

# Aseptic Technique for Cell Culture

This unit describes some of the ways that a laboratory can deal with the constant threat of microbial contamination in cell cultures. Microorganisms are ubiquitous. Bacteria can be isolated from nearly any surface including inanimate objects and human skin. Fungal spores and bits of vegetative hyphae drift into a laboratory from air conditioning ducts and open doors. Mycoplasma infections most frequently originate from improperly sterilized media or serum. At the risk of eliciting paranoia in the novice cell culture user who has no training in microbiological techniques, the possibility for microbial contamination exists everywhere. Inherent with successful manipulation of cell cultures is the basic understanding that everything that comes into contact with the cells must be sterile or noncontaminating. This includes media, glassware, and instruments, as well as the environment to which the cultures are briefly exposed during transfer procedures. Because cleaning up a contaminated culture is too frequently a disheartening and unsuccessful experience, the best strategy is to employ procedures to prevent microbial contamination from occurring in the first place.

This unit begins with a protocol on aseptic technique (see Basic Protocol 1). This catch-all term universally appears in any set of instructions pertaining to procedures in which noncontaminating conditions must be maintained. In reality, aseptic technique cannot be presented in one easily outlined protocol, but rather encompasses all aspects of environmental control, personal hygiene, equipment and media sterilization, and associated quality control procedures needed to ensure that a procedure is, indeed, performed with aseptic, noncontaminating technique. Although cell culture can theoretically be carried out on an open bench in a low-traffic area, most cell culture work is carried out using a horizontal laminar-flow clean bench (see Basic Protocol 2) or a vertical laminar-flow biosafety cabinet (see Alternate Protocol). Subsequent units within this chapter address these diverse considerations—e.g., sterilization and disinfection, use of antibiotics, and quality control. Where applicable, use presterilized, disposable labware and other equipment. The wide availability and reliability of these products has simplified cell culture, particularly for small-scale laboratory needs.

## ASEPTIC TECHNIQUE

This protocol describes basic procedures for aseptic technique for the novice in cell culture technology. One basic concern for successful aseptic technique is personal hygiene. The human skin harbors a naturally occurring and vigorous population of bacterial and fungal inhabitants that shed microscopically and ubiquitously. Most unfortunately for cell culture work, cell culture media and incubation conditions provide ideal growth environments for these potential microbial contaminants. This procedure outlines steps to prevent introduction of human skin flora during aseptic culture manipulations.

Every item that comes into contact with a culture must be sterile. This includes direct contact (e.g., a pipet used to transfer cells) as well as indirect contact (e.g., flasks or containers used to temporarily hold a sterile reagent prior to aliquoting the solution into sterile media). Single-use, sterile disposable plastic items such as test tubes, culture flasks, filters, and pipets are widely available and reliable alternatives to the laborious cleaning and sterilization methods needed for recycling equivalent glass items. However, make certain that sterility of plastic items distributed in multiunit packages is not compromised by inadequate storage conditions once the package has been opened.

## **BASIC PROTOCOL 1**

Ideally, all aseptic work should be conducted in a laminar cabinet (see Basic Protocol 2 and Alternate Protocol). However, work space preparation is essentially the same for working at the bench. Flame sterilization is used as a direct, localized means of decontamination in aseptic work at the open bench. It is most often used (1) to eliminate potential contaminants from the exposed openings of media bottles, culture flasks, or test tubes during transfers, (2) to sterilize small instruments such as forceps, or (3) to sterilize wire inoculating loops and needles before and after transfers. Where possible, flame sterilization should be minimized in laminar-flow environments as the turbulence generated by the flame can significantly disturb the sterile air stream.

### **Materials**

Antibacterial soap  
70% ethanol or other appropriate disinfectant  
95% ethanol

Clean, cuffed laboratory coats or gowns  
Latex surgical gloves  
Clean, quiet work area  
Shallow discard pans containing disinfectant  
Bunsen burner or pilot-activated burner (e.g., Touch-o-Matic, VWR)

### **Take personal precautions**

1. Just prior to aseptic manipulations, tie long hair back behind head. Vigorously scrub hands and arms at least 2 min with an antibacterial soap.

*Superficial lathering is more prone to loosening than removing flaking skin and microbial contaminants. Loosely adhering skin flora easily dislodge and can potentially fall into sterile containers.*

2. Gown appropriately. For nonhazardous sterile-fill applications, wear clean, cuffed laboratory coats and latex gloves.

*Greater stringencies may be necessary depending upon laboratory regulatory requirements. Work with potentially hazardous agents certainly mandates additional considerations for safety. Front-closing laboratory coats are not recommended for work with hazardous biological agents. Safety glasses should be worn by laboratory personnel when manipulating biological agents outside the confines of a biosafety cabinet.*

3. Frequently disinfect gloved hands with 70% ethanol while doing aseptic work.

*Although the gloves may initially have been sterile when first worn, they will no doubt have contacted many nonsterile items while in use.*

*Note that 70% ethanol may not be an appropriate agent for latex glove disinfection when working with cultures containing animal viruses, as studies have shown that ethanol increases latex permeability, reducing protection for the wearer in the event of exposure. In this case, quarternary ammonium compounds are more appropriate.*

4. Dispose of gloves by autoclaving after use. Do not reuse. Bag and autoclave single-use laboratory coats after use. Bag, autoclave (if necessary), and wash other laboratory coats within the laboratory facility or send out for cleaning at a laundry certified for handling biologically contaminated linens.

*Never take laboratory clothing home for washing.*

5. Thoroughly wash hands after removing protective gloves.

### ***Prepare and maintain the work area***

6. Perform all aseptic work in a clean work space, free from contaminating air currents and drafts. For optimal environmental control, work in a laminar-flow cabinet (see Basic Protocol 2 and Alternate Protocol).
7. Clear the work space of all items extraneous to the aseptic operation being performed.
8. Wipe down the work surface before and after use with 70% ethanol or other appropriate disinfectant.
9. Wherever feasible, wipe down items with disinfectant as they are introduced into the clean work space. Arrange necessary items in the work space in a logical pattern from clean to dirty to avoid passing contaminated material (e.g., a pipet used to transfer cultures) over clean items (e.g., flasks of sterile media).
10. Immediately dispose of any small contaminated items into a discard pan.
11. When the aseptic task has been completed, promptly remove any larger contaminated items or other material meant for disposal (e.g., old culture material, spent media, waste containers) from the work space and place in designated bags or pans for autoclaving. Disinfect the work space as in step 8.

### ***Flame sterilize the opening of a vessel***

12. For a right-handed person, hold the vessel in the left hand at ~45° angle (or as much as possible without spilling contents) and gently remove its closure. Do not permit any part of the closure that directly comes in contact with the contents of the vessel to touch any contaminating object (e.g., hands or work bench).

*Ideally, and with practice, one should be able to hold the closure in the crook of the little finger of the right hand while still being able to manipulate an inoculating loop or pipettor with the other fingers of the hand.*

*Holding the vessel off the vertical while opening will prevent any airborne particulates from entering the container.*

13. Slowly pass the opening of the vessel over the top of (rather than through) a Bunsen burner flame to burn off any contaminating matter.

*Be careful when flaming containers of infectious material. Any liquid lodged in the threads of a screw cap container will spatter as it is heated. Aerosols thus formed may actually disseminate entrapped biological agents before the heat of the flame is hot enough to inactivate them.*

14. While still holding the vessel at a slant, use a sterile pipet and pipettor to slowly add or remove aliquots to avoid aerosol formation.
15. Flame-sterilize again as in step 13, allow the container to cool slightly, and carefully recap the vessel.

### ***Flame sterilize small hand instruments***

16. Dip critical areas of the instrument (i.e., those that come into contact with the material of concern) in 95% ethanol.

*Make certain that the alcohol is in a container heavy enough to support the instrument without tipping over.*

**CAUTION:** 95% ethanol is flammable; keep the container at a safe distance from any open flame.

17. Remove the instrument from the alcohol, being careful not to touch the disinfected parts of the instrument. Allow excess ethanol to drain off into the container.
18. Pass the alcohol-treated part of the instrument through the flame of a Bunsen burner and allow residual alcohol to burn off.
19. Do not let the sterilized portion of the instrument contact any nonsterile material before use. Let the heated part of the instrument cool for ~10 sec before use.
20. After use, return the instrument to the alcohol disinfectant until needed again.

***Flame sterilize inoculating loops and needles***

21. Hold the inoculating wire by its handle and begin in the center of the wire to slowly heat the wire with the flame of a Bunsen burner. Proceed back and forth across the wire's full length until it glows orange.
22. While still holding the handle, allow the inoculating wire to cool back to room temperature (~10 sec) before attempting any transfer of material.

*If transfers are made while the inoculating wire is hot, cells will be killed by the hot wire, and aerosols created from spattering material can disperse biological material throughout the work space.*
23. After the transfer is made, reheat the inoculating wire as in step 21 to destroy any remaining biological material. Let cool to room temperature before putting aside for next use.

**BASIC  
PROTOCOL 2**

**USE OF THE HORIZONTAL LAMINAR-FLOW CLEAN BENCH**

Laminar-flow cabinets (hoods) are physical containment devices that act as primary barriers either to protect the material being manipulated within the hood from worker-generated or environmental sources of contamination, or to protect the laboratory worker and laboratory environment from exposure to infectious or other hazardous materials that are present within the hood. Cell culture applications utilize two types of laminar-flow hoods: (a) the horizontal-flow clean bench (described here) and (b) the biological safety cabinet (see Alternate Protocol). Both types of hoods use a high-efficiency particulate air (HEPA) filter and blowers that generate a nonmixing stream of air.

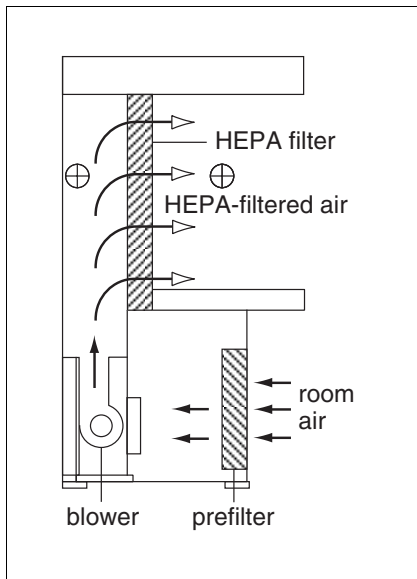
The horizontal laminar-flow clean bench is used to provide a near-sterile environment for the clean (i.e., noncontaminating) handling of nonhazardous material such as sterile media or equipment. Because the air stream pattern directs the flow of air within the hood directly back to the hood operator and the room (Fig. 1.3.1), horizontal flow hoods are never to be used with infectious agents or toxic chemicals.

***Materials***

- 70% ethanol or other disinfectant
- Horizontal laminar-flow hood, certified for use
- Swabs (e.g., cheesecloth, paper towels)
- Pilot light-activated Bunsen burner (e.g., Touch-o-Matic, VWR)

1. Completely clear the bench of the laminar-flow hood and disinfect the bench working surface and the left and right sides of the hood with 70% ethanol or other disinfectant. Do not spray the back (gridded) wall where the HEPA filter is housed.

*Resist the urge to leave frequently used items (e.g., pipet canisters or a bag of disposable plastic tissue culture flasks) in the hood between uses. Their presence makes thorough disinfection of the work space difficult.*



**Figure 1.3.1** Horizontal laminar-flow clean cabinet. Solid arrows, dirty room air; open arrows, clean HEPA-filtered air; circled +, positive pressure with respect to room air.

2. Turn the hood blower and lights on and let the air circulate within the hood 10 min before use.
3. Place items needed for the specific procedure into the hood, wiping each item with 70% ethanol or other disinfectant just before introducing it into the laminar environment.

*Do not overcrowd the work space. For horizontal laminar-flow effectiveness, maintain a clear path between the work area and the back wall of the cabinet where the HEPA filter is located.*

4. Wash hands well before working in the hood and wear a clean laboratory coat and surgical gloves to further protect the work from shedding of skin flora that can contaminate any product (see Basic Protocol 1).
5. While working in the hood, perform all work at least 4 in. back from the front opening, and avoid rapid movements that might disrupt the laminar air flow. Avoid moving materials or hands in and out of the cabinet as much as possible.
6. If flame sterilization is needed in the hood for a particular application, use a burner that can be activated by a pilot light when needed, rather than one that burns constantly.

*The open flame of a Bunsen burner causes turbulence that disrupts the unidirectional laminar air flow.*

7. When work is completed, remove all material from the laminar work bench, clean any spills, and disinfect the bench working surface by wiping with 70% ethanol or other disinfectant.
8. Turn off hood blower and lights.

## USE OF THE VERTICAL LAMINAR-FLOW BIOSAFETY CABINET

Biological safety cabinets provide a clean, safe environment for both the worker and the product. The Class II, Type A biosafety cabinet (Fig. 1.3.2) is frequently encountered in cell culture laboratories, and this protocol describes the use of this type of barrier device. The Class IIA biosafety cabinet is suitable for work with low- to moderate-risk biological agents in the absence of toxic or radioactive chemicals.

### *Materials (also see Basic Protocols 1 and 2)*

Class II, Type A Biosafety Cabinet (BSC), certified for use  
Pilot light-activated Bunsen burner (e.g., Touch-o-Matic, VWR) or electronic incinerator (e.g., Bacti-Cinerator III, VWR)  
Closed-front laboratory gowns (for personnel working with biological agents)

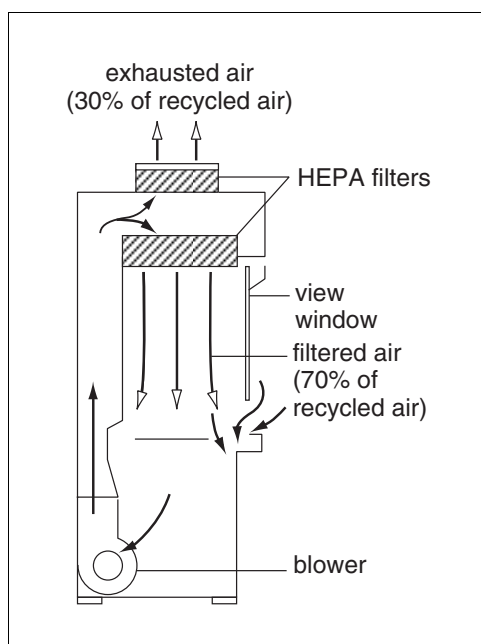
1. Turn the hood blower on and verify air flow by feeling (by hand) the current near the front grill of the work surface. Turn the germicidal UV light off if it is on. Turn the fluorescent light on.

*Before use, the cabinet should already be empty and clean from prior activity. The view window should be lowered to the proper operating height (normally 8 in.) or as specified by the cabinet manufacturer.*

*UV light is effective only for decontaminating clean, solid surfaces with which it comes in contact. It is not effective in decontaminating the cabinet air flow. UV light is not effective against bacterial spores. UV germicidal light tubes should be replaced frequently (at least every 6 months for biosafety cabinets in use on a daily basis) to assure that they are emitting light at 254 nm and at an intensity appropriate for decontamination.*

**CAUTION:** *UV light is harmful to the eyes. Laboratory personnel should not be near the cabinet or looking at the UV light when it is in use.*

2. Wash and gown as required for the operation (see Basic Protocol 1, steps 1 to 5).
3. Wipe down the entire interior cabinet work surface area with 70% ethanol or other appropriate disinfectant.
4. Let blower run for 10 min to filter the cabinet air of any particulates.



**Figure 1.3.2** Biological safety cabinet, Class II, Type A. Note that filtered air is contaminated after passing through the work space, and is filtered again whether it is recycled to the workspace (70%) or exhausted (30%). Solid arrows, dirty (room/contaminated) air; open arrows, filtered air.

5. Raise the front view window as needed to bring necessary items into the cabinet. Wipe each item with 70% ethanol or other disinfectant as it is placed in the cabinet.

*Do not crowd the work space and make sure no air vents are blocked by supplies or equipment. Do not position material so that it obscures any of the air vents at the front edges of the laminar hood. One frequent source of air flow restriction in biosafety cabinets is "lost" paper towels that have been drawn into the air ducts at the back of the work surface.*
6. Organize the work surface for a clean-to-dirty work flow. Place clean pipets, flasks, and sterile media bottles at one side of the cabinet; place discard pans, spent cultures, and other wastes on the other side.
7. Return the view window to the 8-in. operating level. Wait ~10 min for the blowers to filter the disturbed cabinet air before starting work.
8. While working, keep all material and perform work  $\geq 4$  in. back from the front opening of the cabinet, and minimize rapid movements or activity. Keep the view window opening as close to 8 in. as allows reasonable access to the work surface and equipment.

*These precautions assure that any drafts caused by arm movements will not disrupt air flow or churn room air currents into the clean work area.*
9. If direct flame sterilization of items within the cabinet is necessary, use an electric burner or pilot light-activated flame burner located at the back of the work space.

*A constant open flame in the cabinet can disturb the laminar air flow.*
10. At the end of the procedure, enclose all contaminated materials. Clean the cabinet work surface with 70% ethanol or other disinfectant, being especially careful to wipe any spills of culture suspensions or media that can serve as future contamination points. Clear all material from the cabinet.
11. Let the blower run for  $\geq 10$  min with no activity to remove any aerosols that were generated. During this period, turn off the fluorescent light and turn on the germicidal UV light. Allow the UV light to operate  $\geq 30$  min.

## COMMENTARY

### Background Information

#### *Aseptic technique*

The dictionary definition of asepsis simply implies freedom from pathogenic organisms. However, the practical definition of the term for cell biologists, as well as other biotechnologists working with pure cultures, has come to be synonymous with sterile or noncontaminating conditions. The successful manipulation of cell cultures under any circumstance inherently relies upon the ability to maintain rigorous aseptic (i.e., noncontaminating) working conditions. The concept of aseptic technique is simple in theory: prevention of sterile or uncontaminated material and objects from coming into contact with any nonsterile or contaminated material.

Practical application of the theory is often illusive for beginning students. However,

breaches in aseptic technique can also cause significant problems for even well-experienced laboratories, particularly when the source of contamination is not readily evident. A single incident of culture contamination is frustrating in its own right, but repeated contamination (particularly by the same type of organism) invariably results in expensive losses and delays until the localization and source are identified.

The critical areas of concern with respect to successful aseptic technique include environmental conditions (laboratory or work space), source material (cell lines, media, and reagents), equipment (labware, instruments, and apparatuses), sterilization procedures and equipment (autoclave, dry heat, filtration), and human (laboratory personnel) considerations. Budgetary constraints aside, technological aids exist to greatly simplify the hardware needed

for aseptic work. Laminar-flow cabinets create clean working environments (see below); clean, certified cell lines are available from cell repositories; media manufacturers and biotechnology supply companies provide sterile media, sera and reagents; and presterilized disposable labware to satisfy most cell culture needs is available from any large distributor of scientific supplies.

Despite all the technological advances, the one weak link remaining in successful laboratory applications of aseptic technique is the human factor. Too frequently, contamination occurs because of the desire to work a little too quickly, the urge to eliminate an “unimportant” step, or lapses in concentration during mundane procedures. The only advice to offer as protection against the human factor is to work slowly and deliberately when performing procedures under aseptic conditions, don’t eliminate procedural steps, and pay attention! Establishing a standard routine of procedures and of placement of materials can help prevent the omission of steps.

#### ***Laminar-flow cabinets***

Laminar-flow cabinets or hoods have replaced the open laboratory bench for aseptic work in almost all cell culture and microbiology laboratories. Their effectiveness as physical barriers to contamination relies on a cabinet design incorporating high-efficiency particulate air (HEPA) filters to trap airborne contaminants, and blowers to move the filtered air at specified velocities and in a nonmixing (laminar) stream across a work surface.

As noted in each protocol for the particular type of laminar-flow application, the proper choice of cabinet is imperative. Horizontal laminar-flow cabinets are never used with biological or toxic chemical agents as they are not containment devices but rather serve to provide a strong stream of near-sterile air for particle-free working conditions. As this air is blown directly from the HEPA filter (at the back of the cabinet) across the work surface and out of the cabinet (directly into the operator’s face and the room), the restricted use of the horizontal flow cabinet to nonhazardous material is obvious.

The Class IIA biosafety cabinet is a laminar containment device that (1) protects the material being manipulated within the cabinet by HEPA-filtered incoming air and (2) protects the operator and room environment from potentially hazardous material in the cabinet with an air curtain at the front of the cabinet (the view screen) and HEPA-filtered cabinet exhaust air.

As Class IIA biosafety cabinets are not totally leak-proof, they cannot be used for high-risk biological agents (see current Center for Disease Control and NIH guidelines for the status of any biological material used in the laboratory; Richmond and McKinney, 1993). Because Class IIA cabinets operate with ~70% recirculated air within the cabinet (Fig. 1.3.2), the potential for accumulation of chemicals within the laminar work space limits use to low-level toxic or radioactive material.

Laminar-flow cabinets are not replacements for good microbiological aseptic technique and must be used in conjunction with standard concerns for asepsis if full efficiency of the equipment is expected. Similarly, there is a limit to the protection a laminar cabinet can provide if it is operated in an environment not conducive to clean work conditions. The cabinets should be installed and operated in a relatively clean, quiet laboratory environment. Laboratory doors should be kept closed while the cabinet is in use to minimize strong room air currents that could break the laminar air stream within the cabinet. The units should not be located directly near room air ducts or anywhere a strong environmental air flow exists. Additionally, air flow disturbance by personnel or equipment, particularly within a few feet in front of the cabinets, should be limited when the laminar device is in use.

Because of the critical nature of their function (particularly for the biosafety laminar cabinet), these devices must be certified at installation by professional laminar flow technicians in accordance with National Sanitation Foundation Standard No. 49 for Class II (laminar flow) Biohazard Cabinetry (NSF International, 1992) or other applicable regulatory and safety guidelines. As HEPA filters are brittle and will crack with normal usage of the unit, laminar cabinets must also be recertified annually or after 1000 hr use, and whenever they are moved.

### **Critical Parameters and Troubleshooting**

#### ***Human sources of contamination***

As noted above, bacterial shedding from human skin is a natural occurrence. However, under times of physiological or psychological stress, a human may shed so excessively that routine gowning procedures are inadequate. A clue to this condition can be the veteran technician who suddenly can’t seem to transfer anything without contaminating it, especially when contamination is repeatedly bacterial and

by species of *Staphylococcus*, *Micrococcus*, or coryneforms.

Alleviation of the problem may be achieved by simply controlling the temperature of the laboratory. Gowned personnel sweat in 27°C (80°F) rooms, and people who sweat shed more than people who don't. Rigorous attention to gowning details as well as liberal washing of hands and arms with an antimicrobial soap just prior to aseptic work may alleviate the situation. If the problem involves psychological stress or physiological stress due to illness or medication, more rigorous gowning procedures may help. Use fresh, clean laboratory coats for each round of aseptic work and make sure laboratory coat sleeves are tucked inside gloves to prevent exposed wrists. Use disinfectants liberally. For worst-case incidences of excessive shedding, the only recourse may be to move the individual to nonaseptic procedures until the condition clears.

#### ***Decontamination of a laminar-flow cabinet***

Any mechanical failure of a laminar cabinet must be evaluated by qualified, trained personnel. Increased incidences of microbial contamination (particularly by the same organism) could originate from (1) poor cleaning and disinfection of the cabinet work space, (2) a source of contamination lodged in the ducts within the cabinet (e.g., media or culture material spilled into the cabinet ducts), or (3) a crack in the HEPA filter.

Disinfect the catch basin if culture material has spilled through the vents in the work surface into the catch basin below. Use a strong disinfectant (such as 5% to 10% bleach in a sufficient volume to thoroughly contact the spilled material) and allow the disinfectant to stay in contact with the spill for 30 min. Drain the contents of the catch basin into a container suitable for final sterilization by autoclaving.

Visually inspect the working interior of the laminar cabinet for evidence of dried culture material or media, especially in the corners of the cabinet. Clean the interior of the cabinet with a laboratory detergent, rinse with water, dry, and treat the area with an appropriate disinfectant. Be very careful not to wet the exposed HEPA filter located on the back wall of horizontal flow cabinets, as this can compromise the filter integrity. Be careful not to let cleaning solutions enter any vents of the cabinet.

After thorough cleaning of the cabinet work surface, operate the cabinet (as detailed in Basic Protocol 2 or Alternate Protocol) using a control procedure for localizing the source of any

remaining contamination. This can be achieved with a series of opened plates of trypticase soy agar and Emmons' modification of Sabouraud's agar systematically coded and placed across the work surface. Leave the media plates open and the cabinet operating for 30 min. Close the lids of the agar plates and incubate them at 26°C for 5 days. If significant microbial contamination appears in the plates, consult with a qualified laminar technician. The resolution to the problem will require either caulking leaks in the HEPA filter or sealing the cabinet for total interior decontamination of filter and ducts with formaldehyde gas.

A final source of frequent contamination in a laminar working condition can be the "sterile" equipment, labware, or solutions used. A poorly filter-sterilized phosphate-buffered saline solution can give rise to significant numbers of pseudomonad bacteria within weeks when stored at room temperature. Insufficiently processed autoclaved or dry heat-sterilized labware frequently results in contamination of cell culture material by spore-forming bacteria.

#### **Anticipated Results**

When proper aseptic techniques are used, it should be possible to maintain cell cultures without contamination.

#### **Time Considerations**

It takes ~1/2 hr to properly prepare oneself and the cell culture area for culture procedures and a similar amount of time to properly clean up afterward.

#### **Literature Cited**

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*Chapter provides an overview of general concerns for working with biological agents, from a classic publication on general methods in bacteriology that often overlaps to satisfy the technical needs of cell biologists.*

Chatigny, M.A. 1986. Primary barriers. *In* Laboratory Safety: Principles and Practices (B.M. Miller, D.H.M. Gröschel, J.H. Richardson, D.Vesley, J.R. Songer, R.D. Housewright, and W.E. Barkley, eds.) pp. 144-163. American Society for Microbiology, Washington, D.C.

*Offers detailed considerations on the types and uses of laminar-flow barrier technology. The main publication is well worth its price for anyone (staff, supervisors, administrators) responsible for safety in a biological laboratory.*

Freshney, R.I. 1994. Culture of Animal Cells: A Manual of Basic Technique, 3rd ed., pp. 51-69. Wiley-Liss, New York.

*Offers suggestions for maintaining aseptic conditions while working with cell cultures. A classic cell culture publication that surveys the field while providing enough detail for an individual with intermediate knowledge of microbiology and cell biology.*

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