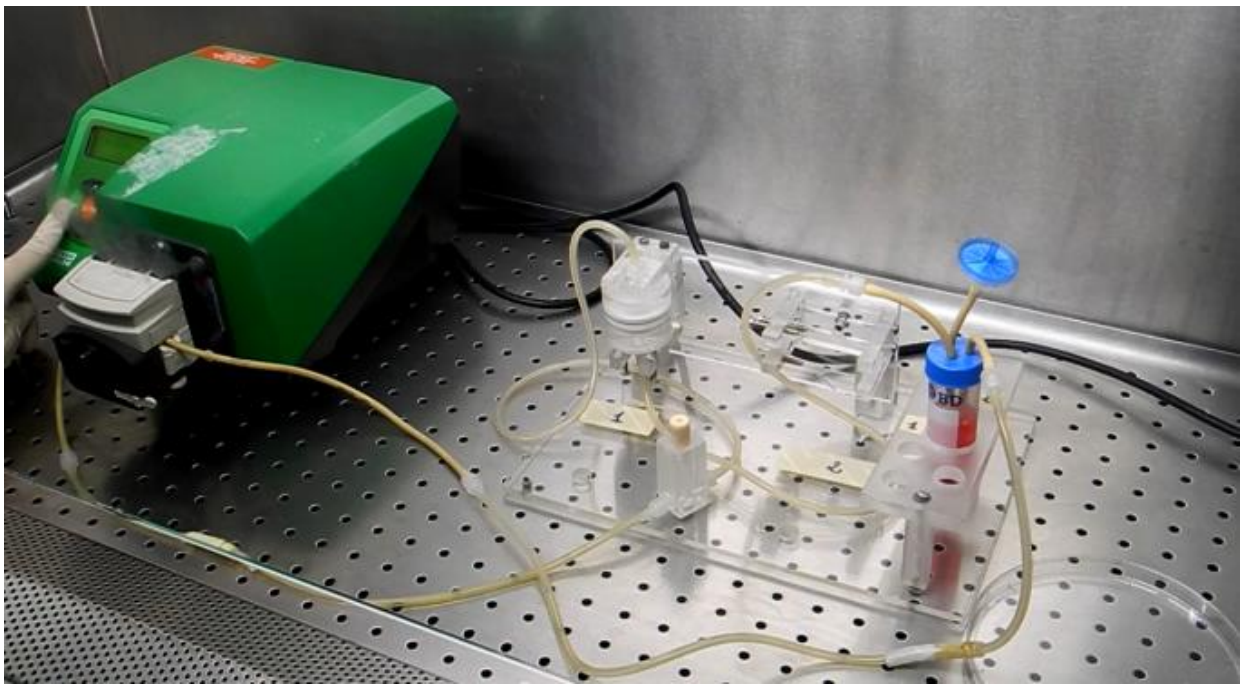


Figure 12a

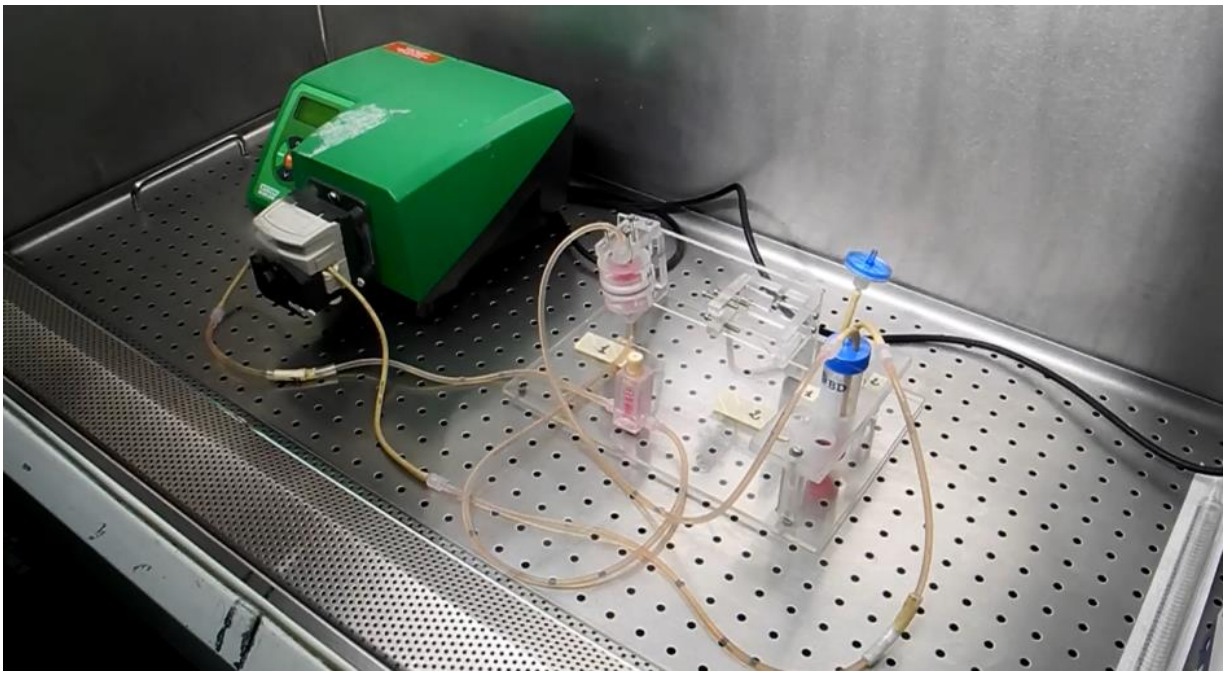


Figure 12b

- When the circuits are completely filled and de-bubbled, rotate the chambers in horizontal position, insert the culture system in the incubator and place the roller pump on the incubator top (Fig. 13).

Figure 13

- Check for the presence of bubbles in each chamber once a day, if there are bubbles rotate the chamber in vertical position for two hours to allow de-bubbling and then rotate it back to a horizontal position.

BIOREACTOR FOR 3D TISSUE CULTURE UNDER COMBINED REGIMENS OF HYDROSTATIC PRESSURE AND INTERSTITIAL PERFUSION (BRAD)

The bioreactor is made of AISI 316L steel and is able to apply high hydrostatic pressure regimens (Fig. 1).

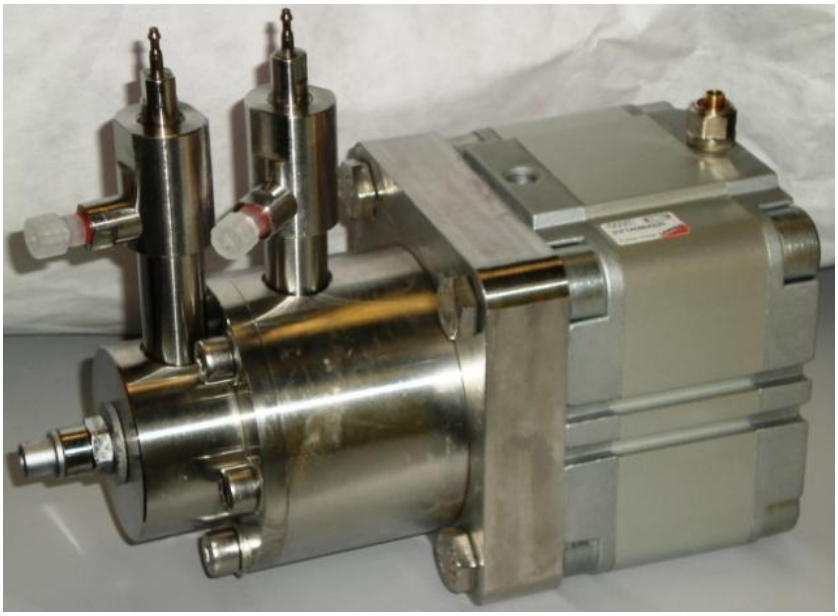


Figure 1. Component of the perfusion bioreactor applying hydrostatic pressure cycles (culture chamber, pneumatic actuator and hydraulic valves), not yet connected to other components.

Its prototype is made of poly(methylmetacrilate) and can be used only for perfusion (Fig. 2). The design of the perfusion bioreactor culture chamber is reproduced in Fig. 3.

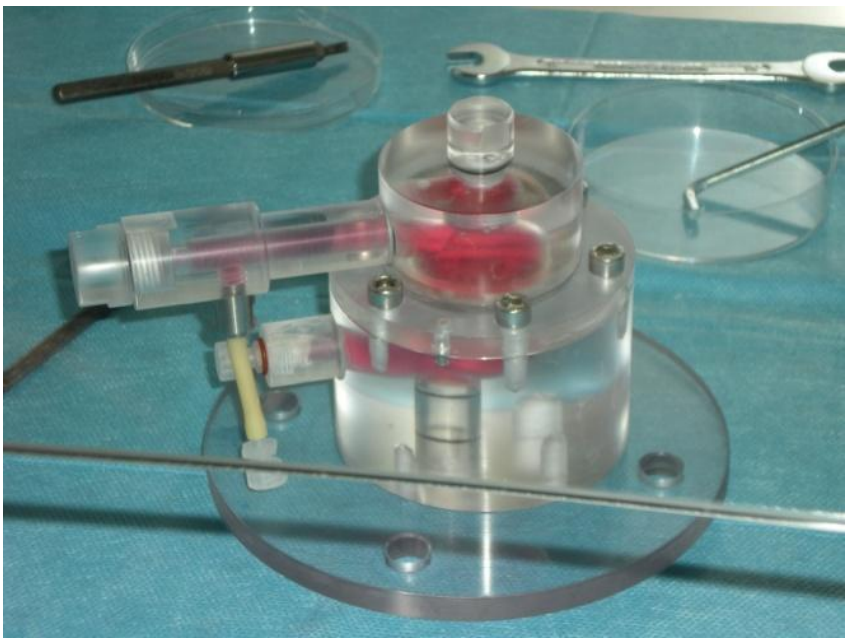


Figure 2. Perfusion bioreactor culture chamber, not yet connected to the hydraulic network.

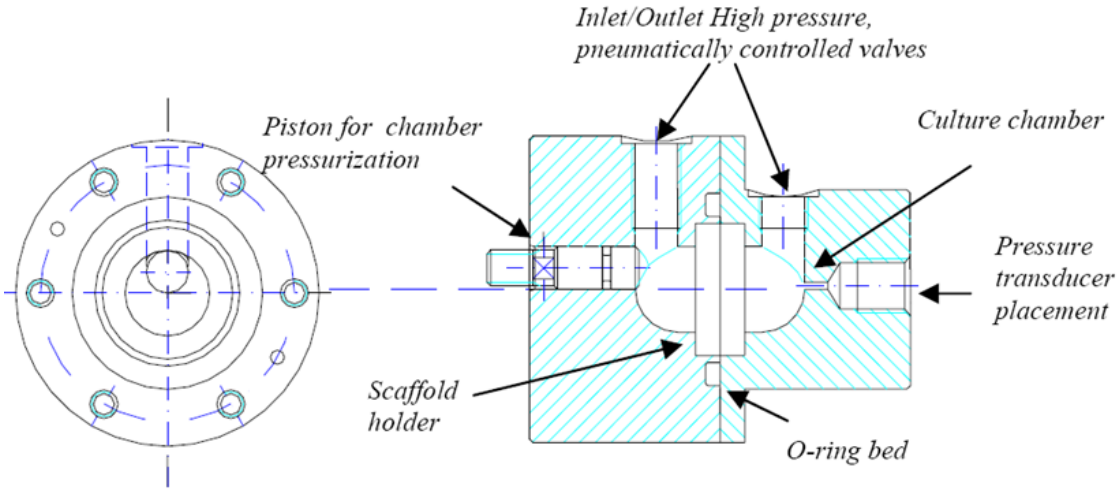


Figure 3. Design of the perfusion bioreactor culture chamber.

The geometry of the chamber containing the sample holders and perfusion parameters are the same for both bioreactors, so the only difference between experimental groups P and P+H is the application of pressure stimulus.

The pressure bioreactor is composed of 4 subsystems (Fig. 4):

- Culture chamber with sample holder housed on it.
- Hydraulic circuit for medium recirculation, composed of a roller pump and two channels.
- Pneumatic circuit to activate the piston that pressurizes the culture chamber and to regulate special valves able to isolate it during pressurization cycles.
- Electronic control unit to set the entire culture system.

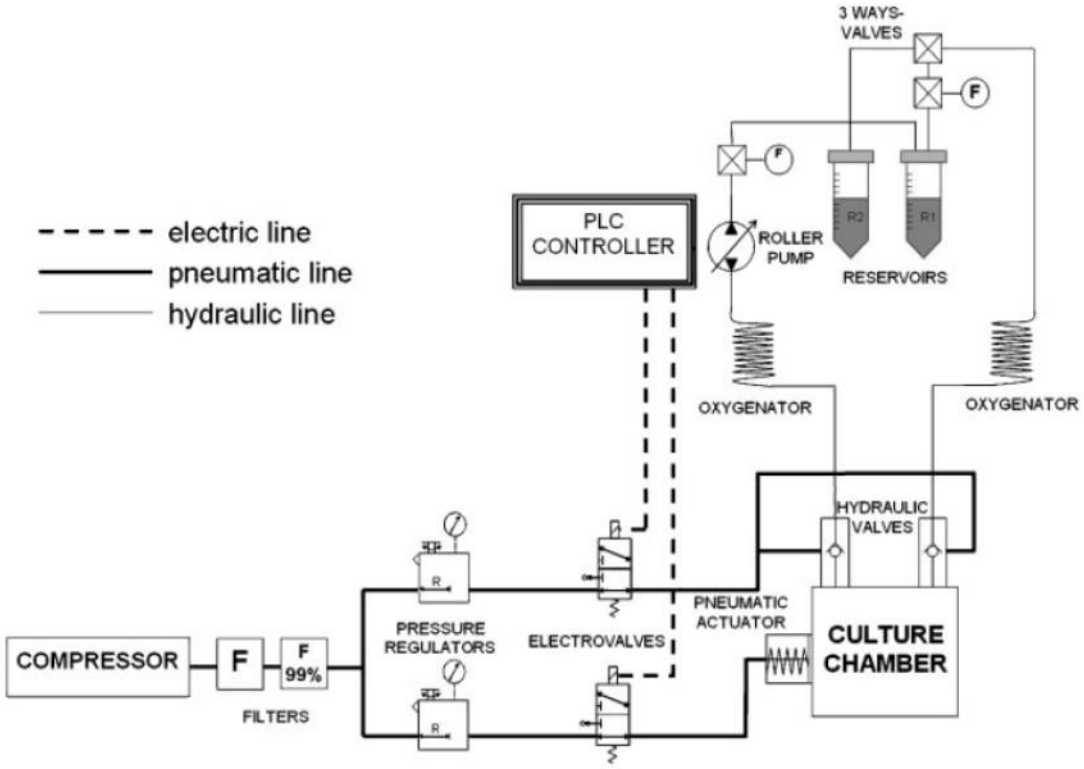


Figure 4. Scheme of the 4 subsystems composing the pressure bioreactor.

CULTURE CHAMBER

The culture chamber constitutes the central system of the device and its internal geometry satisfies the following prerequisites.

- Low filling volume (20 ml): useful because it minimizes the medium recirculation time during perfusion, so that the constructs are exposed to the medium coming from the oxygenators; moreover the low filling volume of the whole system reduces costs deriving from the culture medium turnover.
- Symmetry conditions in both the fluid flowing directions.
- Equal distribution of medium inside the chamber.
- Easy air bubbles expulsion (if present): essential for the correct working of the system; air would hinder culture medium pressurization, because of its high compressibility and because of the limited piston stroke. Furthermore, high amount of air could influence perfusion, because it would empty oxygenators and avoid the correct gas exchange between medium and incubator atmosphere.

HYDRAULIC CIRCUIT

The hydraulic circuit is made of two oxygenator tubes (Silastic 508-004, I.D 0.76 mm O.D 1.65 mm, DOW CORNING) 4 m long and respectively connected upstream and downstream the culture chamber, to improve the oxygenation of medium entering the culture chamber in case of reverse flow direction.

The oxygenator edges not connected to the culture chamber valves are grafted with luer lock junctions to the subpump tube and to the reservoir.

The reservoir constitutes the medium reserve necessary to constantly keep the entire system filled, it dampens the flow pulsatility deriving from the roller pump and allows expulsion of air bubbles coming from the culture chamber. Moreover, it allows the operator to make a total or partial circulating medium turnover. A HEPA filter 0.22 μm long is assembled on the reservoir, so that balance with ambient air pressure is maintained, without allowing contamination agents to enter.

The subpump tube (Marprend #13, Watson Marlow) closes the circuit and is the deformable element mashed by the roller pump, so that it allows the culture medium motion. Input and output culture chamber valves (CPRA2506005A-The Lee Company) are of aeronautic origin, entirely made of inox steel, autoclavable and able to assure the chamber seal for pressure higher than 150 bar; they are regulated by the pneumatic system through a pin actuator, which acts on the valve shutter.

A double channel roller pump (Watson Marlow) allows the flow motion inside the circuit; it is managed by the system PLC controller (LOGO!, Siemens) which reverses the rotation of rolls, and so even the perfusion direction, at established times.

PNEUMATIC CIRCUIT

The pneumatic circuit is divided into two branches:

The first one drives the pneumatic piston (CAMOZZI series 31: 80 mm bore and 20 mm piston stroke), used to pressurize the fluid inside the culture chamber.

The second one activates the hydraulic valves placed respectively at the entrance and at the exit of the culture chamber.

A compressor, equipped with a filter used to eliminate air impurities, allows the functioning of the piston and controls the hydraulic valves.

Each of the two branches includes a pressure regulator and its correspondent manometer, because the pressure used for the piston (2.5 bar) is different from the one necessary to command the hydraulic valves (5.5 bar). The high pressurization of the culture chamber (100 bar) is reached thanks to the hydraulic lever, granted by the ratio between the pneumatic piston section and the much smaller section of the little piston, which acts on the culture chamber medium. An interesting aspect is that air coming from the compressor cannot in any way be in contact with the culture medium, because the hydraulic valves are controlled by mechanical actuators and are in contact only with them.

ELECTRONIC CONTROL UNIT

The electronic control unit (LOGO! 12:24V DC Siemens) coordinates the bioreactor functioning. The ECU can be programmed from a PC and it commands the roller pump and the electrovalves equipped on the two branches of the pneumatic circuit. When the ECU inactivates the pump, the hydraulic valves are automatically closed, the culture chamber is isolated from the perfusion circuit and the compressed air is sent to the pneumatic piston. At the end of the pressure cycle set, valves reopen and roller pump is reactivated to restart perfusion. So, it is possible to set different combinations of perfusion times and pressurization times.

PERFUSION BIOREACTOR

The perfusion bioreactor works in a similar way to the perfusion bioreactor applying hydrostatic pressure cycles; the differences consist in the missing of the mechanic actuator, of the hydraulic valves and of the related control circuit.

SAMPLE HOLDER

The bioreactor described above did not include a sample holder, functional to house the cellular pellets, so we decided to realize a specific device.

The new sample holder, now a pellet holder (Fig. 5), is composed of 12 housings with 3 mm diameter. It is a turned inox steel AISI 316L cylinder, sectioned into 3 parts connected by screws: the central part, containing the pellets, is closed between two square mesh wire cloths (AISI 316L), with a 30 μm mesh light (inter-axis between two consecutive wires). The mesh dimensions are such as to allow the perfusion but not the extrusion of the pellet. On the external side of the cylinder there are two O-ring housings; these gaskets hinder the culture medium to bypass the pellet holder, forcing the fluid flowing through the pellet housings.

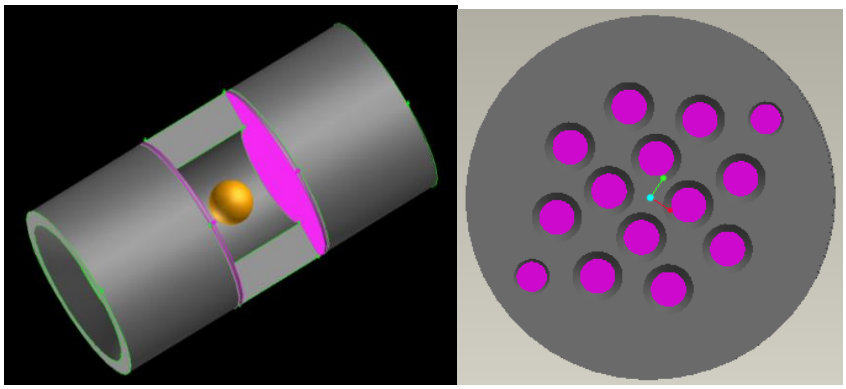


Figure 5. Front view of the pellet holder and sectioned detail of a pellet housing, closed between two metal wires.

CHEMICALS AND REAGENTS

Cells: depending on the application

Culture medium: depending on the cellular type chosen

STERILIZATION ENVELOPES

The components must be grouped in sterilization envelopes (Fig. 6)

- Envelope 1: piston with gasket for high pressure + wrench n.9
- Envelope 2: bioreactor base
- Envelope 3: 4 big screws + hex screw n.8
- Envelope 4: gasket for bioreactor base
- Envelope 5: tower with long thread (with gasket) + cannon (with gasket) + 2 luer lock adapter (female, with gasket).
- Envelope 6: wrench n.11 + wrench n.22
- Envelope 7: scaffold holder

- Envelope 8: 4 small screws for scaffold holder + hex screw n.2
- Envelope 9: bioreactor head + 6 screws with 6 washer + hex screw n.5
- Envelope 10: manometer support with gasket + wrench n.18
- Envelope 11: tower with short thread (with gasket) + cannon (with gasket) + 2 luer lock adapter (female, with gasket)
- Envelope 12: 1 reservoir + 1 cap + 3 male luer lock.
- Envelope 13: under pump tube + 2 male luer lock.
- Envelope 14, 15, 16: 3 oxygenator tubes (three different envelopes) + 4 female luer lock + 2 male luer lock + 1 female-female luer lock.
- Envelope 0: this is an envelope for emergency if any component loses its sterility or is broken. Fill it with luer lock, gaskets and gray metal grid.

EXTRA MATERIAL

- 1 syringe with central hole
- 1 HEPA filter
- 1 pair of sterile gloves

Figure 6a Material

PROCEDURE

BEFORE STERILIZATION

- Connect luer lock to each tube.
- The shortest tube (female+female) connects the under pump tube (male+male) to the reservoir (male+male+male).
- One of the longest tube (male+female) connects the under pump tube (male+male) to the lowest bioreactor tower (female). The other tube (male+female) connects the reservoir (male+male+male) to the highest bioreactor tower (female).

ASSEMBLY OF THE CULTURE CHAMBER

- Assembly must be done by two operators under micro-flow biological safety cabinet. One with sterile gloves (“MAIN” operator) and one with normal gloves (“SUPPORT” operator) who has the task to open the sterile envelopes and pass them to the main operator (Fig.7).

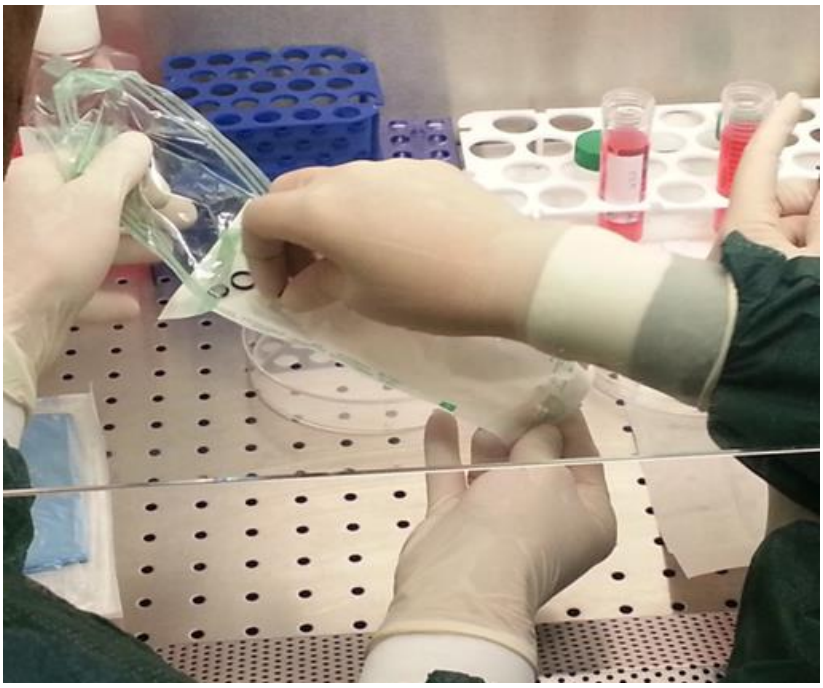


Figure 7

- Wear sterile hand gloves
- Leave the sterile packaging paper of the gloves on the workplace (Fig.8).
- The support operator places the piston base (not sterile) on the workplace.

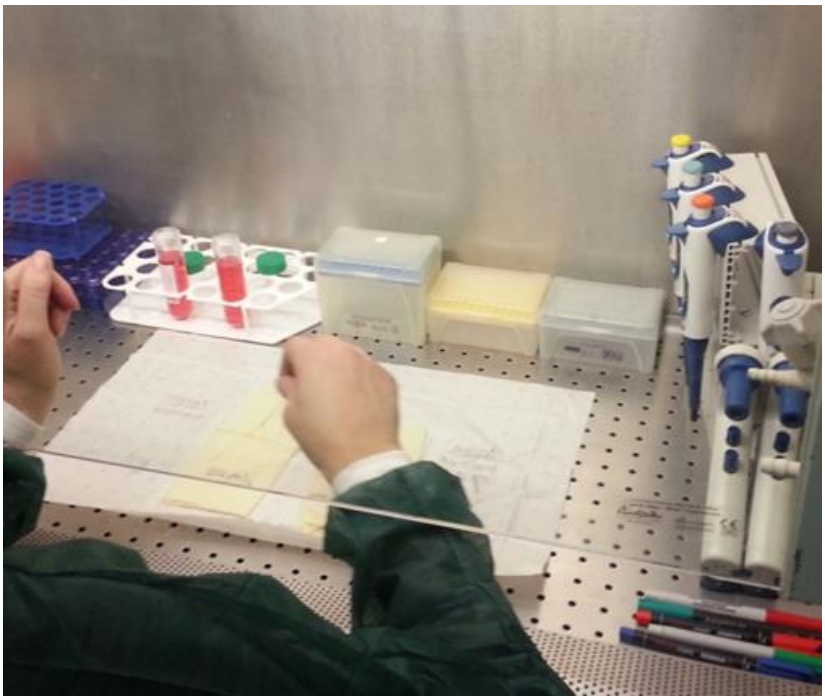


Figure 8

- Extract the sterile components from **envelope 1** (Fig.8).

Figure 9

- Insert the piston on the top of the piston base and screw it using wrench n.9 (Fig.9).
- The support operator must hold the piston base which is not sterile while the main operator tightens the piston (Fig.10).

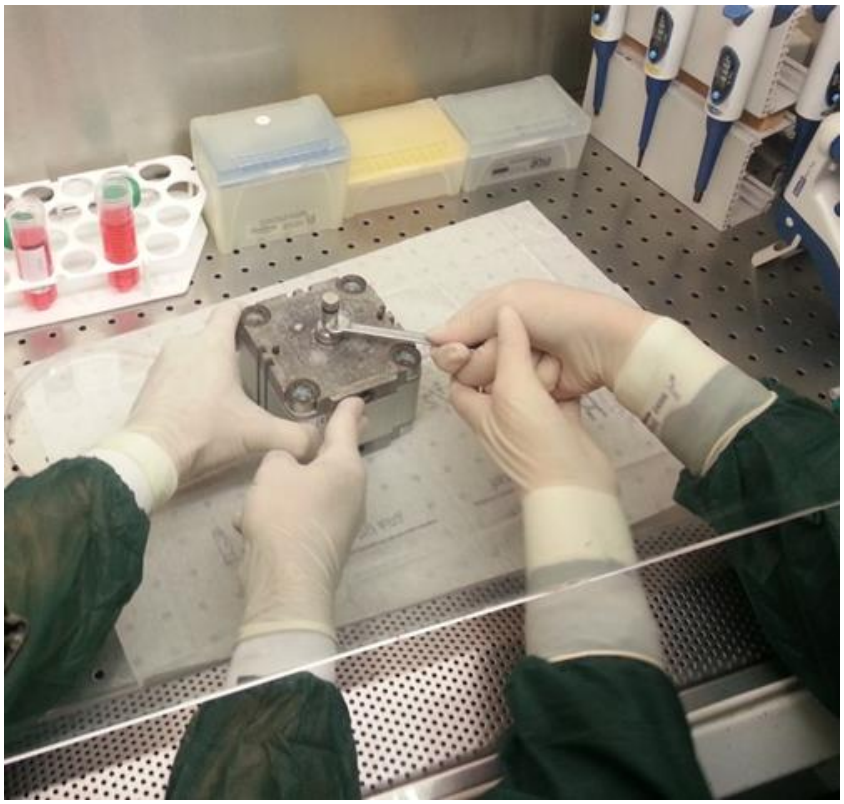


Figure 10

- Extract the sterile components from **envelope 2 and 3**. Place the bioreactor base on the piston base. Align on the same side the air pressure inlet hole of the piston base and the hydraulic inlet hole of the bioreactor base (Fig.11).
- Insert the 4 big screws and start screwing them manually and then tighten them with hex screw n.8 acting contra laterally.

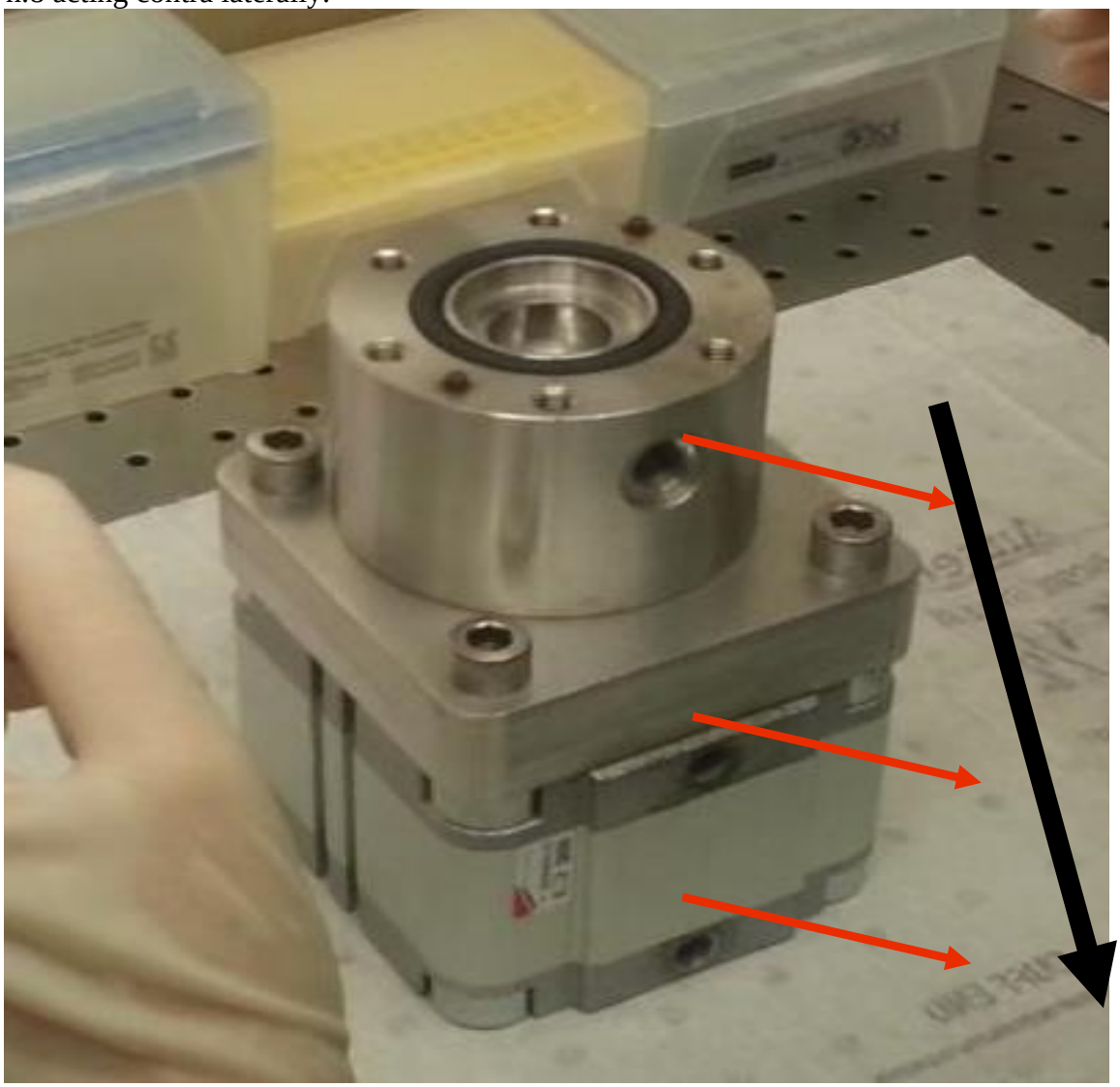


Figure 11. Holes alignment.

- Extract the sterile components from **envelope 4**.
- Insert the big gasket inside the bioreactor base (Fig.12).

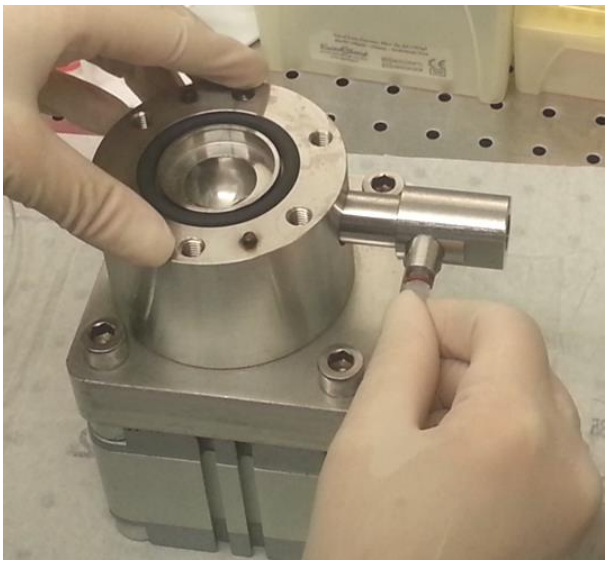


Figure 12

- Extract the sterile components from **envelope 5**.
- Screw the tower (verify that you are applying the one with longer thread) into the bioreactor base. Then screw the cannon to tower and the luer lock using wrench n.8 for the luer lock and wrench n.10 for the cannon. (n.8 from **envelope 1** and **n.10** from **envelope 6**).
- Check if all the gaskets are in place (Fig.12).
- Fill bioreactor base with approximately 10 ml of culture medium.
- Recline the structure while injecting to help de-bubbling (Fig.13).

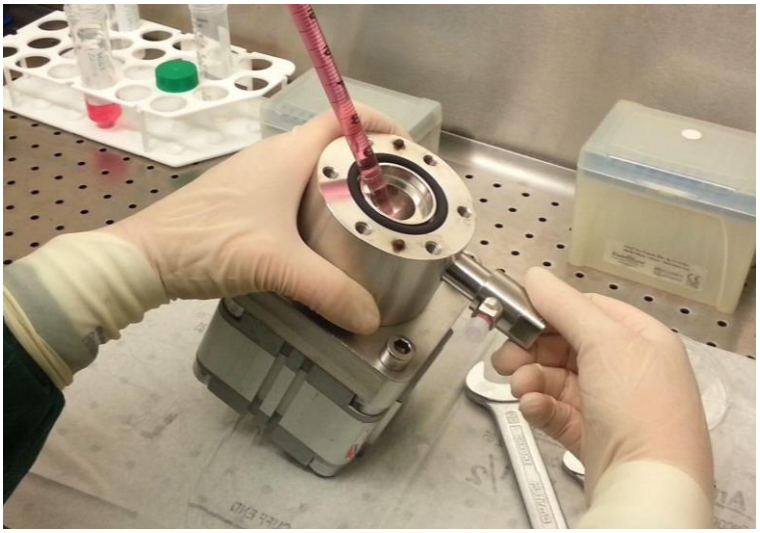


Figure 13

NOTE: If you intend to use cell pellets follow this point, otherwise skip and proceed above the figure 14**.**

- Extract the sterile components from **envelope 7** and **envelope 8**.

- Assemble the scaffold holder.
- Take the bottom of the scaffold holder and lay the first gray metal grid on the top of it (to avoid cells leaking during perfusion).
- Lay the central part of the scaffold holder on the bottom part and screw the 2 small screws with hex screw n.2 (envelope 8). Align the screw holes on the bottom scaffold holder.
- Insert the cell pellets in each of the 12 wells (of volume 21 mm^3 each).
- Place the top part of the scaffold holder on the top of the second gray metal grid.
- Fix it with the last 2 small screws using hex screws n.2 (Fig.14).
- Insert the scaffold holder into the bioreactor base (Fig.14).

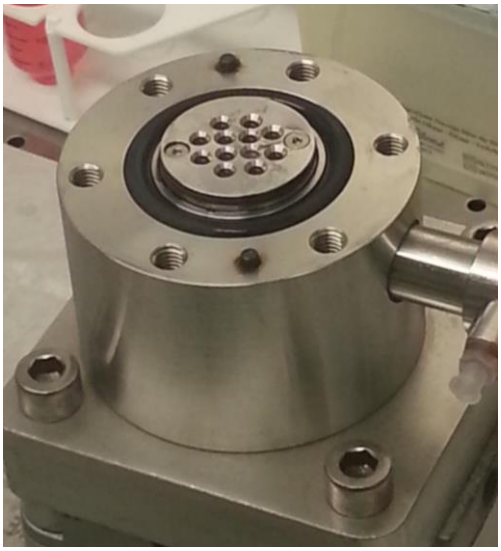


Figure 14

- Extract the sterile components from **envelope 9**.
- Place the bioreactor head on the bioreactor base (align the inlet hole properly – Fig.15).
- Insert its 6 screws, each with a screw ring.
- Start screwing the screws manually and then tighten them with hex screw n.5 acting contra laterally (Fig.15).

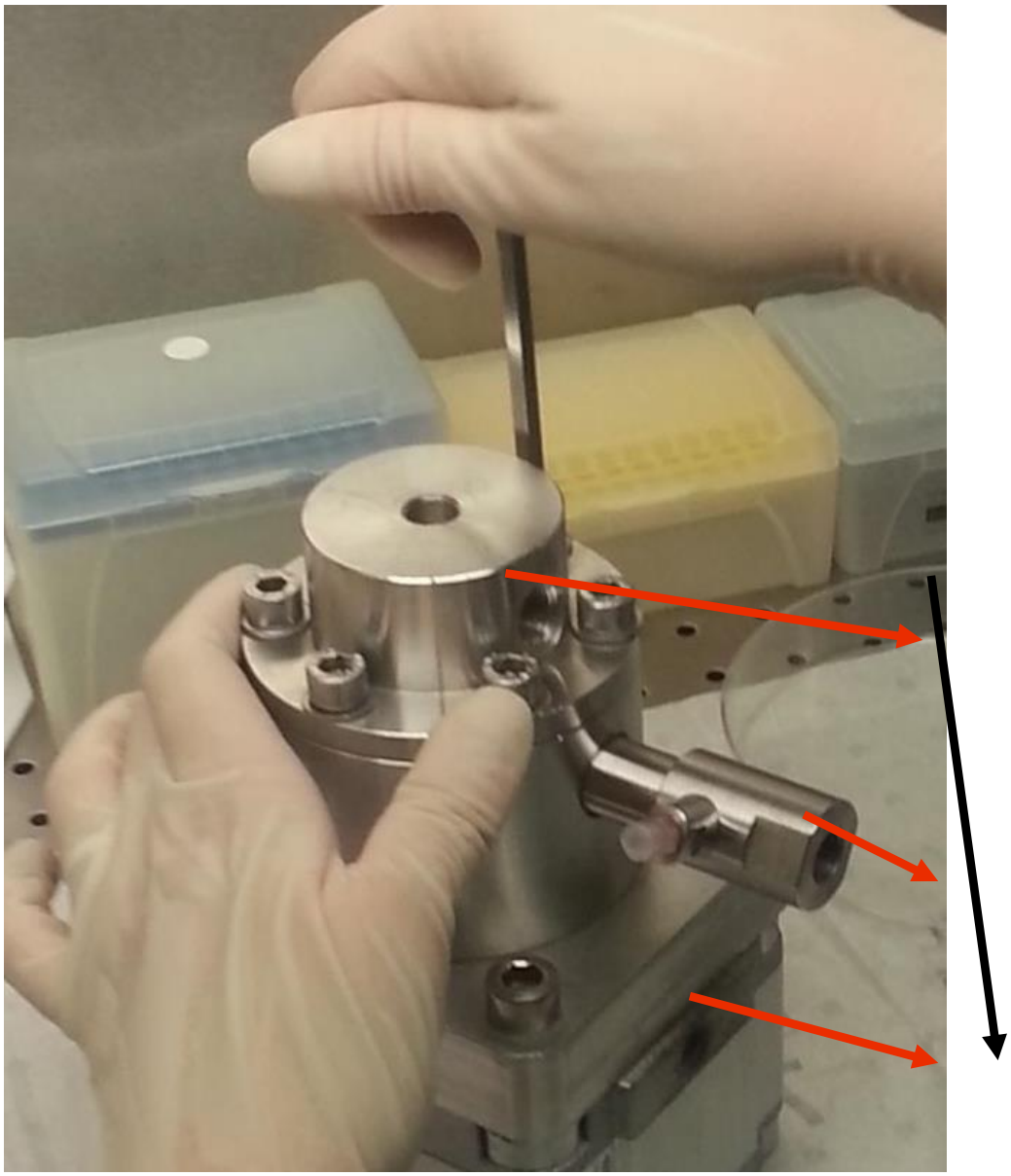


Figure 15. Align holes on the same side.

- Extract the sterile components from **envelope 11**.
- Screw the tower with short thread into the bioreactor head (Fig.16).
- Then insert the cannon and its luer lock using wrench n.8 for the luer lock and wrench n.10 for the cannon.
- Check if all the gaskets are in place (Fig.16).

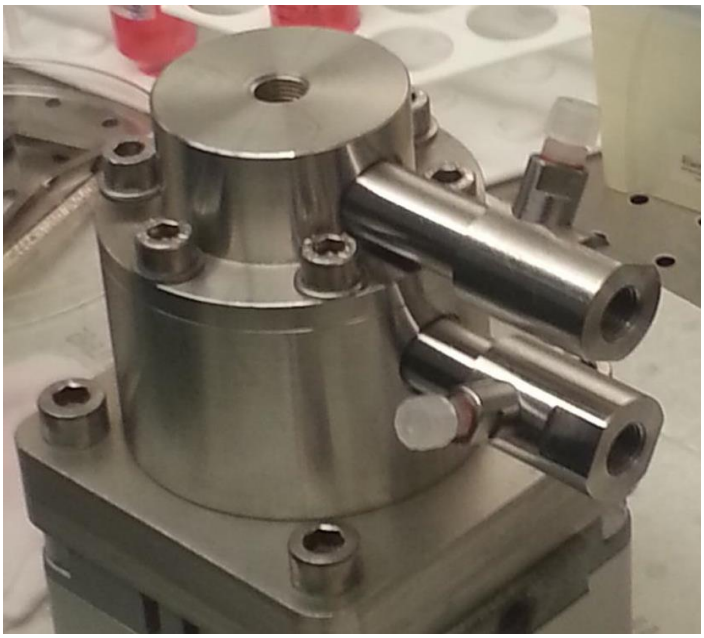


Figure 16

- Extract the sterile components from **envelope 10**.
- Insert the manometer support on the top of the bioreactor head and screw it with wrench n.18.
- Inject around 15ml of culture medium in the bioreactor (Fig.17).

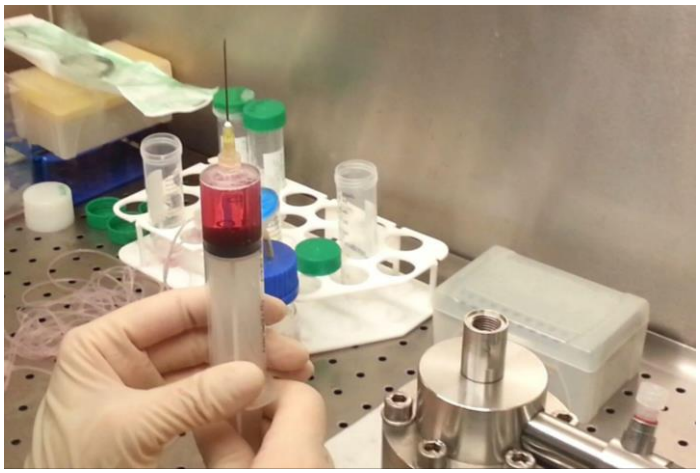


Figure 17

- Screw the manometer on the manometer the support and tighten it manually or using wrench n.18 (Fig.18).

Figure 18

ASSEMBLY OF THE PERFUSION CIRCUIT

- Fill reservoir with about 50 ml of culture medium.
- Close the reservoir with its cap and connect one HEPA filter (0,22 μm).

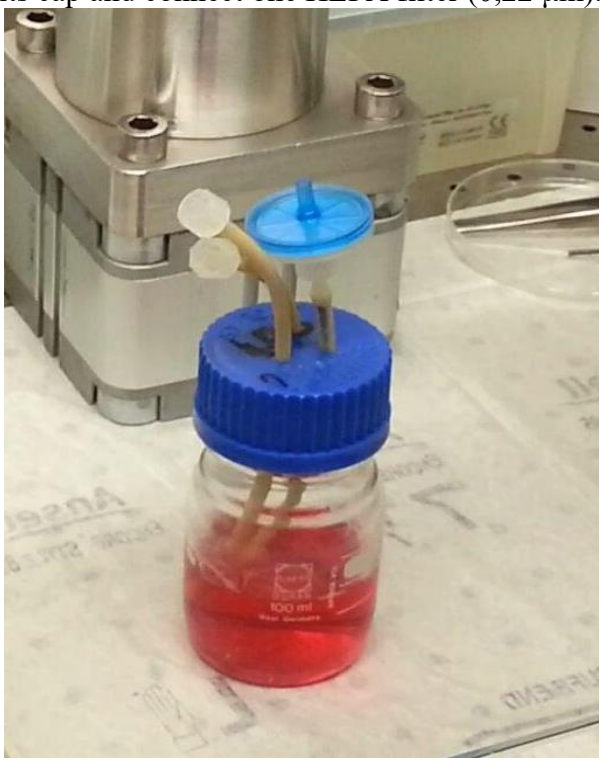


Figure 19

- Take the shortest oxygenator and connect one extremity to the under pump tubing and the extremity to the reservoir.
- Take the other two oxygenators tubes and connect them through the female-female luer lock.
- Connect the long oxygenator to the pump tubing and the other oxygenator tube to the reservoir.
- In this way, you will have a closed circuit excluding the bioreactor.
- When switching on the pump, it will be possible to fill the oxygenators with culture medium.
- Once the tubes are filled, disconnect the two oxygenators.
- The oxygenator connected to the pump tubing must be connected to the lowest bioreactor tower.
- The oxygenator connected to the reservoir must be connected to the highest bioreactor tower.

AT THIS POINT THE PERFUSION CIRCUIT IS SEALED.

- Connect the high pressure valves to the two bioreactor towers.
- Manually screw the actuator to the lowest hole of the piston base.
- Switch on the bioreactor control unit, the compressor and the pump.
- Transfer the bioreactor inside the incubator (Fig.20).

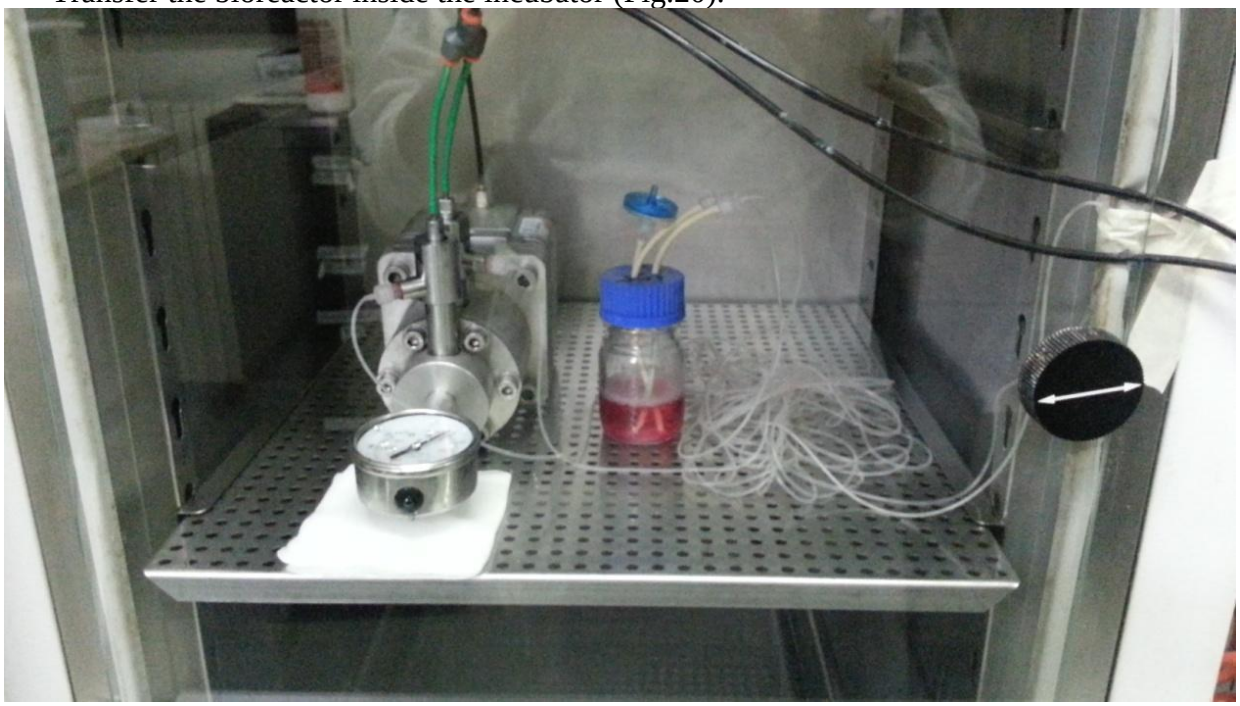


Figure 20

- On the control unit display move the cursor down using the arrows until “Start” and press the button “ok”
- **START:** After the control unit is activated, press the button “Start” on the pump.

NOTE

The manometer pressure sensor, is disinfected with 70% Ethanol before assembly.

In general, every time an envelope with hand tool is opened, place the tool on a sterile petri dish to be re-used during the assembly (Fig.3b).

It is better to use syringe with central needle to remove the bubbles from it (Fig.11b).

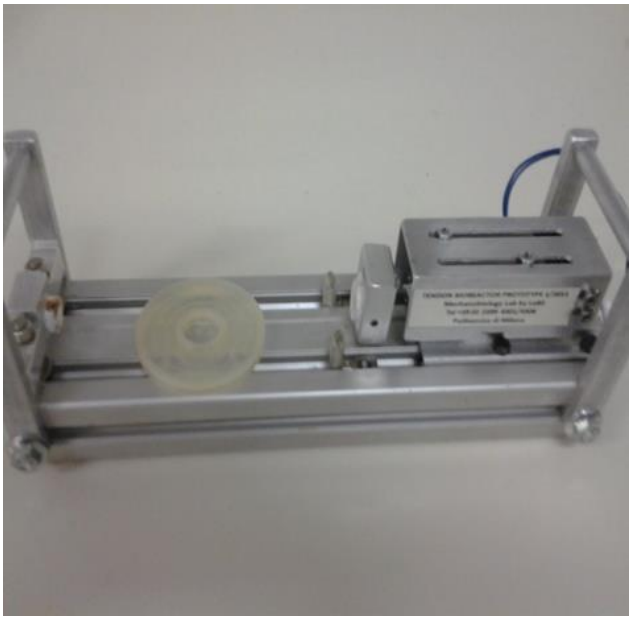
REFERENCES

Laganà K, Moretti M, Dubini G, **Raimondi MT**. A new bioreactor for controlled application of complex mechanical stimuli for cartilage tissue engineering. Proceedings of the Institution of Mechanical Engineers, Part H, Journal of Engineering in Medicine. 2008. 222(H5):705-715, ISSN 0954-4119, DOI:10.1243/09544119JEIM383

BIOREACTOR FOR 3D TISSUE CULTURE UNDER PULSATILE STRETCH

DESCRIPTION

The bioreactor applies a cyclic strain stimulation on cell constructs in order to encourage the production of bioartificial tissue with mechanical and biochemical properties as more similar to those of native tissue, in particular, this bioreactor was born to engineer tendon tissue *in vitro*.



MATERIAL

Petri dish (PS, Ø= 15 cm);

Scaffold stretch unit (sterilized with Sterrad®. Envelope 1);

External supporting structure for scaffold stretch unit;

Electronic devices with display;

Forceps (sterilized in autoclave, 121°C, 1 bar, 24 minutes. Envelope 2);

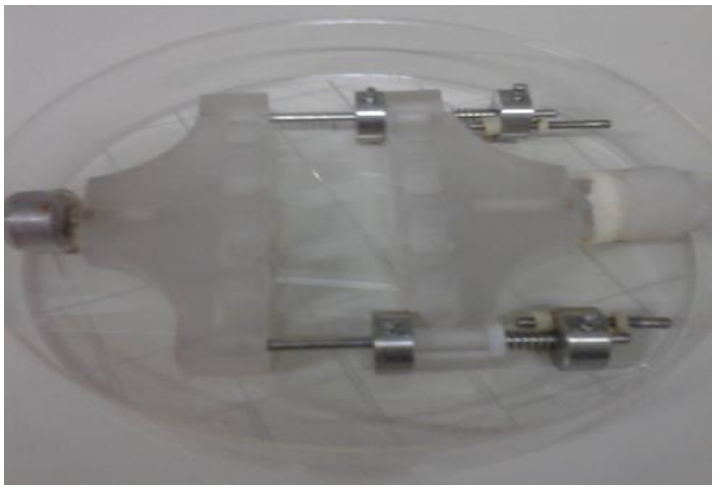
Max 4 cell-seeded scaffolds (resistant to traction, e.g. collagen fibres, etc);

Max 8 holders (PTFE, sterilized in autoclave, 121°C, 1 bar, 24 minutes. Envelope 3);

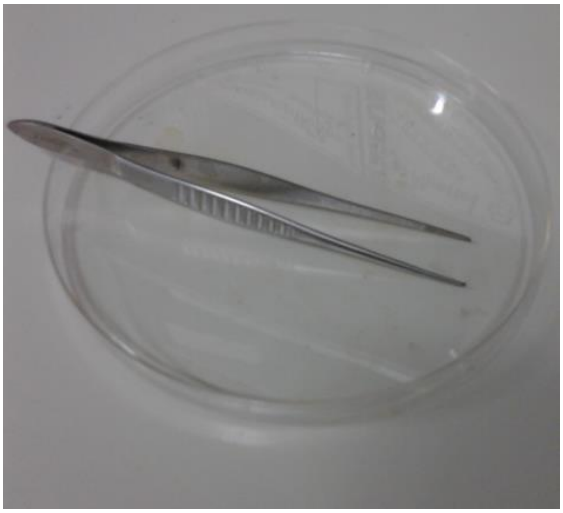
Complete Medium (60 ml);

Rubber support (see Other Unsterilized Materials figure).

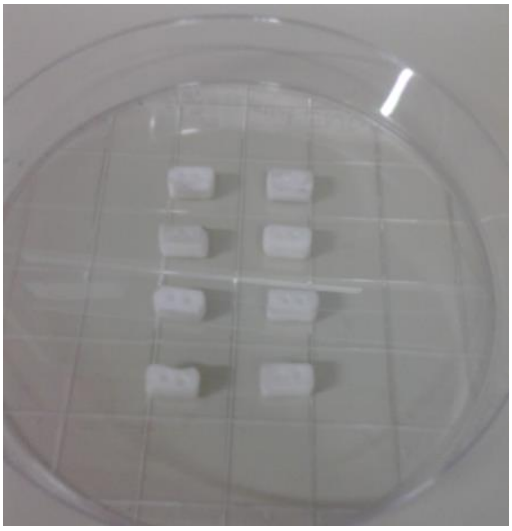
STERILIZATION



Envelope 1



Envelope 2



Envelope 3

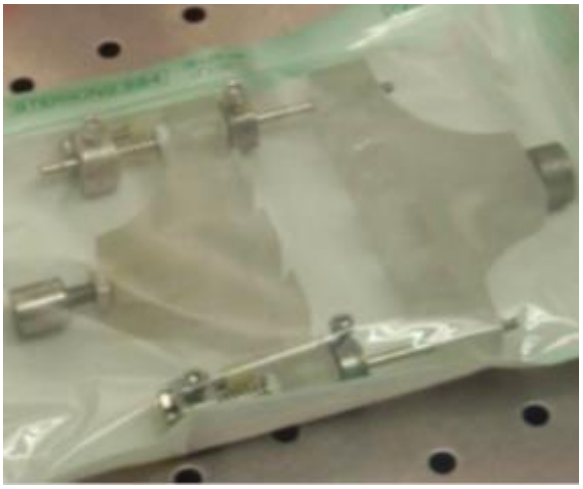
PROCEDURE

Use sterilized glows and work under biological safety cabinet

Open a sterile Petri dish maintaining sterility also of the dish cover (Fig.4).

Figure 4

Pull the scaffold stretch unit out from its envelope and place it in the Petri dish in sterile conditions (Fig.5).



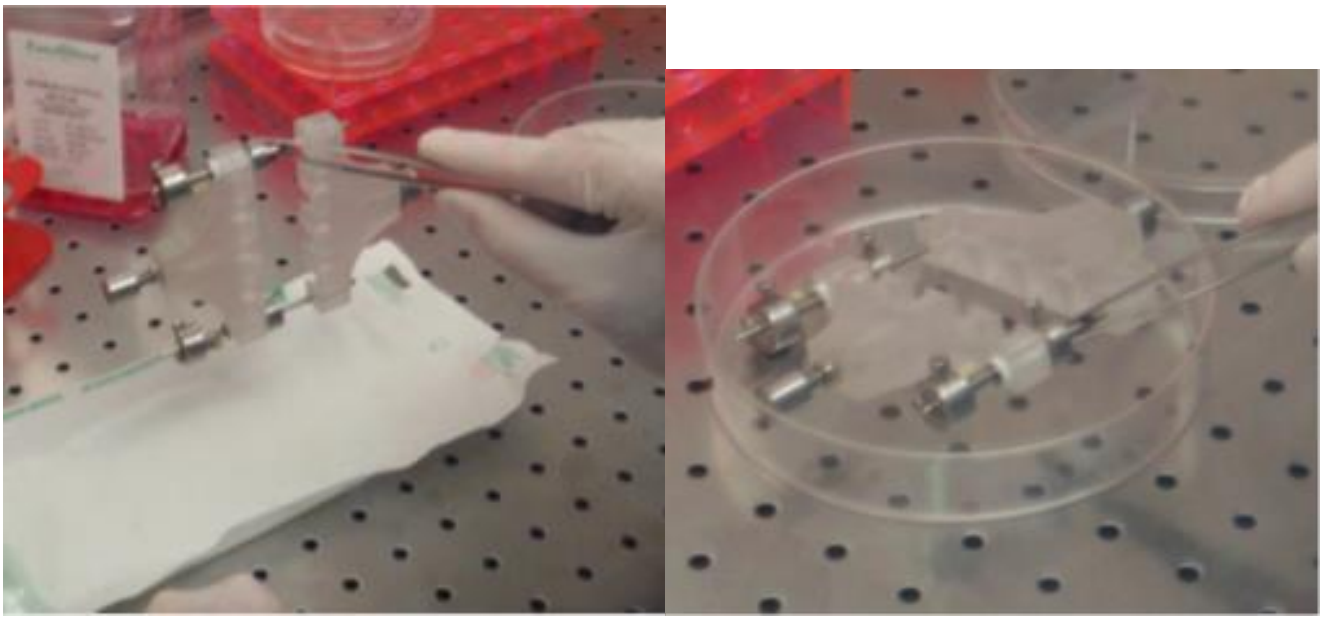


Figure 5

Take a cell-seeded scaffold from the culture medium with sterile forceps and place it extended from one housing to the opposite, being careful not to break it (Fig. 6).

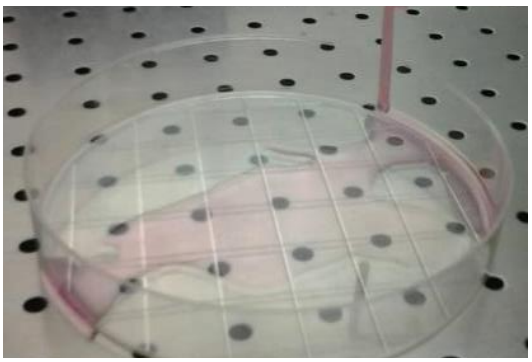


Figure 6

Take a PTFE holder by inserting the tip of the forceps into its top holes and insert it at one end of the scaffold (Fig.7).

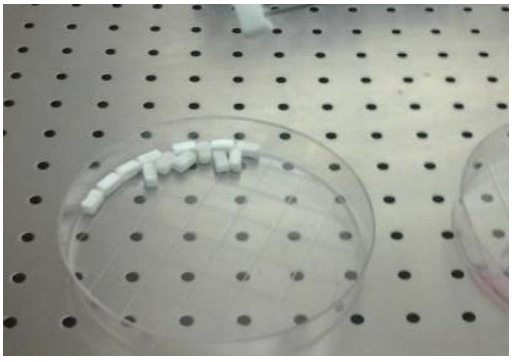


Figure 7

Firmly press down the holder with the help of the sterile forceps until it completely wedges into the cavity (Fig.8).

Don’t lean the forceps outside of the sterile zone.

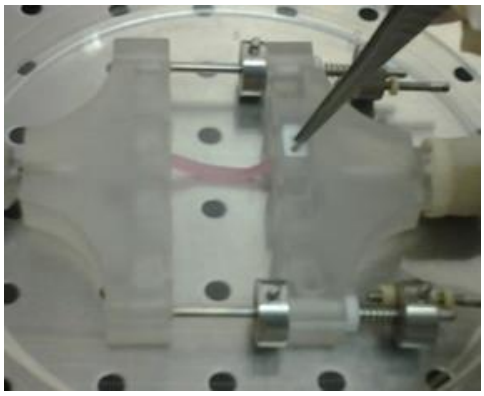


Figure 8

Repeat the procedure to fix the other end of the scaffold to the scaffold stretch unit.

Repeat the procedure to house other scaffolds (maximum 4 scaffolds).

Fill the Petri dish with culture medium up to a maximum thickness of 1 mm above the superior surface of the scaffold (use approximately 60 ml of culture medium for a 15 cm diameter Petri dish).

Do not employ more medium because this causes splurts during bioreactor operation.

Do not employ a smaller volume of medium, to avoid ipoxia (Fig.9).

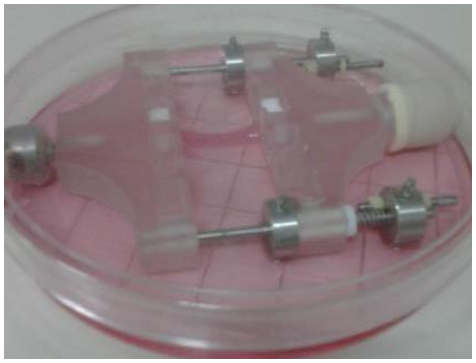


Figure 9

Place the rubber support on the supporting structure.

Close the Petri dish with its cover, extract it from the safety cabinet and put it on the rubber support. The largest magnet of the scaffold stretch unit shall be interfaced to the actuation magnet.

The smallest magnet of the scaffold stretch unit shall be interfaced to the fixed magnet.

A proper alignment of the magnets is crucial to exercise the proper traction force on the scaffolds (Fig.10).

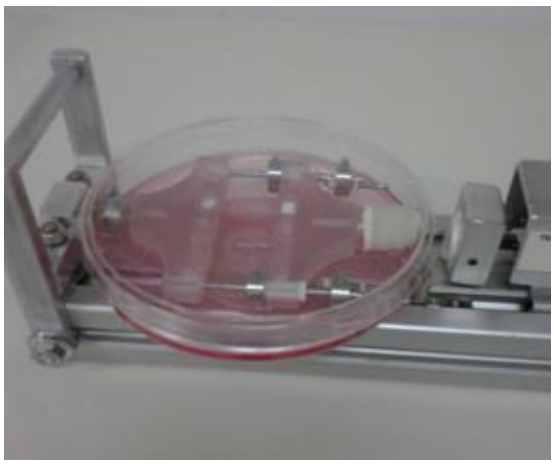


Figure 10

Connect the bioreactor control device to the external structure and connect the power line.

Turn on the electronic instrumentation using the “ON/OFF” switch located on the side of the control device (Fig. 11).

Set stimulation parameters pressing down “SET” repeatedly until the desired value of frequency is reached.

Confirm with “SAVE”.

Repeat the procedure to set the duration of the stimulation.

Start the stimulation with “START”.

To stop the stretching actuation for a while, e.g. to manually change the medium, press “PAUSE”. To definitively stop the actuation press “STOP”.

Figure 11

MICROFLUIDIC BIOREACTOR FOR 3D TISSUE CULTURE WITH OPTICAL ACCESSIBILITY

DESCRIPTION

Standard set-up procedure for a Mini-bioreactor with controlled perfusion system and O_2 sensor. The Bioreactor (Materials: glass and PDMS) and Scaffold (Material: PET) are highly transparent allowing an easy inspection. Magnets are a useful system to open and close the Bioreactor any time in an easy way. The O_2 sensor is a technologically advanced system for O_2 real time consumption monitoring.

MATERIALS (sterilized, *2 pieces each + 1 in case of emergency):

Oxygenators*

Reservoirs* (small glass bottles with tubes)

Syringe Filters [0.22 µm]*

Forceps

Syringes ($N°_{syringes} = N°_{perfusion\ chambers}$)

Bioreactor

Petri dishes (Ø= 15 cm)

Pipette's Tips

Oxygen Sensor (disinfected, in Petri dish)

Seeded Scaffolds (Multiwell, extract only after point 5)

Glass Slide (PDMS 20:1, "Large&Long")

Sterile Gauze

Tubes: Oxygenator (EVA), Long, Short

Complete Medium

Sterile Needle

Figure 1

PROCEDURE

Use non sterile gloves.

FILL THE SYRINGES WITH COMPLETE MEDIUM (fig.2)

In case of 30 ml Syringes (not fitting in Falcon) previously set up the Oxygenator tube.

Otherwise fill first the Syringe and then connect the Oxygenator tube.

Use sterile forceps to extract the tube.

Protect the long tube from contamination using the Petri dish.

Avoid bubbles.

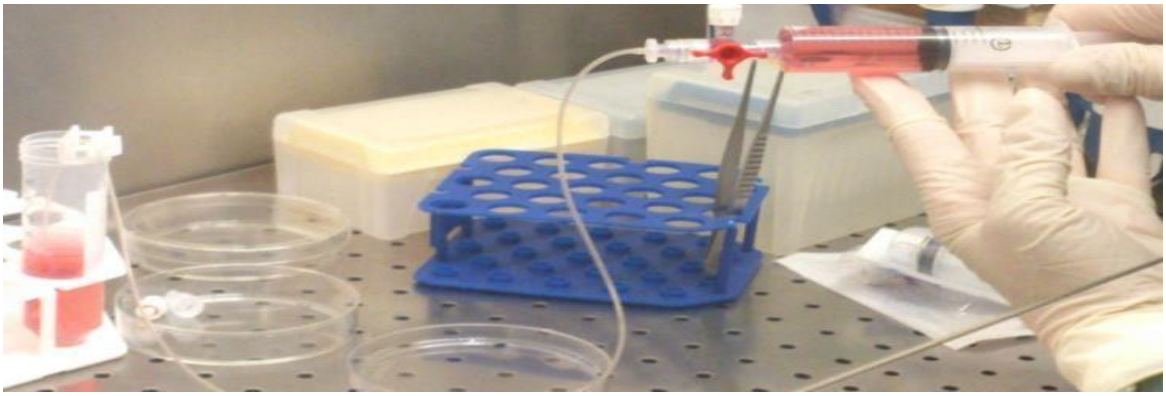


Figure 2

CONNECT BYPASS TUBE AND PERFUSION TUBE TO SYRINGE (Fig.3)

Protect the long tube from contamination using the Petri dish.

Use sterile forceps to extract the tube.

Connect Filter to Reservoir.

First shut the Reservoir (sterilized open).

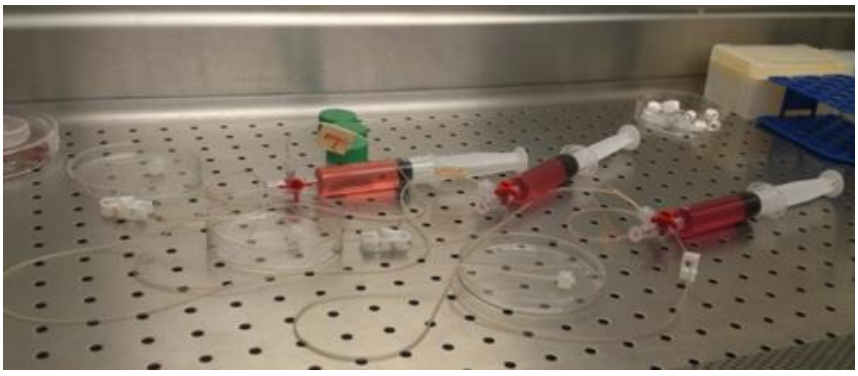


Figure 3

CONNECT BYPASS TUBE TO RESERVOIR (Fig. 4)

Figure 4

PUT THE BIOREACTOR IN PETRI DISH

PUT COMPLETE MEDIUM IN BIOREACTOR CHAMBERS (Fig. 5)

Figure 5

Use the Micro-Pipette (50 µl each chamber).

Fill the duct avoiding bubbles.

SCAFFOLD INSERTION IN BIOREACTOR (Fig. 6)

One for each chamber.

Avoid Medium leakage.

Figure 6

OXYGEN SENSOR INSERTION IN BIOREACTOR (Fig. 7)

Opaque side in contact with scaffold (operator should distinguish a glossy side and a opaque side).

Use a sterile Needle and Forceps to pick up the Sensor (**DO NOT** clamp the Sensor with the Forceps).

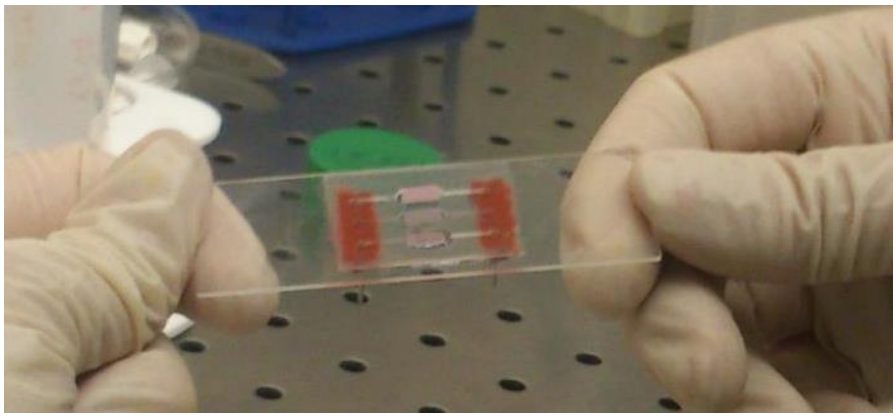


Figure 7

OVERLAP THE PDMS (20:1) GLASS SLIDE

PDMS side facing the culture chamber.

PLACE THE MAGNETS TO SHUT THE BIOREACTOR (Fig. 8)

Caution: Magnets are not sterilized (Autoclave damages the magnets).

Don’t touch metal connections.

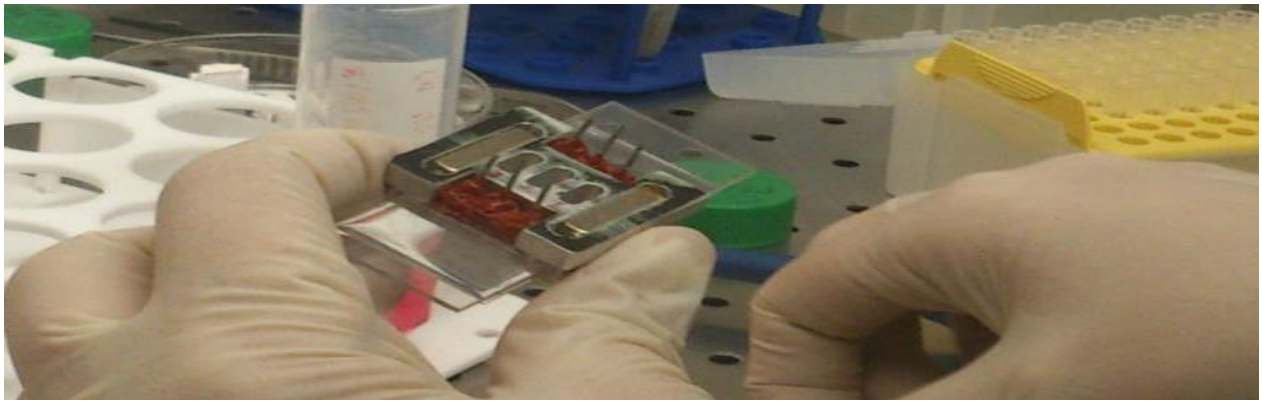


Figure 8

CONNECT SYRINGES (LEFT SIDE) AND RESERVOIRS (RIGHT SIDE) TO THE BIOREACTOR (Fig. 9)

Close clamps avoiding unwanted flows through cells

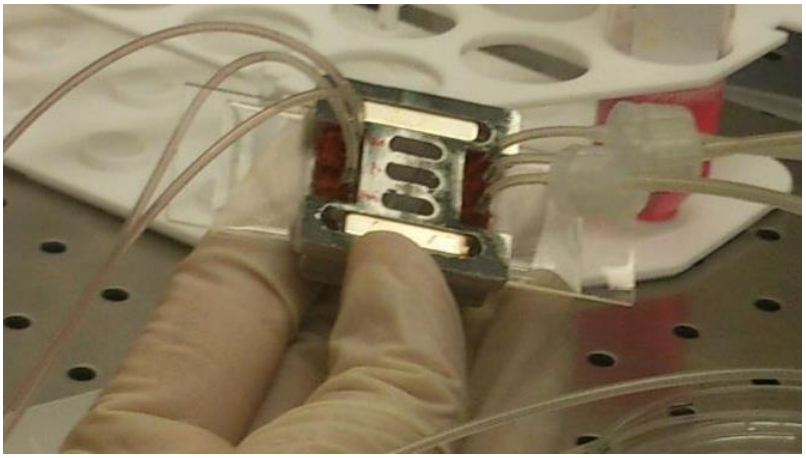


Figure 9

POSITION THE SYSTEM IN THE INCUBATOR (Fig 10, 11)

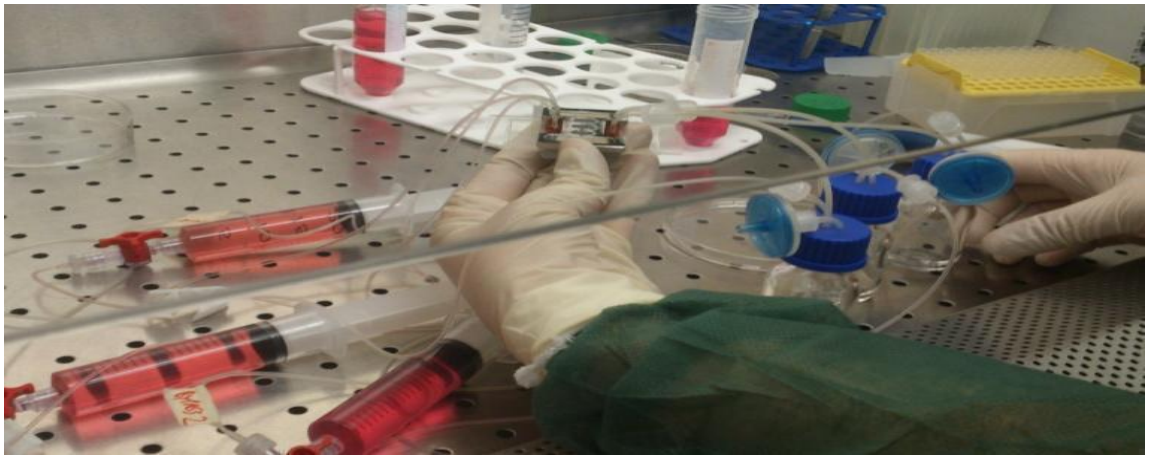


Figure 10

Place Syringes on the pump

Set acquisition system for O_2 sensor.

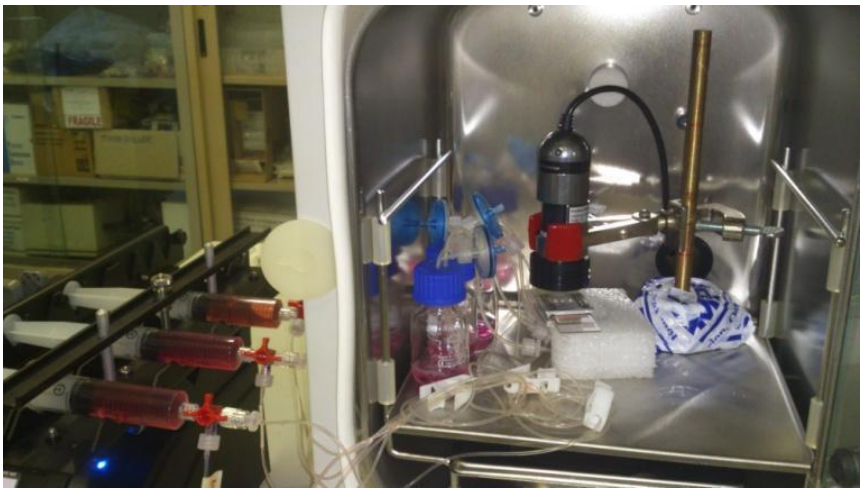


Figure 11

MICROBIOLOGY PROCEDURES

PREPARATION OF AGAR-ENRICHED WITH BACTERIAL MEDIUM

CHEMICALS AND REAGENTS

Reagents	**Quantities**	**Storage**
Agar	25 mg/ml	Room temperature
Bacterial medium	Defined by the supplier	Room temperature

EQUIPMENT, GLASSWARE AND CONSUMABLES

Material	**Storage**
Autoclavable bottles with hermetic caps	locker
Petri dishes 60 x 15 mm	shelf

PROCEDURE

- Prepare a solution of 1.5% agar in ddH2O. Add the specific medium for the bacterial culture (for example LB or MH) to the agar solution in a quantity defined by the supplier (for example, 2.5% LB when supplied by Sigma Aldrich)
- Autoclave the agar enriched with specific medium
- After autoclaving, shake the liquid agar solution enriched with medium
- Pour 7-8 ml of the medium-enriched agar solution into 60x15 mm Petri dishes
- Place the Petri dishes under the hood with the cover slightly shifted, thus preventing condensation during agar gelation.
- Once gelation is completed, close and turn upside down the Petri dishes with agar to prevent condensation during storage
- Store the Petri dishes in the fridge (upside down).

INOCULUM

CHEMICALS AND REAGENTS

Reagents	**Quantity**	**Storage**
Bacterial culture medium	10 ml	Fridge
Bacterial strain (vial)		Freezer -80 °C

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**Storage**
Centrifuge tubes (50 ml)	Locker
Loops for microbiology	Locker
Vial holder	Freezer -80 °C

PROCEDURE

- Take a frozen vial and carry under a hood in a refrigerated vial holder.
- With a loop, scratch frozen bacteria and suspend in 10 ml liquid medium.
- Repeat this procedure 3 times.
- Stir the inoculum overnight at 37 ° C.

BACTERIAL CULTURE ON GEL

CHEMICALS AND REAGENTS

Reagents	**Quantity**	**Storage**
Bacteria inoculum	10 ml	incubator
Bacterial medium	10 ml	fridge
Ethanol		Safety cabinet

EQUIPMENT, GLASSWARE AND CONSUMABLES

Material	**storage**
spectrophotometer	shelf
Strain-specific growth curve	wardrobe
ruler	wardrobe
96-well microplate, with hydrogels	fridge
micropipettes	locker
Tips	locker
cuvettes	locker
Eppendorf tubes	locker

PROCEDURE

The bacteria to be seeded are those inoculated the day before and left at 37 ° C overnight.

For instance, in the case of *E. coli*, when bacteria reach a sufficiently high concentration, the suspension turns green in the case of LB and turbid yellow in the case of DMEM.

We consider the final concentration of bacteria that is desired (for example 10^4 bacteria /ml), or a seeding of 1000 bacteria (every 100 µl).

- The absorbance (OD) of the suspension at 600 nm must be measured to count the bacteria.

- For the spectrophotometer analysis, use glass cuvettes. These cuvettes are reused every time; hence, before each use they must be washed with ethanol. After use, bacteria are thrown away, while the cuvettes are soaked in bleach.
- For the spectrophotometer analysis, prepare 3 eppendorf tube:
  - 1 eppendorf tube with more than 1 ml of inoculum.
  - 1 eppendorf tube to make a 1:10 dilution of the bacteria (100 µl bacteria + 900 µl of medium)
  - 1 eppendorf to make a 1:20 dilution of the bacteria (50 µl bacteria + 950 µl of medium)
- Prepare 4 cuvettes:
  - 2 blanks filled with 1 ml of pure medium
  - 1 filled with 1 ml of the 1:10 dilution
  - 1 filled with 1 ml of the 1:20 dilution

- Insert the blanks cuvettes into the spectrophotometer. Press Autozero and repeat 3 times. Check that the OD readings are close to 0.
- A cuvette is replaced with one of the samples and the OD is read.
- When finished reading, the software is closed and the spectrophotometer is turned off.
- To calculate the concentration of the bacteria, take the calibration line calibrated on the instrument. On the y axis there is the concentration of bacteria (in the order of 10^8), while in the x axis there is the OD. For instance, assuming that we have measured an OD of 0.2334 (for the 1:10 dilution), we consider only the OD interval containing the measured value, i.e. 0.2 and 0.3. Taking the ruler, we measure that the distance from 0.2 to 0.3 is 4 cm. Hence, having measured 0.2334, we want to know how many centimeters there are from 0.2 to 0.2334. Making the proportion, one has: X=1.336 cm
- X is the length needed to identify the abscissa of a parallel line with respect to the ordinate with which to intersect the calibration line. The zero to define this line is 0.1 if the measured OD value is between 0.1 and 0.2, 0.2 if the value is between 0.2 and 0.3 (as in the example case), etc. the ordinate of the intersection point with the calibration line is the (diluted) concentration of bacteria in the inoculum.
- The resulting value for 0.2334 is $0.88x10^8$ bacteria/ ml. Since we have obtained OD from a 1:10 dilution, we have to multiply $0.88x10^8$ by 10. Therefore, our initial suspension concentration is $8.8x10^8$ bacteria/ml.
- We need to get 10^4 bacteria / ml.
- We make a first dilution to reach 10^8 bacteria/ml (Vfinal: 1 ml. So 114 µl of bacteria + 886 µl of medium).
- We dilute 1:10 to reach 10^7 bacteria/ml (Vfinal: 1 ml. So 100 µl of bacteria +900 µl of medium).
- We dilute 1: 100 to reach 10^5 bacteria/ml (Vfinal: 1 ml. So 10 µl of bacteria +990 µl of medium).
- We dilute 1:10 to arrive at 10^4 bacteria/ml (Vfinal: 5 ml. Then 500 µl of bacteria + 4500 µl of medium).
- Take the 96- well microplate containing the gels.
- 100 µl from the eppendorf tube with 10^4 bacteria/ml are seeded on each well with the hydrogels. Wells with plankton bacteria (floating forms of bacteria) are also seeded, i.e. control wells that do not have gels. The bacteria are poured directly on the plate.
- The 96-well microplate is placed in an incubator at 37 ° C for 24 h.

COUNT OF THE COLONY FORMING UNITS (CFU)

CHEMICALS AND REAGENTS

Reagents	**Quantity**	**Storage**
Sodium citrate 50 mM	10 ml	fridge
Sterile 0.9% NaCl solution in ddH_2O	50 ml	fridge
Bacterial medium	10 ml	fridge

EQUIPMENT, GLASSWARE AND CONSUMABLES

Materials	**storage**
vortex	shelf
96-well microplates with infected hydrogels	fridge
Micropipettes and tips	locker
Eppendorf tubes	locker
Petri 60x15 cm with agar enriched with bacterial medium	fridge
Loops for microbial culture	locker

- Take the plate containing the infected gels and the planktonic control bacteria. The bacteria must be seeded on the medium-enriched agar plates only after they have been diluted appropriately.
- Perform a progressive dilution of the suspension. Vortex, at each dilution to homogenize the suspension.
- If the gel is present, eliminate the supernatant. Dissolve the gel without damaging the bacteria. In this case, it is sodium citrate (150 µl, 50 mM). Wait for 2 minutes. Then, take the suspension (remember to have altered the initial volume).
- The serial dilutions are:
  10^{-2} = 10 µl of bacteria suspension from the well (fine pipetting) + 990 µl NaCl 0.9%

  10^{-4} = 10 µl mix 10^{-2} + 990 µl NaCl 0.9%

  10^{-5} = 100 µl mix 10^{-4} + 900 µl NaCl 0.9%

  10^{-6} = 100 µl mix 10^{-5} + 900 µl NaCl 0.9%

10^{-7} = 100 µl mix 10^{-6} + 900 µl NaCl 0.9%

- From the dilutions to be seeded, take 10 µl and distributed homogeneously on the agar Petri with the loop. The choice of dilutions must be made empirically. The more vital the starting suspension is, the more it is recommended to seed only high dilution. For example, in the case of gels infected with *E. coli*, 10 ul are taken from the 10^{-5} and 10^{-6} dilutions (final dilution 10^{-7} and 10^{-8}, respectively). Differently, 10 ul are taken from the 10^{-6} and 10^{-7} dilutions in the planktonic controls (final dilution 10^{-8} and 10^{-9}, respectively).
- After pouring 10 ul of the desired dilution on the agar-enriched dishes, the loops are used to homogeneously spread the drop on the surface.
- Each dilution is seeded in duplicate (technical duplicate).
- At the end of the seeding, place the dishes in an incubator at 35 ° C. Move the dishes to the incubator at the last available moment of the day and take the next day, as soon as possible. This is done to allow the colonies growing and be visible, but not overlapping one to the others and become difficult to count.