



Ten commandments for preventing contamination of primary cell cultures

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Abstract. Procedures for preventing contamination in primary cell cultures must be carefully defined and strictly followed in order to obtain healthy cells. Protocols have been developed and refined in our laboratory for establishing primary cultures of muscle and fat stem cells without contamination from a variety of animals. Contamination of cell cultures is not only frustrating, but is also very expensive both

in time and loss of materials. Through the consistent use of proper aseptic techniques, most instances of contamination may be avoided. We suggest that the basic principles detailed here will find wide applicability in the culturing of primary cells without contamination from many different types of animals and tissues.

Key words: Aseptic technique, Cell culture, Contamination, Sterilization, Tissue collection

1. Introduction

Techniques for the successful isolation and culture of muscle [6] and fat stem cells [33] from animal tissue have been developed and refined [7, 9, 10, 12, 28, 31, 32, 34] in this laboratory over the past seventeen years. Through trial and error, success and failure, original protocols have been modified and fine-tuned to minimize deterioration of the fresh tissue and maximize the number and viability of the isolated target cells. Healthy stocks of primary cells have been used for a variety of *in vitro* studies in this laboratory [7–9, 23, 31]. Isolated cells have also been cloned and propagated into cell strains [12, 24, 32], which have become unique tools for characterizing cellular and molecular mechanisms regulating muscle [9, 17, 21, 24] and fat [10, 33, 34] development. As our research has expanded to incorporate a variety of animal species [7, 8, 17, 28], the use of well-tested methods for cell isolation has increased our rates of success in isolating the cells of interest.

From the moment a biological sample is removed from its natural environment within the animal, it is susceptible to deterioration from external factors such as moisture loss and temperature fluctuation [3] and excessive passage of time [15]. Another threat to the success of any cell isolation method is contamination from microbial sources including the host's own natural flora [3]. All phases of the isolation process offer opportunities for contamination to occur unless strict adherence to proper aseptic techniques is consistently followed [3].

Contamination of primary cell cultures can be devastating to subsequent experimentation and is

costly in terms of time and materials [15]. The focus of this paper is to provide information on the isolation and culture of cells, derived from tissue, without contamination (Figure 1). The incorporation of these basic commandments will minimize tissue degeneration, maximize cell yield, and keep the cells viable until they can be plated in culture.

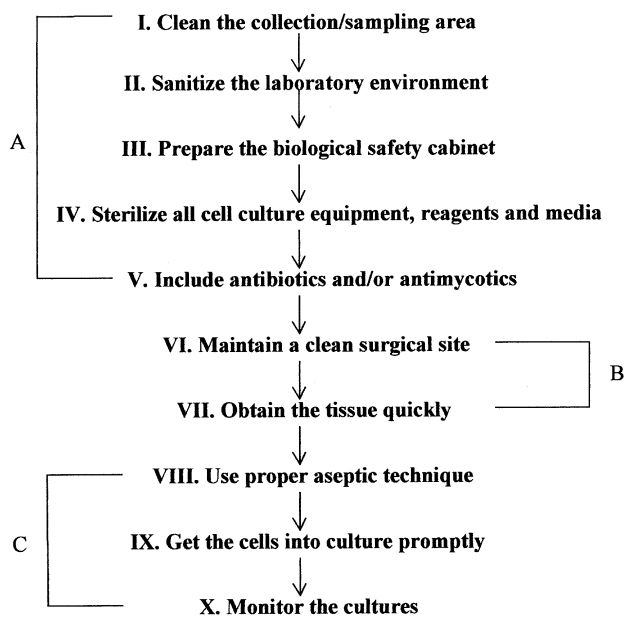


Figure 1. Ten commandments for preventing contamination of primary cell cultures; A) Steps involved prior to surgery/tissue collection; B) Steps involved in collection of tissue from the animal and transportation to the laboratory; C) Steps involved in obtaining the cells from the tissue and placing them into culture.

2. Materials

Materials utilized in our laboratory for the successful and contamination-free isolation of a variety of tissues for subsequent harvest of primary cells are summarized as follows:

A. Major equipment

1. Laminar flow, biological safety cabinet, Labgard Class II, Type A/B3, Model NU-425-4000.¹
2. Autoclave, gravity air removal type, P-89501-091.²
3. Dry heat gravity oven, Model 1370 GM.³
4. Peristaltic pump, Model XX80 200 00.⁴
5. Centrifuge, Model TJ-6.⁵
6. CO₂ water-jacketed incubator, Model NU-4500.¹
7. Water bath, stainless steel, Model 185.⁶
8. Bernz-O-Matic propane torch, TS2000.⁷

B. Disinfectants, detergents, soaps (used in this laboratory)

1. Ethyl alcohol, 200 proof, No. 0000518.⁸
2. Roccal[®]-D Plus, NDC 0009-7308-01.⁹
3. Betadine[®], NDC 0034-2100-01.¹⁰
4. Cliniscrub[®], No. 4471.¹¹
5. Liquinox[®], No. 1201.¹²
6. Calgon Vestal[®] hand soap.¹³

C. Antibiotics/antimycotics (used in this laboratory)

1. Penicillin-Streptomycin, liquid; contains 10,000 units of penicillin G (sodium salt) and 10,000 µg of streptomycin sulfate/ml in 0.85% saline; Product No. 15140-122¹⁴ (refer to Material Safety Data Sheet from manufacturer for information on safe handling of this product).
2. Gentamicin, liquid, contains 10 mg/ml gentamicin sulfate, Product No. 15710-015¹⁴ (refer to Material Safety Data Sheet from manufacturer for information on safe handling of this product).
3. Antibiotic-antimycotic, liquid; contains 10,000 units of penicillin G (sodium salt); 10,000 µg/ml of streptomycin sulfate and 25 µg/ml of amphotericin B in 0.85% saline; Product No. 15240-062¹⁴ (refer to Material Safety Data Sheet from manufacturer for information on safe handling of this product).

D. Filtration devices

1. Filter bells, AcroCap[®], 0.2 µm, Product No. 4480.¹⁵
2. Filter units, 115 ml, 0.2 µm, Product No. 122-0020.¹⁶
3. Syringe filters, Acrodisc[®], 0.2 µm, Product No. 4192.¹⁵

E. Reagents

Phosphate buffered saline (PBS)

1. NaCl: 10.0 g/liter, S-5889.¹⁷
2. KCl: 0.25 g/liter, P-5405.¹⁷

3. Na₂HPO₄: 1.44 g/liter, S-5136.¹⁷

4. KH₂PO₄: 0.25 g/liter, P-5655.¹⁷

3. Procedures and discussion.

The following ten commandments describe protocols for the prevention of contamination of primary cell cultures.

Commandment I. Clean the collection/sampling area. There can be wide variations in the physical properties of the area designated for tissue collection. These extremes can range from a meat processing plant to the surgical suite of a research institution. Opportunities for contamination are greatly increased in facilities with limited environmental controls [3]. Regardless of the dynamics of the surgery facility, proper methods of disinfection must be employed.

Disinfection is the chemical process that destroys infectious agents and pathogens, including bacteria, fungal spores, and viruses [18, 27]. Disinfectants are used primarily on inanimate objects, whereas anti-septics can be used on living tissue [27], although some chemicals may be classified as both a disinfectant and an antiseptic (Table 1). Some disinfectants also have detergent capabilities (Table 1), and all require a specific amount of contact time to be effective [18, 27]. The selection of a disinfectant is determined by factors such as microbial killing efficiency, toxicity to animals, and contact time [18]. The presence of organic matter greatly reduces the effectiveness of a disinfectant, so the area must be cleaned of organic debris before the disinfectant is applied [18, 27]. It is essential to use the concentration recommended by the manufacturer to avoid subsequent contact toxicity to animals [18].

In the worst case scenario, such as a livestock barn or meat processing operation, the surgery site should be cleaned of debris including straw and fecal material. Next, the area should be scrubbed with a detergent/disinfectant such as Cliniscrub[®] or Roccal[®], rinsed with hot water to remove the detergent and organic material, and treated with a disinfectant such as Weladol to eliminate residual bacteria and fungal spores [18]. If a surgery room is the tissue procurement site, all surfaces including floors, tables, and lower walls should be thoroughly cleaned with a detergent/disinfectant, rinsed, and treated with a disinfectant [1, 27].

Commandment II. Sanitize the laboratory environment. In the cell culture laboratory, precautions can be taken to reduce possible sources of contamination prior to cell isolation and cell culturing. The ideal scenario is to have a separate room dedicated specifically to cell culture. Regardless of the physical arrangement of the facility, monitoring the airflow is of primary importance in maintaining a clean

Table 1. Comparison of common disinfectants [13, 18, 27]

Classification	Example	Effective on			Activity with organic matter	Comments
		Bacteria	Fungi	Yeasts		
Phenolics	Lysol® Pine-Sol® Vesphene II®	Yes	Yes	Yes	Good	Stable, remain active after drying Can irritate tissue Strong pine-tar odor
Quaternary ammonium compounds	Roccal® Germex® Zephiran® Conflikt® Coverage Plus®	Yes	Yes	Yes	Reduced	Have detergent action Some are inactivated by soaps Can irritate tissue
Iodophors	Betadine® Weladol® Isodyne® Clinis scrub®	Yes	Yes	Yes	Poor	Low toxicity May stain porous surfaces May corrode instruments Good antiseptics Clinis scrub® has detergent action
Chlorine compounds	Clorox® Chloramine-T® Halazone®	Yes	Poor	Mod	Reduced	Irritating to skin Will corrode metal surfaces Inexpensive
Alcohols	Ethanol Isopropanol	Yes	Yes	Yes	Poor	More useful as antiseptics Become ineffective upon evaporation Use at 70% concentration or above

working environment [15]. It is highly desirable that the overall ventilation system for the room utilize HEPA filters to remove particulate material from the common circulating air [2]. Traffic into and out of the cell culture area should be restricted to prevent air drafts. If windows are present in the room, they must remain closed to avoid the introduction of airborne contaminants. A positive pressure air balance to the room will help reduce dust inflow [15].

Potential sources of microbial contaminants within the tissue handling/processing laboratory should be eliminated. For example, all nonessential equipment should be removed from the cell culture area near the laminar flow hoods. Floors should be swept and then cleaned with a suitable detergent/disinfectant [27] (see Table 1). Supply carts and surfaces of ancillary equipment that will be used during tissue handling should be wiped down with 70% ethanol or another appropriate disinfectant to reduce the surface microbe population. Pipet jars used for soaking dirty pipets should be emptied, thoroughly cleaned, and refilled with fresh water and a detergent such as Liquinox® that will not form residues on the glass pipets. Water baths used for warming enzyme solutions, reagents and media should be emptied, cleaned with a detergent/disinfectant solution, refilled with distilled or deionized water [29] and re-equilibrated to the desired temperature. Adding a disinfectant such as Roccal® to the water bath will prevent microbial growth [29]. Well in advance, CO₂ incubators should be wiped down with an appropriate disinfectant such as 70% ethanol (no chlorinated or halogen disinfectants)

[26], replenished with distilled or deionized water in the humidity pans, and allowed to re-equilibrate. A disinfectant such as Roccal® may be used at a 2% concentration in the humidifier trays without any deleterious effect on cell cultures [15]. Centrifuges, including buckets and tube holders, used for separating cells from tissue should also be cleaned with an appropriate disinfectant.

Commandment III. Prepare the biological safety cabinet. Before use, the biological safety cabinet or laminar flow hood should be switched on and allowed to equilibrate for a minimum of 15 minutes to filter airborne particles from the cabinet environment [25] and to ensure that the working area is clean [14]. Immediately after the hood is switched on, and intermittently during use, all interior surfaces should be sanitized with a non-corrosive chemical disinfectant such as a 70% ethanol solution to inactivate surface contaminants [1, 25]. A bottle with a non-aerosol trigger spray is useful for ethanol application. Chlorinated or halogen materials should not be used on stainless steel surfaces [25] (Table 1), because they are corrosive to metal [18]. In order to reduce airborne contamination, the HEPA filters, air flow rates, and air balance of a laminar flow unit should be checked and re-certified annually [25]. Exterior grills and prefilters of the cabinet should be kept free of dust and debris by vacuuming and/or washing, according to the manufacturer's instructions

Some laminar flow units are equipped with an ultraviolet light, which can be used to sterilize the

working surface when the hood is not in use. However, other disinfecting agents such as 70% ethanol can more effectively reach the crevices of the cabinet than the UV rays [15]. Chronic use of the UV light in a biological safety cabinet can cause small cracks to appear in plastic panels and will discolor any other plastic parts on equipment, so items such as pipeting devices should be removed before using UV sterilization [15]. When UV light is employed, precautions should be taken to protect eyes and skin from exposure [15].

Commandment IV. Sterilize all cell culture equipment, reagents and media. The goal of all sterilization procedures is to kill microorganisms and eliminate resistant bacterial and fungal spores [15]. Before any sterilization procedures are conducted, glassware, instruments, and specific equipment that will make direct contact with the tissue must be cleaned with a detergent that will not leave toxic residues [1, 14, 15] and rinsed with ultra pure, double distilled water that is free from undesirable organic and inorganic compounds [11]. It is recommended that specific glassware should be dedicated solely for cell culture and not be used for any other biochemical procedures [15]. In some situations the use of sterile, disposable cell cultureware is more cost effective and precludes the need for other methods of sterilization. Other laboratories rely heavily on routine sterilization techniques to prepare materials for use in cell culture.

Sterilization of glassware and other heat stable cell culture materials may be accomplished by either dry heat sterilization or autoclaving. Dry heat sterilization involves heating the items to a temperature of 180 °C for two hours or longer [15, 27]. Most glassware and pipets can be effectively sterilized using dry heat, except for items that contain heat labile components such as rubber, teflon, or plastic [11] that would melt at a temperature of 180 °C. Dry heat sterilization is preferred for surgical instruments, because it preserves sharp cutting edges [4]. Items of glassware can be capped with foil to provide an effective barrier for maintaining sterility after sterilization. Timing of the sterilization cycle should not begin until the desired temperature has been reached. When pipets in metal cans are being sterilized by dry heat, the temperature should be held at 180 °C for four hours to allow heat to penetrate to the interior of the cans. Dry heat sterilization indicators are available from commercial suppliers to ensure that the desired internal temperature is reached.

Autoclaving, or steam sterilization, is the method of choice for sterilizing materials containing rubber or plastic that cannot tolerate the higher temperatures of the dry heat process. Autoclaving utilizes steam under pressure (100 kPa or 15 lb/in²) at a temperature of 121 °C [15]. It is important that the water source for the steam is of high quality and free from

metallic ions which, if deposited on glassware, could prove toxic to cells [4]. Most materials should be autoclaved on a fast exhaust/dry cycle to remove any residual water from the items [27]. All articles should be wrapped in packaging that will allow steam to penetrate easily and still provide protection from dust or external contamination. There are various types of autoclavable pouches, wraps, and bags available from commercial sources. The length of time for autoclaving will depend on the density of the article and the wrapping material used [27]. Autoclaving of different types of wrapped containers or packages can be monitored with commercially available indicator strips, which indicate if the materials have reached the desired temperature and have been held for a length of time that is adequate to achieve sterilization.

If steam cannot reach the interiors of containers, sterilization will not occur [27]. Because it is difficult for external steam to penetrate through narrow openings, items such as bottles should have glass distilled water added to them (0.5 ml per 100 ml of volume) to generate internal steam. Before autoclaving, these bottles can be loosely capped with plastic screw caps, which are not tightened until after cooling.

Autoclaving can be used to sterilize heat stable liquids such as buffers and balanced salt solutions. All containers of solutions should be placed in a pan (to catch spillage) and autoclaved with a slow or liquid exhaust cycle, which will prevent the solution from boiling over into the interior of the autoclave as the pressure is reduced. The length of time for autoclaving a liquid is a function of its volume: 100 to 500 ml – 25 min minimum, 500 to 1000 ml – 35 min minimum [27]. All caps on bottles should be loosened and not tightened until after the solutions have cooled down to room temperature.

Sterilization of certain pieces of equipment cannot be accomplished by either dry heat sterilization or autoclaving. An example is a meat grinder, which is commonly used in processing muscle tissue [6]. The blades and small parts of the grinder can be wrapped and sterilized by dry heat. However, the larger throat/feeder tube section is best sterilized by immersion for several hours in a container of 70% ethanol, followed by evaporation of residual ethanol in a sanitized and operating laminar flow hood [15].

Media and other liquid reagents containing heat labile components, such as small proteins and metabolic agents, can be sterilized by filtration through 0.22 µm filters [15, 22]. Use of a positive-pressure system, such as with a peristaltic pump, can prevent foaming (protein denaturation) and loss of CO₂ from the media [11, 15]. Disposable, sterile filters are readily obtainable from many commercial sources and are easy to use when filtering up to 20 or more liters of media (Table 2). Most disposable filter/storage units and bottle top filters require

Table 2. Commercially available, disposable cell culture filtration devices^{a, b, n}

Classification	Product	Source	Membrane	Comments
Filter bells	Millex [®]	Millipore	PES ^c	+ press ^l , up to 4 liters
	Polycap [®]	Whatman	NYL ^d , PES	+ press, up to 25 liters
	Sterivex [®]	Millipore	PES, PVDF ^e , MCE ^f	+ press, 100 ml to 2 liters
	Acrocap [®]	Gelman	PES	+ press, up to 3 liters
Bottle top	Stericap [®]	Millipore	PES	Dual-mode:vacuum ^m / + press, 2 liters
	Sterivac [®]	Millipore	PES	Vacuum, up to 20 liters
	Steritop [®]	Millipore	PES, PVDF	Vacuum
	MSI [®]	Micron Separations	CA ^g	Vacuum, 250–750 ml
	Nalgene	Nalgene	SFCA ^h or PES	Vacuum, 150–1000 ml
	Corning	Corning	CA, PES	Vacuum, 125–1000 ml
	Steriflip [®]	Millipore	PES	Vacuum, 50 ml or less
	Mini-Miser [®]	Corning	CA	Vacuum, 50 ml or less
	VacuCap [®]	Gelman	PES	Vacuum, up to 5 liters
Filter units	Stericup [®]	Millipore	PES, MCE, PVDF	Vacuum
	MSI [®]	Micron Separations	CA	CA, 250–500 ml, vacuum
	Nalgene	Nalgene	CN ⁱ	Vacuum, 115–1000 ml
	Corning	Corning	CA, NYL, CN, PES	Vacuum, 150–1000 ml
Syringe filters	Millex [®]	Millipore	PES, PVDF, MCE, PTFE ^j	
	MSI-Cameo [®]	Micron Separations	CA	
	Corning	Corning	CA, NYL	
	Nalgene	Nalgene	PES, PVDF	
	Acrodisc [®]	Gelman	NYL, PES, PS ^k , PVDF, PTFE	
	GD/X13	Whatman	NYL, PVDF, PES, CA	

^a All are 0.22 μm pore size.

^b Information taken from VWR, Fisher, Gelman, and Millipore catalogs.

^c Polyethersulfone membrane (Millipore Express[®]-Millipore, Supor[®]-Gelman).

^d Nylon membrane.

^e Polyvinylidene fluoride membrane (Durapore[®]-Millipore).

^f Mixed cellulose ester membrane (MF-Millipore[®]).

^g Cellulose acetate membrane (AcetatePlus VT[®]-Micron Separations)

^h Surfactant-free cellulose acetate membrane.

ⁱ Cellulose nitrate membrane.

^j Polytetrafluoroethylene (Fluoropore[®] and Mitex[®]-Millipore).

^k Polysulfone membrane (HT Tuffryn[®]-Gelman).

^l + press = positive pressure filtration.

^m Vacuum or suction filtration.

ⁿ The use of a product name implies no approval of the product to the exclusion of other products that may also be suitable.

negative-pressure filtration (vacuum or suction). Syringe filters are efficient for filtering smaller volumes under 50 ml. Autoclavable, stainless steel filter holders (positive pressure) cost more initially but are economical when filtering large volumes of media [15].

A variety of filter membranes are commercially available for different disposable filtration devices used to filter cell culture media and reagents (Table 2). Cellulose acetate and cellulose nitrate membranes usually contain a wetting agent to make them hydrophilic and are used for general purpose filtration. Polysulfone and polyethersulfone membranes have higher flow rates, lower extractables, and greater strength than cellulose acetate and nitrate but are more expensive. Polyvinylidene fluoride mem-

branes are advertised to have the lowest protein binding capacity available [13]. Both nylon membranes and polytetrafluoroethylene (PTFE) membranes can be used to filter DMSO, a cryogenic agent used in freezing cells [13]. The catalogs of the major manufacturers of filters contain helpful information regarding membrane and filter selection.

Commandment V. Include antibiotics and/or antimicrobials. When a tissue is removed from its normal environment, a loss of immune surveillance takes place, allowing any inherent microbial or fungal populations to begin increasing in number. Microorganisms on the skin or hide of the animal, or in the surrounding physical environment, can also threaten the sterility of the sample [1, 4]. Use of

antibiotics and antimycotics during the initial isolation and cell plating procedures may greatly reduce the potential for contamination of the cultures [3].

It is recommended that the use of antibiotics be carefully monitored and not used indiscriminately over long periods of time in cell cultures [3]. Such a policy will help to prevent the creation of antibiotic-resistant strains of microorganisms [1, 15] and the production of genetic changes in the cells [4]. Chronic use of antibiotics can also mask poor cell culture techniques or the presence of microbial contaminants such as mycoplasmas [16]. Mycoplasmas are difficult to detect macroscopically even when present in high concentrations and often do not cause visible deterioration of the cultures [14, 15, 20]. However, because mycoplasmal infection can alter the metabolism and behavior of the cells in culture, its detection and elimination is important [14]. Numerous methods for testing for the presence of mycoplasmas have been reported [14, 15, 20].

When selecting an antibiotic, it is important to follow the working concentrations recommended by the manufacturer to avoid cytotoxic effects on the cells of interest [11]. Because antibiotics are often labile and have a finite, limited time period of activity at 37 °C [11], buffers and media containing antibiotics must be freshly prepared. Most commercial suppliers of antibiotics publish information regarding the properties of the antibiotics they sell including solubility, mode of action, what type of microorganism is affected, and stability at 37 °C [16, 30]. In many cases, penicillin and streptomycin are used to suppress gram-positive and gram-negative bacteria, respectively [1, 11], and gentamicin is used to prevent both broad spectrum bacterial infection [30] and mycoplasmal growth [11, 30]. Amphotericin B is effective in controlling fungal or yeast contamination but can be toxic to cells if it is used long term [11]. This anti-fungal agent is minimally soluble in water [30] and is commonly used in combination with penicillin and streptomycin as an antibiotic/antimycotic solution [16, 30].

Commandment VI. Maintain a clean surgical site.

There are two types of tissue collection regimens: recoverable (non-terminal) and non-recoverable (terminal) surgery. The following procedures detail non-recoverable tissue collection/surgery performed by a veterinarian or experienced animal scientist following the appropriate animal care and use guidelines. Personnel involved in the tissue collection should wear clean surgical-type gowns or laboratory coats, masks, sterile gloves, clean footwear, and hair covers. The incision area is prepared by removing any fur or hair with scissors or clippers [1, 19]. Next the site is thoroughly scrubbed with a detergent/disinfectant such as Cliniscrub[®], working from the inner to the outer area. The site is then wiped with gauze soaked in 70% ethanol. This scrubbing/ethanol

procedure is repeated at least three times and is followed with a final application of Betadine[®] or other suitable disinfectant [19]. As the surgery begins, the skin or hide of the animal should be retracted and clamped with hemostats to prevent it from contaminating the prepared area. Any instruments used in the surgery should have been properly sterilized and during the surgical procedure can be rinsed in 70% ethanol followed by a rinse in sterile PBS [15].

Commandment VII. Obtain the tissue quickly. In order to lessen deterioration of the tissue once it leaves its natural environment, it is important that only a minimal amount of time is allowed to lapse between tissue collection and processing for cell isolation [15]. During transport, the tissue should be maintained at room temperature or at 4 °C if there will be a delay in reaching the processing laboratory [3, 15]. Dehydration of the tissue and potential loss of cell viability is prevented by carrying the tissue to the laboratory in a sterile container (such as a beaker) filled with a sufficient volume of balanced salt solution plus antibiotics/antimycotics to cover the sample [3]. A foil cap or some type of covering for the transporting container is mandatory to prevent airborne contamination. Once in the laboratory, the tissue should be considered contaminated and handled accordingly. First, the tissue should be rinsed several times by immersion in sterile PBS plus antibiotics/antimycotics, changing physical containers with each rinse. When only interior tissue samples are collected, for example, muscle from a fish, the whole tissue (fish) can be rinsed in 70% ethanol to eliminate surface microbial flora [31]. Only after surface contaminants are removed should the tissue be placed in a sterile tray in the biological safety cabinet.

Commandment VIII. Use proper aseptic techniques.

Most cases of contamination in cell cultures can be traced to poor aseptic practices by personnel [1, 3]. Common sense and experience are major factors in perfecting good aseptic technique [15]. Following a consistent regimen of precautions maintains an effective barrier between the sterile environment in the cell culture dish and microorganisms in the outside environment [1, 15].

Personnel who will be handling tissues during cell isolation processing should take appropriate steps to ensure aseptic conditions [1]. The first line of defense is proper hand washing with an antibacterial soap such as Vestal[®], which will remove skin bacteria and loose pieces of dry skin and hair that could fall into open culture vessels. Long hair should be tied back to prevent it from entering the hood [15], and beards should be covered with disposable guards. Sterile gloves should be worn during the initial handling (rinsing, trimming, and grinding) of the muscle or fat

sample but are not absolutely necessary once the ground tissue is placed into culture tubes. Laboratory coats should be reserved specifically for cell culture use and cleaned regularly.

Precautions should be taken prior to placing the tissue into the biological safety cabinet. The work surface of the cabinet should be swabbed with 70% ethanol before beginning work and also following any spills. The sliding sash (if present) of the cabinet should not be raised above the designated operating position. Unobstructed airflow in the laminar flow unit is a primary objective and crucial for maintaining sterility in cultures [25]. Articles should always be arranged so that the airflow is not blocked around any item. Manipulation of cultures and sterile supplies/equipment should always take place within this unobstructed air flow area. The outsides of all bottles, especially those coming directly from a water bath, should be rinsed with 70% ethanol before being placed in the hood [1]. Flaming the openings of bottles and flasks should be done judiciously if at all, because the flame can disrupt the airflow and also create a fire hazard [3, 15, 25]. A propane torch performs more satisfactorily as a flaming source than a continually burning bunsen burner, because it can be removed from the hood when not in use.

When working with a tissue sample or cell culture in the biological safety cabinet, talking should be minimal because of the potential for contamination from oral aerosols [15]. When sneezing and coughing are unavoidable, disposable facemasks should be worn [1]. Reaching across any open container should always be avoided. Sterile surgical instruments used to process the tissue in the hood can be resterilized on site by rinsing in ethanol, flaming, and allowing to cool briefly. Bottles should not be left uncapped in the hood for any extended period of time [15]. The sterile lip of a cap should never be allowed to touch the surface of the interior of the cabinet. If a cap is inadvertently dropped, a different sterile cap should be obtained to replace it. Pouring sterile liquids from one container to another should be done carefully, because this process can introduce contamination when the liquid forms a bridge between the non-sterile outside of the bottle and the sterile inside, allowing bacteria to enter the sterile solution [15]. Even with a careful pouring technique, droplets of sterile liquid can form on the lip of the container and run down into a non-sterile area. If this occurs, the droplets should be carefully wiped away with alcohol-soaked cotton balls or gauze.

Commandment IX. Get the cells into culture promptly. The viability of cells residing in a tissue is at risk from the moment that tissue is removed from its normal environment in the animal. A well thought-out and rehearsed plan of action ensures that the tissue samples are processed quickly and efficiently in the biological safety cabinet before any

deterioration or contamination can occur. The processing equipment, such as grinders, centrifuge tubes, plates, etc., should be organized in the correct order of usage. A primary goal is to get the isolated cells into an artificial environment that mimics *in vivo* circumstances as quickly as possible. It is important that the dissecting, grinding, and digesting of the tissue and the placement of the cells of interest into culture dishes occur in an organized, efficient, and timely fashion. While some cells may be isolated from animals under less than ideal circumstances, contamination problems are directly linked to time.

Commandment X. Monitor the cultures. After the cells have attached, usually within twenty-four hours, the cultures should be rinsed repeatedly with sterile media to remove blood cells and other tissue debris which may contain microorganisms [6]. Plates should first be rinsed gently to remove pieces of tissue and then more aggressively with subsequent washes [6]. During the first forty-eight hours of culture, the plates or dishes should be maintained with media containing antibiotics plus antimycotics [6]. After this time, if the cultures remain uncontaminated, the antimycotic can be eliminated. Our laboratory continues to use antibiotics, at least until the cultures are subpassaged or cryopreserved.

Healthy cultures display consistent cell morphologies with no cell deterioration. Signs of contamination in cell cultures [14, 15] include cloudiness or an abrupt pH change in the medium, uniform particulate material visible under the microscope, deterioration of the cells [15], or in some instances, detachment of the cell monolayer (may indicate mycoplasma contamination) [5]. When contamination occurs, the best course of action is to remove the involved cultures immediately from the cell culture area and treat them with a disinfectant and/or autoclave them [14]. All reagents and media bottles used with the contaminated cultures should be discarded. Cell culture equipment such as incubators and water baths should also be cleaned and disinfected. Decontamination of cultures by treatment with high levels of antibiotics has a very low success rate and should not be attempted unless the contaminated cells are irreplaceable and absolutely critical to the research [15].

5. Summary

The experiences and knowledge gained in this laboratory are offered to those researchers either starting up a new cell culture operation or wishing to compare or modify their own protocols. Our procedures were specifically designed for use in obtaining muscle-derived satellite cells and preadipocytes, but the basic principles described may be applicable to the isolation of other types of cells

from other tissues. Noncontaminated, healthy, primary cultures of cells do not occur by accident or chance but rather are the result of the consistent application of proven protocols. It is our hope that the commandments outlined in this paper will provide a practical and easily duplicated framework of procedures for others to use in isolating cells free from contamination.

Notes on suppliers

1. Nu Aire Inc., 2100 Fernbrook Lane, Plymouth, MN 55447, USA
2. American Sterilizer Company, 2425 West 23rd St., Erie, PA 16512, USA
3. VWR Scientific Products, P.O. Box 1002, So. Plainfield, NJ 07080, USA
4. Millipore Corporation, 80 Ashby Road, Bedford, MA 93235, USA
5. Beckman Instruments, Inc., 1050 Page Mill Road, Palo Alto, CA 94304, USA
6. Precision Scientific Inc., 3737 W. Cortland St., Chicago, IL 60647, USA
7. The Newell Group, Medina, NY 14103, USA
8. McCormick Distilling Co. Inc., Weston, MO 64098, USA
9. The UpJohn Company, Kalamazoo, MI 49001, USA
10. The Purdue Frederick Company, Norwalk, CT 06856, USA
11. The Clinipad Corporation, Charlotte, NC 28161, USA
12. Alconox Inc., 9 E. 40th St., New York, NY 10016, USA
13. Merck Inc., St. Louis, MO 63110, USA
14. Gibco BRL Products, Life Technologies, Grand Island, NY 14072, USA
15. Gelman Sciences, 600 S. Wagner Road, Ann Arbor, MI 48106-1448, USA
16. Nalgene Company, Box 20365, Rochester, NY 14602-0365, USA
17. Sigma, P.O. Box 14508, St. Louis, MO 63178, USA

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