BIOSAFETY STANDARD FOR SORTING OF UNFIXED CELLS

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abstract

Background: Cell sorting of viable biological specimens has become very prevalent in laboratories involved in basic and clinical research. As these samples can contain infectious agents precautions to protect instrument operators and the environment from hazards arising from the use of sorters are paramount. To this end the International Society of Analytical Cytology (ISAC) took a lead in establishing biosafety guidelines for sorting of unfixed cells (Schmid et al., Cytometry 28:99-117, 1997). However, since these recommendations were generated the field of cytometry has advanced and an update of current cell sorting safety standards is needed.

Methods: Initially, suggestions about the document format and content were discussed among the current members of the ISAC Biosafety Committee and were incorporated into a draft version that was sent to all committee members for review. Comments were collected, carefully considered, and incorporated as appropriate into a draft document that was posted on the ISAC web site to invite comments from the flow cytometry community at large. The revised document was then submitted to ISAC Council for review and approval.

Results: A safety standard for performing viable cell sorting experiments has been generated. The document contains background information on the biohazard potential of sorting and the hazard classification of infectious agents as well as recommendations on 1) sample handling 2) operator training and personal protection 3) laboratory design 4) cell sorter set-up, maintenance, and decontamination, and 5) testing the instrument for the efficiency of aerosol containment.

Conclusions: The standard constitutes an up-dated and expanded revision of the 1997 biosafety guideline document. It is intended to provide laboratories involved in cell sorting with safety practices that take into account the enhanced hazard potential of high-speed sorting, safety features of novel instrumentation, new options for personal protective equipment, and recently developed methods for testing the efficiency of aerosol containment.

Keywords: flow cytometry, occupational health, biohazards, cell sorting, biosafety, aerosol containment
Introduction

In 1994 the International Society of Analytical Cytology (ISAC), the pre-eminent association representing researchers involved in cytometry, recognized the need to formulate safety guidelines for sorting and analysis of unfixed cells to provide laboratories with recommendations for practices to reduce the potential for biohazard exposure of instrument operators. To this end ISAC initiated the formation of a Biohazard Working Group charged with this task. After extensive discussions of the contents of the draft document among working group members and members of the flow cytometry community who had indicated an interest in participating in a review of the proposed safety recommendations, ISAC adopted the document as the official guidelines, and they were published in 1997 (1). Many concepts addressed in the 1997 document are valid today, however, the field of cytometry and safety practices have progressed since 1997:

Advances in cell sorter technology made high-speed cell sorting more prevalent and changed the biohazard potential of cell sorting experiments.

New and less expensive options for personal protection of operators have become available.

Instrument manufacturers responded to the need for improved operator protection and have introduced instrumentation containing novel safety features.

Newly designed safety attachments for cell sorters have become commercially available.

With the availability of compact sorters many more laboratories have incorporated cell sorting into their experimentation, but often do not have dedicated operators to perform cell sorting experiments.

Simpler, bead-based techniques for measuring the efficiency of aerosol containment during cell sorting have been developed.

Advances in cell biology have increased the need for live infectious cell sorting for cell culture and experiments involving molecular genetics

Hence, the previously-published guidelines are out-dated and the generation of a new standard has become a pressing issue for the flow cytometry community world-wide, particularly as more and more laboratories conduct cell sorting experiments involving samples with variable and sometimes complex levels of biohazard potential such as genetically engineered cell preparations. The current ISAC Biosafety Committee has
been charged with generating up-dated guidelines that reflect the present knowledge and occupational safety practices.

A. Purpose

The purpose of this document is to provide written recommendations that modify or expand the 1997 guideline document for handling and sorting of potentially biohazardous specimens and includes methods to assess the risk of exposure of laboratory personnel to biological aerosols that may be produced by deflected-droplet fluorescence-activated cell sorters. The possibility exists that operators of cell sorters could become infected with biological agents contained in the specimens they are sorting (2-5). Laboratories following these guidelines aid in preventing exposures of laboratory personnel to infectious agents from sorting of unfixed cells. Recommendations in this document focus on cell sorting of live, unfixed samples. However, it is important to note that functional measurements on cells, e.g., evaluation of calcium flux or membrane potential, certain apoptosis assays, cytokine assays, or live DNA or RNA staining, preclude cell fixation, and when performed on jet-in-air flow cytometers, can also expose operators or others involved in these experiments to potentially hazardous aerosols and sample splashes. Therefore, these guidelines apply to all procedures whenever unfixed samples are run through a jet-in-air flow cytometer or a cell sorter which combines a flow cell with jet-in-air sorting.

1. Biohazard potential of unfixed cells

Typical biological specimens that are subjected to cell sorting include but are not limited to peripheral blood leukocytes, bone marrow, splenocytes, thymocytes, sperm cells, cells from primary and immortalized cultures from humans, non-human primates, other species, and transgenic animals. These samples can harbor known and unknown infectious agents such as hepatitis viruses (A, B, C, D[delta]), human immunodeficiency viruses (HIV-1, -2), or cytomegalovirus. Hepatitis B, C, D and HIV viruses have been classified as carcinogenic for humans by the International Agency for Research on Cancer as have other viruses which are encountered in biological specimens, e.g., Epstein Barr virus, human T-lymphotropic viruses, Kaposi sarcoma herpesvirus/human herpesvirus 8, Herpesvirus saimiri, and simian virus 40 (6-10). Samples may also consist of genetically engineered cells which contain genomic sequences of potentially infectious organisms. Occupational transmissions across species to humans of retroviral agents such as simian type D retrovirus are of particular concern as these animal viruses could be introduced into the human population by this route (11). Most known pathogens encountered when sorting clinical or research samples are transmitted by inoculation,
by direct exposure of broken skin or mucous membranes, or by ingestion. Some may be transmitted by inhalation of organism-containing droplets (see Appendix 1, Table 1) that are generated either through laboratory manipulations (12) or the sorting process (13). Although HIV viruses and Hepatitis viruses are primarily transmitted through inoculation, infection through aerosolization of virus particles has been documented for Hepatitis B (14) but not for HIV. However, transmission of the HIV virus has been described through ingestion of HIV-infected breast milk (15). Therefore, HIV can potentially infect an individual via the oral mucosal route.

Biological particles of 0.1 μm to 60 μm sizes (i.e., aerosols) were found to be important in the spread of infectious diseases (16-20). Submicrometer particles formed through dehydration of small droplets (droplet nuclei) can contain inorganic, organic material, or infectious agents and may stay suspended in air for prolonged periods of time. During inhalation, larger particles are deposited mainly into the nasal passages, particles in the size range from 3-7 μm into the tracheal area and pharynx, and ≤ 3 μm particles are deposited into the lung of the exposed individual (19). Droplets that fall out of suspension in air will fall onto surfaces and pathogens they may contain can then be transmitted by exposure to broken skin or mucous membranes, or by ingestion. For instance, exposed eye mucosa creates a risk during laboratory work. Consequently, protection of all laboratory workers from exposure is critical, in particular during high-risk procedures such as droplet-based cell sorting using instruments with high system pressures.

2. Creation of droplets and aerosols during cell sorting

Jet-in-air technology used for cell sorting involves a liquid stream carrying the cells through a nozzle vibrating at a high frequency. At a given distance from the nozzle orifice the stream is broken into individual droplets passing by high voltage plates. Droplets containing the cells that were pre-selected by the operator are electrostatically charged and deflected into sort sample receptacles. Overall droplet size depends on the instrument operating pressure and the size of the nozzle orifice and its vibration frequency. High-speed cell sorters utilize higher system pressures and sort frequencies (21) and thus produce a higher number of smaller droplets compared to older instruments designed for low speed separations (22). All sorters also generate microdroplets, i.e., satellite droplets, 3-7μm. Due to the high fluid pressure produced in high-speed cell sorters large amounts of secondary aerosols of various and undefined droplet sizes can occur during instrument failures, for instance, when a
partial clog in the nozzle causes a deflection in the fluid stream that is hitting a hard surface, e.g., the waste catcher. Droplets larger than 80 µm constitute the majority of the droplets generated during sorting and settle quickly out of the atmosphere, smaller droplets however may be aerosolized, particularly when they are elevated by air currents. Because of the potential health risk to sorter operators and the environment if aerosols escape into the room, aerosol containment of a cell sorter must be verified by using appropriate testing methods. Refer to section VI of this document for recommendations for the assessment of aerosol containment.

B. International Society for Analytical Cytology (ISAC) Biosafety Committee

In 1997 ISAC under the presidency of Joe Gray established a permanent Biosafety Committee with the goal of providing guidance to its membership and the scientific community at large in safety issues related to cytometry experimentation. The following is a list of the current members:

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II. Level of containment

“Containment “ refers to safe methods for managing infectious agents in the laboratory based on the type of organism and the type of risk due to the nature of the procedure. The four biosafety levels (BSL-1, BSL-2, BSL-3, BSL-4) consist of a combination of laboratory facilities, laboratory practices and techniques, and safety equipment as outlined in the Center for Disease Control and Prevention Publication “Biosafety in Microbiological and Biomedical Laboratories”, 4th Edition, 1999 (23), which is also available on line at www.cdc.gov/od/ohs. The purpose of containment is to reduce or eliminate exposure of laboratory personnel or others and the environment to hazardous agents contained in the samples to be processed. Risk assessment of the experiments to be performed on the equipment available will define the appropriate combination of the different safety elements.

Considering the potential for exposure to aerosols during cell sorting, it is incumbent on the investigator who wishes to have such live, unfixed cells sorted or analyzed to determine the appropriate biosafety level (BSL), and in conjunction with the flow cytometry laboratory director or manager and the sorter operator, review all the appropriate safety procedures for the particular pathogen for risk assessment. Such a review must also take into account the National Institutes of Health (NIH) Guidelines for
Research involving Recombinant DNA Molecules (24). An extensive list of biological agents and their BSL assignment is found in the CDC Publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999.

Risk assessment is based on pathogenicity of the infectious or suspected infectious agent and its route of transmission. It is prudent to consider the potential for aerosol transmission when working with a relatively uncharacterized agent. Greater aerosol potential means greater risk. Origin of the potential infectious material pertaining to a geographic location or a host is also critical for assessing the risk of performing a given experiment. Other factors include agent stability, concentration, infectious dose, data from animal studies, and availability of effective prophylaxis or therapy. Clinical samples may contain unknown pathogens; in these cases in the absence of hard data a cautious approach and adaptation of a higher biosafety level is advisable. Risk assessment is likely to be most difficult for samples containing recombinant DNA molecules. In recent years technologies have evolved that lead to the generation of modified viruses, bacteria, yeast, and other microorganisms. Common recombinant viruses include adenoviruses, alphaviruses, retroviruses, vaccinia, and herpesviruses designed to express heterologous gene products. The challenge faced when selecting the appropriate biosafety level for such work begins by establishing the classification of the non-modified virus and then proceeds to an evaluation for a possible increase in hazard potential associated with a given genetic alteration. Of particular concern are modifications that result in expression of a toxin or a known oncogene, or of sequences that alter the host range or cell tropism, or allow the virus to integrate into the host genome. If needed, advice from a virologist should be sought to determine the proper BSL for planned flow cytometric experiments.

BSL-1 containment is used for work with agents not known to cause disease in humans. BSL-2 practices, safety equipment and facilities are used for pathogens which are known to cause disease in humans but can be easily contained and are not usually transmitted by aerosols. BSL-3 containment is applicable to biological agents which cause serious and potentially lethal disease as a result of exposure by the inhalation route. The ultimate level of containment, BSL-4, is reserved for work with exotic and highly dangerous organisms transmitted by aerosols for which no vaccination or therapies exist. These agents pose a high individual risk of life-threatening disease, therefore, viable sorts should never been done when working with these pathogens. Refer to Appendix 1 Table 1 for the recommended biosafety containment levels for selected agents and to Appendix 1 Table 2 for the corresponding work practices. All laboratory manipulations that can generate aerosols (pipetting, vortexing, etc.) should be performed in a Class I or Class II Biosafety Cabinet. Many cell sorters do not fit within a biosafety cabinet, therefore specimens must be handled on the open bench during cell sorting. Furthermore, for any number of reasons, aerosol containment of a sorter may be breached during the cell sorting procedure and expose the operator to potentially biohazardous aerosols.

It is therefore recommended that viable, unfixed samples that are potentially infectious be sorted at a minimum in a BSL-2 facility (for details refer to the
Environmental Control section of the document) using BSL-3 work practices and BSL-3 personal protective equipment. However, because of the increased hazard for a quick release of large amounts of fluid or aerosols into the environment, it is highly recommended that high-speed sorting be performed in a BSL-3 laboratory facility under complete BSL-3 containment.

Alternatively, BSL-3 containment can also be achieved without the need for building a BSL-3 sort facility by enclosing a cell sorter into a biosafety cabinet. Newly developed cell sorters, e.g., BD FACS Aria (BD Biosciences), JSAN (Bay bioscience Co., Ltd, Japan), are small enough to be completely enclosed into a Biocontainment Biological Safety Enclosure (The Baker Co., Sanford, ME), and more recently compact high-speed sorters such as the inFlux\textsuperscript{TM} (Cytopeia, Seattle, WA) and the Reflection\textsuperscript{TM} (iCyt Visionary Bioscience, Champaign, IL) which can be integrated into a biosafety cabinet have become commercially available.

III. Recommendations for containment controls

A. Standard precautions and other regulatory requirements

In the United States of America all laboratory personnel who handle human cells and other potentially infectious materials such as specimens from experimentally infected animals are required to follow universal precautions and procedures as outlined in the Occupational Safety and Health Administration document *Occupational Exposure to Bloodborne Pathogens* (25) and put forth in additional specific local and institutional safety regulation documents. Laboratories also must comply with federal code regulations for possession, use, and transfer of select agents and toxins (26). All recombinant DNA experiments have to be performed in compliance with the specific NIH guidelines (24) and have to be approved by Institutional Biosafety Committees. All institutions receiving grant or contract awards from NIH are expected to follow the current health and safety guidelines published at [http://grants1.nih.gov/grants/policy]. Other countries have developed their own stringent regulatory standards and/or have adopted aspects of regulations for work with biological agents as mandated in the US. For instance, the European Parliament published in 2000 the Directive 2000/54/EC on the “Protection of workers from risks related to exposure to biological agents at work” which provides classification of biological agents into four risk groups and their associated containment measures (27). Furthermore, guidelines for specimen handling are published in the document M29-A by the Clinical Laboratory Standards Institute (28). Relevant details for the preparation of infectious samples containing HIV for flow cytometry such as shipping and receiving of specimens, local transport, staining, and disposal were previously described (29). Each laboratory needs to develop or adapt a biosafety operations
manual which specifies practices designed to minimize risks and takes into account the biohazard potential of the specimens that are processed (30). Appropriate safety practices are the responsibility of the laboratory director. Personnel must be trained in the required procedures and strict adherence to the techniques set forth is essential.

Handling of all unfixed human specimens and primary cell cultures as if infectious is recommended. This practice also applies to established cell lines that are in vitro or animal-passaged human explanted tissues transformed by spontaneous mutation or a natural or laboratory infection with an immortalization agent, e.g., Epstein Barr virus. In fact, cell lines from the American Type Culture Collection (ATCC) and other sources bear warnings that they may contain bloodborne pathogens, and ATCC recommends they be accorded the same biosafety level as the ones known to be infected with HIV. Likewise specimens from non-human primates and animal tissues, explants, or cell cultures known to be deliberately infected with human pathogens are subject to safety procedures as outlined in the Bloodborne Pathogen Standard. Only rigorously characterized human cell lines that have been proven by stringent techniques such as PCR, sensitive antigen detection, stimulation and co-culture assays, enzyme analysis etc. to be completely devoid of bloodborne pathogens could be excluded. However, as most laboratories are not able to provide reliable confirmation that the samples are pathogen free before they are subjected to cell sorting using biosafety precautions as outlined in the standard is strongly advised.

If samples are fixed, appropriate methods must be selected to reliably inactivate potentially biohazardous agents. Concerns exist about the effectiveness of standard fixation methods to reduce the level of infectivity in samples containing high titers of known viruses or unknown infectious agents resilient to inactivation (31,32). Fixation procedures must be performed carefully, otherwise, samples that are considered inactivated, but in fact are not, can pose a serious health risk to laboratory personnel.

B. Cell sorter operator-specific precautions

The protection of operators from infection and biohazard exposure during sorting of unfixed cells is of paramount importance (1,13,33-41). The following recommendations also apply to others who may be present in the room during the sort, e.g., scientists involved in the experiment.

1. Immunization
Whenever vaccination against a potential infectious organism that may be present in samples to be sorted becomes available, the sorter operator should consider vaccination. Vaccination against Hepatitis B virus is highly recommended.

2. Personal protective equipment

During sorting of unfixed samples personal protective equipment of the operator must conform to BSL-3 recommendations as outlined in the CDC Publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999. The sorter operator should wear a disposable, wrap-around, solid front, long sleeved laboratory coat made of fluid-resistant material. Examination gloves are required at all times and in some cases double-gloving should be considered. When the outer glove(s) are contaminated by contact with potentially biohazardous material, they must be sprayed with 70% ethanol and discarded. Then, new outer gloves are put on over the inner gloves to prevent cross contamination. Gloves must be changed whenever they are torn. In addition, laboratory manipulations cause gloves to fatigue, therefore, they should be changed often. It is recommended that the operator wear a respirator protective device appropriate for aerosol protection, e.g., N95 NIOSH-approved particulate respirators covering nose and mouth, and safety glasses with side shields. For added splash protection, a full face shield may be placed over the respirator and the glasses. Recently, for an added margin of safety a complete DePuy Bio-Hazard Respiratory System (DePuy Chesapeake Surgical, Ltd., Sterling, VA) consisting of a body suit, a helmet and its battery-powered respiratory system with electrostatic filter media, has become commercially available (36). Protective clothing is not worn outside the laboratory.

3. Specimen handling

All specimen processing prior to cell sorting should be performed in biological safety cabinets. Capped tubes or microtiter plates with sealed covers should be used as sample containers. For local transport place primary collection tubes or sample tubes into a secondary container which is able to contain the specimen in case of breakage of the primary container, e.g., a plastic carrier with a secure lid. For specimen centrifugation, use sealed vessels or safety carriers.

i. Avoid use of “sharps”

Avoid the use of needles, glass pipets, glass transfer pipets, or glass containers or tubes whenever possible for handling
or transferring any biological material and use suitable replacements. Dispose of any “sharps” using a leakproof, puncture-resistant container as specified by local biosafety regulations.

ii. No mouth pipetting

No mouth pipetting is allowed. Manual pipetting devices must be used and must be equipped with filters to prevent infectious liquid from contaminating the pipetting device.

iii. Sample preparation steps to minimize potential aerosol formation

Sort samples need to be prepared as single cell suspensions because aggregated cells can partially or completely clog sort nozzles and stop a sort. Any interruption of a potentially biohazardous sort increases the risk of operator exposure to pathogens contained in the sort sample due to an increased probability for splashes and escape of sort aerosols during the manipulations required to continue sorting. To reduce the formation of cell aggregates during sample preparation samples should be centrifuged at approximately 300 g for 5 to 10 minutes to pellet cells. Higher centrifugation speeds can damage cells and compact them so densely that they are difficult to break apart. Frozen cell samples that are thawed for sorting frequently contain dead cells which may release DNA into the media. DNA binds to the surface of live cells, and after centrifugation these samples form solid aggregates leading to nozzle clogging problems and excessive aerosol formation. In these situations, add 20 µg/ml of RNase-free DNase for 10 minutes at 37°C. Select an optimal solution for sample resuspension to maintain cell viability. Highly concentrated cell suspensions have an increased tendency to clump, therefore, dilute them to the lowest possible density for the sort speed used. Sort samples are often chilled to preserve cellular structures and prevent capping of antibodies bound to cell surface receptors. However, the cold can aggravate clumping, thus, keeping sort samples at an intermediate temperature such as 15°C may be preferable over sorting at 4°C. Immediately prior to sorting all samples should be filtered through filter meshes with the appropriate pore size for the nozzle tip used on the sorter.
iv. Work area clean-up

Work areas must be cleaned routinely. Discard all contaminated materials, e.g., sample and collection tubes, pipets, pipet tips, gloves, laboratory coats, using appropriate biohazard containers. Follow the established procedures at your institution for storage and disposal of biomedical/hazardous waste. Generally, this involves either autoclaving or decontamination with a 1/10 volume dilution of 0.71 M sodium hypochlorite (undiluted household bleach) prior to waste disposal. Wipe off all work surfaces with an appropriate disinfectant solution, taking into account the potential biohazard. Refer to Appendix 1 Table 3 for information on the application of chemical disinfectants. Summary information on the survival and disinfectant inactivation of HIV has been published (42-45), and is reviewed in (29).

v. Disinfection of spills

After any spill of biological material, the protection of personnel is the first priority. In general, for small spills on a non-permeable surface, a disinfecting agent, e.g., a 1/10 volume dilution of 0.71 M sodium hypochlorite (undiluted household bleach) is applied to a paper towel, placed on the spill, and allowed to make contact for an appropriate time to inactivate any biological organisms. Rapid clean-up of spills should be an established laboratory practice. Refer to Appendix 1 Table 3 for a summary of practical applications of chemical disinfectants. For the handling of larger spills or spills on a non-smooth or permeable surface, refer to the Clinical Laboratory Standard Institute document (M29-A) (28) or the biosafety office of your institution.

4. Accidental exposure

It is recommended that all laboratory personnel provide a serum sample when they initiate their employment for storage as a baseline for future assay in the event of accidental exposure. Additional serum samples may be collected periodically, depending on the agents under study in the laboratory. Each laboratory should develop or adapt a written protocol to be
followed in case of a suspected exposure to a biohazardous agent. Current guidelines for postexposure management of healthcare workers should be used and a medical evaluation, surveillance and treatment record must be maintained (46-48). Guidelines for postexposure chemo-prophylaxis against HIV infection are available from CDC and should always follow the latest recommendations available on line at www.cdc.gov/mmwr.

C. Environmental controls

Cell sorting of unfixed samples, especially when using sorters with high operating pressures, should be performed in a BSL-3 laboratory facility because containment of the sorter could be breached any time during a sort due to a variety of reasons and release potentially hazardous materials into the area surrounding the instrument. A BSL-3 type room is only accessible through a passageway with self-closing lockable doors and requires a ducted HEPA-filtered air ventilation system, water resistant interior surfaces (walls, floor, ceiling) and laboratory furniture that can be easily cleaned and decontaminated, sealed windows, and a sink that can be operated without hands (refer to Appendix 1, Table 2). For further details refer to CDC Publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999. However, sort facilities in existing institutions may not have all environmental safety features recommended for BSL-3. Sorting of unfixed cells can still be achieved in a BSL-2 laboratory facility provided the institution can modify the sorter room considering the following requirements:

- The air venting system discharges air towards the outside away from occupied areas or it is HEPA filtered
- The cell sorter is located in a separate, lockable room where no other laboratory activity is performed.
- Air flow in the room is balanced to create airflow into the room with no less than ten changes of air per hour. It is recommended that negative airflow be measured by a visual monitoring device located at the door.
- Access to the sorting room is restricted in order to allow the operator to concentrate on the sort and to maintain regular air flow and negative air pressure in the room. A sign should be placed on the outside of the door to indicate that a potentially biohazardous sort is in progress. This sign should also contain all necessary information for entering the room safely, if needed.
• All other safety recommendations for BSL-3 practices and personal protective equipment are followed rigorously.

Compact cell sorters that can be placed into a biocontainment safety enclosure or biosafety cabinet, or larger sorters that have been integrated into a biosafety cabinet (49) allow for BSL-3 containment without the need for building a BSL-3 room.

Ultraviolet (UV) room light may be used to sterilize the room after each sort at the end of the day after all personnel have left, although its effectiveness against different pathogens may vary and areas where UV rays are blocked will remain unexposed. Therefore, routine cleaning with disinfecting agents is highly recommended.

D. Instrument considerations

1. Proper operation of the cell sorter

Follow all the manufacturer’s recommendations for instrument operation and maintenance carefully. Perform high-speed sorting of unfixed cells only on instruments that were designed for increased sorting rates or have been properly modified by the manufacturer. Never increase the system pressure on a cell sorter designed for low pressure sorting, because the fluidic lines, valves, and fittings cannot reliably withstand the increased pressure.

i. Maintenance

Set up a rigorous sorter preventive maintenance schedule either as part of a service contract offered by the manufacturer of the instrument or performed by laboratory personnel. Routinely perform leak checks on the fluid lines of the cell sorter. To do this, gain access to the fluidic lines. Carefully check for wet areas, indicating leaks in the tubing. Inspect tubing for cracks and signs of stress, particularly around the fittings and where tubing passes through valves. Also inspect sheath lines and waste lines. Replace any leaking tubing.

ii. Sort mechanism

Verify the proper operation of the sort mechanism and the stability of the sort streams and droplet break-off each time immediately before attempting to sort a potentially biohazardous specimen. If the streams and the droplet break-off do not remain stable during
the sort set-up, correct the problem before attempting a potentially biohazardous sort.

iii. Decontamination

After each sort, the instrument should be decontaminated with a disinfecting agent, taking into account the biohazard under study. Sort collection tube holders are heavily exposed to sample droplets and must be carefully decontaminated before handling. As per regulations outlined in the Bloodborne Pathogen Standard (25) appropriate disinfectants to be used for decontamination of equipment or work surfaces exposed to blood or other potentially infectious materials include diluted bleach, Environmental Protection Agency (EPA)-registered tuberculocides, EPA-registered sterilants or products registered to be effective against HIV or HBV as listed on line at http://www.epa.gov/oppad001/chemregindex.html. Common laboratory disinfectants (4,50) applicable for instrument decontamination and their properties are listed in Appendix 1, Table 3. Before designing a cell sorter-specific decontamination protocol check with the instrument manufacturer that all components exposed to the disinfectant can tolerate exposure. Alcohols are not classified as high-level disinfectants, because they cannot inactivate bacterial spores and penetrate protein-rich materials, and isopropanol is not able to kill hydrophilic viruses. Wipe off all surfaces inside the sort chamber, the sample introduction port and holder. Run the disinfectant through the instrument for the appropriate exposure time. Always follow with distilled water to completely remove the disinfectant, because some disinfecting agents are corrosive and residual disinfectant solution can affect the viability of subsequent samples that are run through the sorter.

2. Sample introduction system

Cell sorters pressurize the sample tube once it is placed onto the sample introduction port. While newer generation instruments are equipped with completely enclosed sample introduction chambers for operator safety, some older sorters have an open port requiring careful operator handling. Each time a sample tube is put on the instrument, check the tube seal and its secure fit onto the sample introduction port. Otherwise, once the sample tube is pressurized it could be blown off and splash sample onto the operator. When the tube is removed, the sample line back-drips, creating a potential biohazard through spraying of droplets. Permit the
back-drip to go into a tube until the sample is flushed out of its introduction line to avoid splashing of sample droplets. The catch tray or trough should be decontaminated regularly. Droplet-containment is necessary to eliminate the back-drip from the sample introduction line for prevention of droplet splashes. Alternatively, installation of a plastic shield around the sample introduction port can block droplet spraying from the sample back-drip.

3. Nozzle tip

Since a clogged sort nozzle is one of the major reasons for increased aerosol production on cell sorters, samples should be prepared properly to minimize the formation of cell clumps. If clumped cells are present in the sample to be sorted, they need to be removed. Options include filtration through nylon mesh filters, e.g., different pore size meshes from Small Parts Inc. (Hialeh, FL), tubes with cell strainer caps (Becton Dickinson, Falcon), or individual cell strainers (Becton Dickinson, Falcon). Filter samples immediately before starting to sort to give cells less time to re-aggregate. For large cell numbers distribute cell aliquots into separate tubes and filter each sample individually before placing it onto the sorter. If feasible, put an in-line filter, e.g., from BD Biosciences (San Jose, CA), Cytek Developments (Fremont, CA), or made in the laboratory by heating the end of a clipped-off pipet tip and fusing it with nylon mesh, on the uptake port to prevent cell clumps from reaching the sort nozzle. Select a sort tip with the appropriate nozzle size for the cell size to be sorted. Smaller nozzle sizes provide optimal signal resolution and easy sort setup, however, to avoid clogged nozzles, it is recommended that the nozzle orifice be at least four times bigger than the cell diameter (51). Sort nozzles should be cleaned frequently by sonication between sorts to prevent build-up of cellular debris. Inspection under a microscope can help to determine if the nozzle is clear. However, cells can still aggregate inside a clean sort nozzle at the intersecting surface of the sample injection fluid with the sheath fluid, even during sorting of properly prepared samples. Accumulated cells at this intersection eventually break free and may partially clog the nozzle tip. When this happens, stop sample flow, turn off the high voltage and put the fluidic control into the off position. Modern sorters have safety devices that will stop the sorting process as soon as a clog develops and cover the collection vessels. In any case however, the sort chamber door must remain closed until aerosol has been cleared from the chamber. Visual verification of the actual time needed for aerosol clearance should be performed with bottled smoke (Lab Safety Supply Inc., Janesville, WI, or equivalent). Remove the sample to be sorted first. Then, only after aerosols have cleared, open the sort
collection chamber door and take out the collection vials. Cap all tubes. Clear the clog as appropriate. Before the sort is continued, make sure the stream emitted through the tip is straight and steady, and the droplet break-off and the side streams are stable.

4. Aerosol control measures

A standard safety feature of cell sorters includes an interlocking sort chamber door and a sample collection chamber designed to contain aerosols. These barriers are not always completely sealed and verification of air leaks should be performed with bottled smoke. Sealing of any such openings can aid in achieving aerosol containment. All modern sorters are equipped with a receptacle that is connected with a waste evacuation system for collection of the undeflected center stream in order to reduce aerosol production during cell sorting. Auxiliary vacuum pumps designed to remove aerosols from the sort chamber have become available as optional attachments for sorters and are highly recommended. For custom installation of a generic vacuum pump care needs to be taken that no air turbulences are created that will affect the stability of the sort streams. The effluent vacuum lines must be connected to a cartridge-type HEPA filter for removal of airborne particles and a filter flask containing a disinfectant, usually concentrated household bleach, in such quantity that the full flask contains a 1/10 volume dilution of bleach. For each sorting experiment the flask has to be emptied and refilled with fresh, concentrated bleach. Recently, a removable containment hood that is vented by a high-efficiency particulate air filtration (HEPA) filter/fan unit and covers the sort area and the sample introduction port (Cytek Development, Fremont, CA) has become commercially available to improve containment on FACStar™, FACSVantage™, and FACSDiVa™ (BD Biosciences) cell sorters. Dako Colorado, Inc. (Fort Collins, CO) provides a Class I Biosafety Cabinet attachment for their MoFlo® high-speed cell sorter. An efficient containment system on the sorter will be able to remove aerosols as long as the sort chamber door remains closed. Because of the potential hazard to operators if aerosol droplets escape, the efficiency of aerosol containment on the cell sorter must be verified in regular sorting mode and in instrument failure mode before a potentially biohazardous sort is attempted. If aerosol containment is incomplete, the safety features of the cell sorter must be modified such that no escape of aerosol can be detected. Alternately, sorters can be placed inside a biosafety containment cabinet. Older cell sorters with water-cooled lasers were too large to fit into biological safety cabinets, although successful adaptation of a system to accommodate a biosafety cabinet has been described (49). Recently, instruments are becoming small enough, e.g.,
BD FACSAria (BD Biosciences), JSAN (Bay bioscience Co., Ltd., Japan) to be completely enclosed into a Walk-In Clean Air and Biocontainment Biological Safety Enclosure (BioPROtect II, The Baker Co., Sanford, ME), and novel compact sorters, e.g., inFlux (Cytopeia), Reflection (iCyt), can be accomodated in biological safety cabinets.

5. Stream view cameras

Sort stream viewing cameras are standard on newer sorters. They prevent the sorter operator from coming close to the area of the instrument that poses the greatest potential biohazard. Viewing systems that illuminate the center stream and the deflected streams near the sort collection vials with a low-powered laser beam are recommended as they allow the operator to monitor increased aerosol production due to shifting stream positions and fanning.

IV. Limitations and alternate technologies

Complete BSL-3 containment is required when agents known to be transmitted by the inhalation route are sorted since the greatest potential of exposure or infection from these agents is from aerosolization (e.g., M. Tuberculosis) and they are known to cause substantial morbidity and mortality. The need to perform such viable sorting experiments should be reviewed on a case by case basis with the biosafety office and infectious disease professionals of the institution. Cell fixation and alternate cell separation technologies, e.g., manual or automated magnetic bead separation or cell adherence to coated flasks, that can be readily performed in biological safety cabinets should be considered. Future novel high-speed cell sorting technologies that do not generate aerosols, e.g., ultra rapid fluid switching technology, could offer an alternative to droplet-based sorting (22).

Samples labelled with radionuclides are posing major problems as most sorting laboratories do not have authorization for using sources of ionizing radiation. In addition, the stringent requirements for handling such materials, including monitoring their use and disposal, and the issues concerning instrument contamination and the generation of radioactive aerosols during sorting are making flow cytometry experiments that involve cells labelled with radionuclide tracers not generally feasible.

V. Training and Experience

1. Minimum experience of sorting cells

Only experienced flow cytometry operators should perform potentially biohazardous sorts. The time required to obtain cell sorting proficiency on a given sorter varies, but training periods of 6 months are common. Some novel
sorters do not require the complex alignment procedures required for older type instruments and laboratories do not feel a need for a dedicated instrument operator. It is however important that any operator who performs potentially biohazard sorting be trained carefully in the proper instrument operation and all relevant safety procedures, including aerosol containment testing. The operator should have previous laboratory experience and a minimum of two years of experience in flow cytometry. Ideally, this should include training in performing sorting on deflected-droplet cell sorters using non-infectious control material of the same type that will contain the known biohazard, e.g., peripheral blood mononuclear cell preparations.

2. Experience with potential pathogens

Previous working experience with potentially biohazardous specimens is strongly recommended. Knowledge of the characteristics of common bloodborne pathogens and of the specific infectious organism present in the specimens to be sorted will help the sorter operator to formulate safe working practices (52).

3. Mechanism for institutional evaluation and approval

Each institution should establish a biosafety committee for review of potentially hazardous laboratory protocols. A thorough review of the protocols and procedures for sorting of unfixed human cells will include but not be limited to the procedures used to establish containment of the cell sorter and an evaluation of the containment. Ad hoc review committees could be established for each application. These may consist of the investigator requesting the sort, the sorter operator, a representative of the biosafety office of the institution, and a scientist not involved with the protocol under consideration.

VI. Assessment of aerosol containment

The classic method for assessment of aerosol containment on deflected-droplet cell sorters using aerosolized bacteriophage and a detection system of bacterial lawns has been described in several publications (1,13,33,35). The T4 bacteriophage method for assessment of containment can also be combined with active air sampling for testing room air (1,34,35,41). The T4 settle plate method and the active air sampling method are described in detail in Appendix 2. Tagging aerosol droplets with bacteriophages is an established technique that, provided the titer of the bacteriophage is sufficiently high, insures that all droplets generated during the test sort contain T4. Because Merrill has established that a single phage is sufficient to generate one plaque (13) the assay provides high sensitivity. Furthermore, the readout of containment results by counting plaques is straightforward. However, the method requires intermediate knowledge of
microbiological techniques, depends on the performance of biological materials, and even when all the materials have been pre-prepared, results take overnight.

Recently, a novel assay for measuring the efficiency of aerosol containment has been described (38,39). The method uses a suspension of highly-fluorescent melamine copolymer resin particles (Glo Germ™, Glo Germ Inc., Moab, UT) with an approximate size range between 1 to 10 µm simulating a biological sample during the test sort. Aerosol containment is measured by placing microscope slides around the instrument where aerosols are produced and could escape and examining the slides for the presence of Glo Germ™ particles under a fluorescent microscope. Perfetto et al. (36,40) have increased the assay’s sensitivity and reproducibility by using a viable microbial particle sampler. This device draws room air onto a microscope slide and concentrates the collected Glo Germ™ particles onto the areas on the slide located directly underneath the intake ports. This technique is described in Appendix 2 and is suitable to be performed immediately before starting a potentially biohazardous sort, but for this practice a fluorescent microscope has to be readily accessible. Glo Germ™ particles are highly fluorescent and therefore easily detected. However, careful handling of the microscope slides and the air sampler are important to avoid false positives, and diligent scanning of the entire slide is needed to reliably detect escape of single particles.

Before sorting any potentially biohazardous specimens on a given instrument, it is imperative to validate that aerosols are contained during the regular sorting process and during instrument failure modes (see Appendix 2). If aerosols are detected outside of containment then the cell sorter must be modified such that no aerosols are detectable. Contacting the manufacturer of the cell sorter for instructions and information will be necessary to make these instrument modifications. Testing must also be done whenever changes are made to the cell sorter that may affect escape of aerosols, e.g., installation of a new drive head or flow cell, replacement of the sort chamber door, or alterations in the aerosol management system. For instruments that are placed into biological safety cabinets it is imperative that laboratories validate the efficiency of aerosol containment of the cabinet before any potentially biohazardous sorting experiments are performed.

Since every live infectious sort has the potential to create infectious aerosols, verification of aerosol containment should be performed as often as possible. Therefore, it is highly recommended to perform testing prior to every infectious cell sort and maintain a record of the results. This practice will assure validation of the aerosol management system to contain aerosols containing potentially infectious pathogens.
VII. Conclusions

Laboratories involved in basic or clinical research are faced with increasing demands for sorting of unfixed samples. Biohazardous sorting is often performed for infectious disease studies to separate leukocyte subsets on the basis of cell surface expression patterns. Sorted cell populations can then be examined for their response to the pathogen of interest, for cellular mechanisms of its pathogenesis, or to identify or characterize cells infected with the pathogenetic organism (33). Recent sorting applications include studies of gene expression in cells that either carry a pathogen or have been transfected with fluorescent vectors that contain genetic sequences of an infectious agent (53). Novel applications involve preparative cell sorting of clinical samples for therapeutic interventions (54,55). Clinical cell sorting not only requires protection of instrument operators from known or unknown pathogens contained in the patient samples but sorting has to be performed using Good Manufacturing Practices under clean room conditions to prevent contamination of the sorted product to be re-infused into the patient (21,56,57).

The prevention of exposure of laboratory personnel to biohazards is of great importance. Although up to this point in time no infection due to sorting of unfixed material has been documented, the up-dated recommendations set forth here represent a timely effort of ISAC to provide a set of guidelines for sorting of unfixed cells, including known biohazardous samples. These recommendations may also aid laboratories in obtaining institutional (e.g., Institutional Biosafety Committee, Institutional Review Board) and/or regulatory agency approval for sorting of such unfixed cells. Furthermore, it is hoped that these guidelines will continue to prompt cell sorter manufacturers to design any new instruments with operator safety in mind.

Appendix 1: Table 1: Recommended biosafety containment levels for various agents

Table 2: Laboratory practices associated with biosafety levels

Table 3: Disinfectants

Appendix 2: Procedure for verification of aerosol containment on cell sorters using T4 bacteriophage and a bacterial lawn settle plate detection system

Procedure for assessing escape of ≤5µm particles (droplet nuclei) on cell sorters using T4 bacteriophage and a N6-Andersen single-stage air sampler

Procedure for verification of aerosol containment on cell sorters using Glo Germ™ particles and a particle sampler


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## Appendix 1

### Table 1. Infectious agents associated with laboratory-acquired infections due to manipulation with biological samples*

<table>
<thead>
<tr>
<th>AGENT</th>
<th>SOURCE OF INFECTION</th>
<th>SPECIES</th>
<th>ROUTE OF INFECTION</th>
<th>BIOSAFETY LEVEL PRACTICES, SAFETY EQUIPMENT, &amp; FACILITIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis B, C, D virus</td>
<td>blood, cerebrospinal fluid, urine, tissues</td>
<td>human, naturally or experimentally infected primates</td>
<td>inoculation, exposure of mucosal membranes to aerosols, broken skin</td>
<td>BSL2 in case of aerosol production, large quantities or high concentrations</td>
</tr>
<tr>
<td>Herpes virus simiae (B virus)</td>
<td>primary cultures (Rh monkey kidney cells)</td>
<td>macaque or human</td>
<td>inoculation, ? aerosol inhalation minimal</td>
<td>BSL3</td>
</tr>
<tr>
<td>Herpes Simplex 1,2 varicella virus</td>
<td>ubiquitous</td>
<td>opportunistic pathogen in immuno-compromised host</td>
<td>direct contact or aerosol inhalation</td>
<td>BSL4 for large quantities or high concentrations</td>
</tr>
<tr>
<td>Cytomegalovirus Epstein-Barr virus (EBV)</td>
<td>blood, tissues, EBV-transformed cell lines</td>
<td>human</td>
<td>inoculation, exposure of mucosal membranes to aerosols, broken skin</td>
<td>BSL2</td>
</tr>
<tr>
<td>Herpes 6,7 virus</td>
<td>blood, bronchoalveolar lavage</td>
<td>human</td>
<td>risk not known</td>
<td>BSL2</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>bronchoalveolar lavage, respiratory tissues</td>
<td>human, naturally or experimentally infected animals</td>
<td>aerosol inhalation</td>
<td>BSL2</td>
</tr>
<tr>
<td>Lymphocytic chorio-meningitis virus</td>
<td>blood, cell cultures, nasopharynx secretions, bronchoalveolar lavage, tissues</td>
<td>nude mice, SCID mice, naturally infected macaques or marmosets, possibly man</td>
<td>inoculation, exposure of mucosal membranes to aerosols, broken skin well documented transmission by aerosol inhalation</td>
<td>BSL2 in case of aerosol production, large quantities or high concentrations</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>fluids, tissues, respiratory secretions</td>
<td>naturally or experimentally infected non-human primates, transgenic mice</td>
<td>ingestion, parenteral inoculation,</td>
<td>BSL2 practices by vaccinated personnel WHO guidelines for establishing BSL2/polio and BSL3/polio laboratories after wild polio has disappeared when oral vaccination has stopped BSL4 for work with wild polio</td>
</tr>
<tr>
<td>AGENT</td>
<td>SOURCE OF INFECTION</td>
<td>SPECIES</td>
<td>ROUTE OF INFECTION</td>
<td>BIOSAFETY LEVEL</td>
</tr>
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<td>--------------------------------------------</td>
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</tr>
<tr>
<td>Pox viruses</td>
<td>lesion fluid, tissues, respiratory</td>
<td>infected volunteers or animals</td>
<td>inoculation, exposure of mucosal membranes to aerosols, broken skin</td>
<td>BSL2 practices by vaccinated personnel</td>
</tr>
<tr>
<td>genetically engineered recombinant vaccinia</td>
<td>secretions, bronchoalveolar lavage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>virus</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Human immunodeficiency virus (HIV-1, 2)</td>
<td>blood, body fluids, tissues</td>
<td>human</td>
<td>inoculation, exposure of mucosal membranes to aerosols (containing concentrated virus), broken skin</td>
<td>BSL2</td>
</tr>
<tr>
<td>Retroviral vectors containing full-length</td>
<td></td>
<td></td>
<td></td>
<td>BSL3 in case of aerosol production, large quantities or high concentrations</td>
</tr>
<tr>
<td>infectious genomes</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Simian immunodeficiency virus (SIV)</td>
<td>blood, body fluids, tissues</td>
<td>macaque</td>
<td>inoculation</td>
<td>BSL2</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>blood, body fluids, tissues</td>
<td>infected animals, humans</td>
<td>exposure to infectious droplets or aerosols, direct skin and mucosal membrane exposure</td>
<td>BSL3 for laboratory-adapted strains with demonstrated low virulence</td>
</tr>
<tr>
<td>HTLV-1, 2 virus</td>
<td>blood, body fluids, tissues</td>
<td>human</td>
<td>inoculation, exposure of mucosal membranes to aerosols (containing concentrated virus), broken skin</td>
<td>BSL2</td>
</tr>
<tr>
<td>Rickettsia prowazekii</td>
<td>infected tissues</td>
<td>naturally infected non-human</td>
<td>inoculation, aerosol inhalation</td>
<td>BSL2</td>
</tr>
<tr>
<td>Coxiella burnetii</td>
<td>blood, urine, tissues</td>
<td>infected animals, humans</td>
<td>Inoculation, exposure to infectious aerosols</td>
<td>BSL3 for cell culture and manipulation of tissues</td>
</tr>
<tr>
<td>Brucella</td>
<td>blood, cerebrospinal fluid, tissues</td>
<td>human, experimentally</td>
<td>inoculation, direct skin contact</td>
<td>BSL2</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>blood, cerebrospinal fluid, pleural</td>
<td>naturally and experimentally</td>
<td>exposure of intact and broken skin, inoculation</td>
<td>BSL2</td>
</tr>
<tr>
<td></td>
<td>fluid</td>
<td>infected animals</td>
<td></td>
<td>BSL3 in case of aerosol production, large quantities or high concentrations</td>
</tr>
<tr>
<td>AGENT</td>
<td>SOURCE OF INFECTION</td>
<td>SPECIES</td>
<td>ROUTE OF INFECTION</td>
<td>BIOSAFETY LEVEL</td>
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</tr>
<tr>
<td><em>Chlamydia psittaci</em></td>
<td>blood, tissues</td>
<td>birds, human</td>
<td>exposure to infectious aerosols and droplets</td>
<td>BSL2 BSL3</td>
</tr>
<tr>
<td></td>
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<td>in case of</td>
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<td>aerosol</td>
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<td>production,</td>
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<td>large quantities</td>
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<td>or high</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>concentrations</td>
</tr>
<tr>
<td>Leptospira</td>
<td>blood, tissues</td>
<td>infected animals, human</td>
<td>inoculation, skin and mucous membrane contact</td>
<td>BSL2</td>
</tr>
<tr>
<td>interrogans</td>
<td></td>
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</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>blood, cerebrospinal fluid</td>
<td>naturally or experimentally infected animals</td>
<td>ingestion, eye and skin exposure</td>
<td>BSL2</td>
</tr>
<tr>
<td>Mycobacterium</td>
<td>bronchoalveolar lavage, lesion tissues</td>
<td>human</td>
<td>inoculation, direct skin contact, aerosol inhalation</td>
<td>BSL2</td>
</tr>
<tr>
<td>atypicum</td>
<td></td>
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</tr>
<tr>
<td>Mycobacterium</td>
<td>gastric lavage, cerebrospinal fluid, pleural fluid, urine</td>
<td>human, naturally infected primates</td>
<td>aerosol inhalation</td>
<td>BSL3</td>
</tr>
<tr>
<td>tuberculosis</td>
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</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>sinovial fluid, urine, cerebrospinal fluid</td>
<td>human</td>
<td>inoculation, direct skin contact</td>
<td>BSL2</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>pharyngeal exudates, bronchoalveolar lavage, cerebrospinal fluid, blood</td>
<td>human</td>
<td>inoculation, direct skin contact, aerosol inhalation</td>
<td>BSL2 BSL3</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>blood</td>
<td>human</td>
<td>inoculation, direct skin contact</td>
<td>for large</td>
</tr>
<tr>
<td>Salmonella</td>
<td></td>
<td></td>
<td></td>
<td>quantities</td>
</tr>
<tr>
<td>Treponema pallidum</td>
<td>lesion fluid</td>
<td>humans with primary and secondary syphilis</td>
<td>inoculation, direct skin contact, aerosol inhalation</td>
<td>BSL2</td>
</tr>
<tr>
<td>Toxoplasma</td>
<td>blood</td>
<td>humans or experimentally infected animals</td>
<td>inoculation, aerosol inhalation</td>
<td>BSL2</td>
</tr>
<tr>
<td>Trypanosoma</td>
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<tr>
<td>Leishmania</td>
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<tr>
<td>Plasmodium</td>
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<td></td>
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</tr>
<tr>
<td>Blastomyces</td>
<td>tissues</td>
<td>infected animals</td>
<td>inoculation, aerosol inhalation</td>
<td>BSL2 BSL3</td>
</tr>
<tr>
<td>Coccidioides</td>
<td></td>
<td></td>
<td></td>
<td>for cultures</td>
</tr>
<tr>
<td>Histoplasma</td>
<td></td>
<td></td>
<td></td>
<td>containing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Coccidioides,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Histoplasma</td>
</tr>
</tbody>
</table>

*This table was adapted from US HHS Publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999.
## Appendix 1

**Table 2. Summary of laboratory practices associated with biosafety levels**

<table>
<thead>
<tr>
<th>Biosafety levels</th>
<th>BSL-1</th>
<th>BSL-2</th>
<th>BSL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Hazard levels</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low risk</td>
<td>Low to moderate</td>
<td>Moderate to high</td>
<td></td>
</tr>
<tr>
<td><strong>B. Standard microbiological practices</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Public access while experiments are in progress</td>
<td>Limited</td>
<td>Controlled</td>
<td>Not permitted</td>
</tr>
<tr>
<td>2. Handwashing facilities</td>
<td>Required</td>
<td>Required</td>
<td>Not permitted</td>
</tr>
<tr>
<td>3. Eating, drinking, smoking, application of cosmetics, storing food for human consumption</td>
<td>Not permitted at any time</td>
<td>Not permitted at any time</td>
<td>Not permitted at any time</td>
</tr>
<tr>
<td>4. Pipetting</td>
<td>Mechanical devices</td>
<td>Mechanical devices</td>
<td>Mechanical devices</td>
</tr>
<tr>
<td>5. Safe handling of sharps</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>6. Minimization of aerosol production</td>
<td>Recommended</td>
<td>Recommended</td>
<td>Required</td>
</tr>
<tr>
<td>7. Decontamination of work surfaces</td>
<td>Daily and upon spills</td>
<td>Daily and upon spills</td>
<td>Daily, upon finished work with infectious material, and spills</td>
</tr>
<tr>
<td>8. Infectious waste decontamination</td>
<td>Before disposal, placed in durable leak-proof container for transport</td>
<td>Before disposal, placed in durable leak-proof container for transport</td>
<td>Before disposal, placed in durable leak-proof container for transport</td>
</tr>
<tr>
<td>9. Biohazard sign</td>
<td>Posted at entrance</td>
<td>Posted at entrance</td>
<td>Posted at entrance</td>
</tr>
<tr>
<td>10. Insect/rodent control program</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td><strong>C. Special practices</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Immunization</td>
<td>Not required</td>
<td>Recommended</td>
<td>Recommended; depending on the specific biohazard vaccination may be required</td>
</tr>
<tr>
<td>2. Medical surveillance (e.g., baseline serology, periodic testing)</td>
<td>Not required</td>
<td>Required when appropriate</td>
<td>Required when appropriate</td>
</tr>
<tr>
<td>3. Specific biosafety manual and training in policies and procedures appropriate for hazard potential</td>
<td>Not required</td>
<td>Required with annual up-dates</td>
<td>Required with annual up-dates</td>
</tr>
<tr>
<td>4. Bench top work</td>
<td>Permitted</td>
<td>Permitted</td>
<td>Permitted in some circumstances, but appropriate combinations of personal protective equipment, e.g., face-shields, respirators must be used</td>
</tr>
<tr>
<td><strong>Biosafety levels</strong></td>
<td><strong>BSL-1</strong></td>
<td><strong>BSL-2</strong></td>
<td><strong>BSL-3</strong></td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>D. Safety equipment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Laboratory coats, gowns, uniforms</td>
<td>Recommended, not worn outside the laboratory</td>
<td>Required, not worn outside the laboratory</td>
<td>Solid-front, wrap-around disposable clothing required for all workers with potential exposure to infectious agents</td>
</tr>
<tr>
<td>2. Gloves</td>
<td>Recommended, worn when skin contact with infectious material is unavoidable</td>
<td>Required when skin contact with infectious material is unavoidable; double gloving may be appropriate</td>
<td>Required when skin contact with infectious material is unavoidable; double gloving may be appropriate</td>
</tr>
<tr>
<td>3. Biological safety cabinets</td>
<td>Not required</td>
<td>Required for all aerosol generating processes</td>
<td>Required for all work with infectious agents</td>
</tr>
<tr>
<td>4. Other physical containment</td>
<td>Recommended that equipment be decontaminated immediately after use</td>
<td>Appropriate physical containment devices are used when procedures with high potential for creating infectious aerosols are being conducted&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Appropriate physical containment devices such as centrifuge safety cups, sealed centrifuge rotors are used for all activities with infectious materials that pose a threat of aerosols exposure&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5. Freezers/refrigerators</td>
<td>Recommended that biohazard sign be posted</td>
<td>Biohazard sign must be posted</td>
<td>Biohazard sign must be posted; all agents must be stored in separate, closed, labeled containers</td>
</tr>
<tr>
<td><strong>E. Laboratory facilities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Ventilation</td>
<td>No special requirements</td>
<td>Negative pressure</td>
<td>Ducted exhaust air ventilation system</td>
</tr>
<tr>
<td>2. Laboratory separated from the general public</td>
<td>Not required</td>
<td>Yes, while experiments are in progress</td>
<td>Required</td>
</tr>
<tr>
<td>3. Lockable doors</td>
<td>Not required</td>
<td>Required</td>
<td>Passageway with two self-closing doors</td>
</tr>
<tr>
<td>4. Sink</td>
<td>Recommended</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>5. Laboratory surfaces easy to clean, impervious to water, resistant to chemicals used for disinfection</td>
<td>Work surfaces</td>
<td>Work surfaces</td>
<td>Work surfaces, floors, walls and ceilings</td>
</tr>
<tr>
<td>6. Autoclave inside facility</td>
<td>Not required</td>
<td>Not required</td>
<td>Required</td>
</tr>
<tr>
<td>7. HEPA-filtered vacuum lines</td>
<td>Not required</td>
<td>Recommended</td>
<td>Required</td>
</tr>
</tbody>
</table>

<sup>a</sup> These procedures include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, opening containers of infectious materials whose internal pressures may be different from ambient pressures

<sup>b</sup> These procedures include manipulation of cultures and of clinical or environmental material that may be a source of infectious aerosols

* This table was adapted from US HHS publication “Biosafety in Microbiological and Biomedical Laboratories”, 1999
### Appendix 1

#### Table 3. Summary of practical applications of chemical disinfectants for decontamination*

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Quaternary ammonium compounds</th>
<th>Phenolic compounds</th>
<th>Chlorine compounds</th>
<th>Hydrogen peroxide</th>
<th>Iodophor</th>
<th>Alcohols</th>
<th>Aldehydes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/10-1/100 dilution of 0.71 M sodium hypochlorite, ~50-500 ppm (a)</td>
<td>3-6%</td>
<td>0.0075%-10%, ~25-1600 ppm (a)</td>
<td>70-85%</td>
<td>70-85%</td>
<td>0.2-8%</td>
<td>2%</td>
</tr>
<tr>
<td>Use dilution</td>
<td>0.1-2%</td>
<td>1-5%</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Contact time to lipovirus</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Broad spectrum</td>
<td>not effective</td>
<td>not effective</td>
<td>30 min</td>
<td>30 min</td>
<td>30 min</td>
<td>not effective</td>
<td>not effective</td>
</tr>
</tbody>
</table>

#### Practical requirements

- **Vegetative bacteria**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Lipoviruses**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Non-lipid viruses**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Bacterial spores**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Fungi**: (c) ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Protozoal parasites**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓

#### Inactivation profile

#### Characteristics

- **Stability (d)**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Corrosive**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Flammable**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Residue**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Organic material inactivated (e)**: ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓

#### Potential application

- **Surfaces (f)**: ✓ (g) ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
- **Instrument surfaces and parts**: ✓ ✓ ✓ ✓ ✓ ✓
- **Flow cytometer fluid lines**: ✓ ✓ ✓

---

(a) Available halogen; iodophors may require prolonged contact time for inactivation of bacterial spores, Tubercle bacilli, and certain fungi
(b) Variable results depending on the virus; activity drops sharply when alcohols are diluted below 50%
(c) Weak activity only
(d) Shelf life greater than 1 week when protected from light and air
(e) Prior to decontamination cleaning with lipophilic detergent/disinfectant necessary
(f) Work surfaces, decontamination of fixed or portable equipment surfaces
(g) Usually compatible with optics, but consider interference from residues and effects on associated materials such as mounting adhesives

---

* This table was adapted from “Biohazardous Operations”, Lawrence Livermore National Laboratory (1995) and from [http://www.ianr.unl.edu/animaldisease/g1410.htm](http://www.ianr.unl.edu/animaldisease/g1410.htm).
APPENDIX 2

TESTING OF AEROSOL CONTAINMENT ON CELL SORTERS

1) BACTERIOPHAGE AND E. COLI SETTLE PLATES

2) TESTING ROOM AIR WITH BACTERIOPHAGE AND AN ACTIVE AIR SAMPLING METHOD

3) TESTING AEROSOL CONTAINMENT USING GLO GERM™ PARTICLES AND A MICROBIAL PARTICLE SAMPLER

Introduction: This section contains laboratory protocols developed for aerosol containment testing of sorters. The T4 bacteriophage protocol written by Ingrid Schmid was developed in the laboratory of the late Dr. Janis V. Giorgi and incorporates work performed by Lance Hultin and Dr. John Ferbas. It was expanded from previous publications (1,2,3) and has been published in more detail in a chapter in Current Protocols in Cytometry (4) and in a recent chapter (5). The bead-based aerosol testing method described here was developed by Stephen Perfetto and others in the laboratory of Dr. Mario Roederer (6,7) and incorporates work described in previous publications (8,9).

Purpose: As part of the normal sorting process, deflected-droplet fluorescence-activated cell sorters produce droplets and microdroplets. Secondary aerosols are produced when the fluid streams splash into receptacles and when a clogged nozzle causes a deflection in the streams that is striking an obstacle. Droplets that are smaller than 80 µm can be aerosolized and could escape into the area surrounding the instrument. If these aerosols harbor active infectious agents they pose a potential hazard to the operator. Therefore, the effectiveness of aerosol control measures on commercially available cell sorters has to be evaluated during routine operation and in failure mode. Furthermore, any jet-in-air flow cytometer even when it is not used for cell sorting can produce aerosols, particularly, when the nozzle is partially clogged; therefore, it is advisable to also test instruments that are used for data acquisition of unfixed samples. As most of the
aerosol mass produced by cell sorters consists of droplets of < 5 µm size and thus quickly settles from air through gravity, the settle plate method described below will effectively measure aerosol escape. However, cell sorters can also produce aerosolized droplets of ≤5 µm sizes (droplet nuclei). Droplet nuclei can stay suspended in air for prolonged periods of time and can be deposited into the lung of the exposed individual during inhalation. If testing of the instrument for escape of droplet nuclei is desired an active air sampling method has to be used. The T4 bacteriophage assay has high sensitivity because it has been established that one phage is sufficient to create a plaque and the readout is straightforward. However, intermediate knowledge of microbiological techniques is required, preparation of the biological materials for the test take several days, and results require overnight incubations.

Several years ago a non-biological method for measuring aerosol escape was developed (7) using highly-fluorescent melamin copolymer resin particles (Glo Germ™, Inc., Moab, Utah). Perfetto et al. modified the original method (6,7). A suspension of these particles is run through the instrument at speeds and concentrations simulating a biological sample. Aerosol containment is tested by placing microscope slides into a vacuum-driven air device designed to efficiently sample aerosols from air (2L/sec). This device can be positioned around the instrument testing a variety of areas for potential aerosol escape. Slides are then examined under a fluorescent microscope for the highly-fluorescent resin particles. Readouts produce a “real-time” quantitative result of containment and therefore is suitable to demonstrate protective containment before each infectious sort is performed.

1) BACTERIOPHAGE AND E. COLI SETTLE PLATES

2) TESTING ROOM AIR WITH BACTERIOPHAGE AND AN ACTIVE AIR SAMPLING METHOD

A broth culture of Escherichia coli (E. coli) is set up to create confluent bacterial lawns on petri dishes. These dishes are placed in the working environment while T4 bacteriophage are run through the instrument and are aerosolized. Plaques formed by the bacteriophage landing on the bacterial E. coli lawns and lysing the bacteria are used to indicate the presence of aerosol. For testing of room air, petri dishes with confluent lawns of E. coli are placed into a single-stage N6 Andersen air sampler. Air is drawn onto these dishes while
T4 bacteriophage are run through the cells sorter. Bacteriophage plaques formed on the lawns indicate the presence of droplet nuclei (aerosolized droplets of <5 µm sizes) in the room air. The procedures are relatively simple but must be followed closely in order to insure the success of the experiment. Although concerns exist about utilization of *E. coli* in an environment where sterile cell sorting is to be performed, our laboratory has not encountered problems with the sterility of sorted samples since we have started to test our instruments for aerosol containment. No plaques should be detected on the petri dishes placed outside the sort chamber and on petri dishes that were collected from the air sampler. Otherwise, the instrument has to be modified to achieve aerosol containment. Repeat testing every one to three months.

I. PREPARATION OF MATERIALS

A) Media: All media ingredients are per liter

- **Nutrient broth**
  
  a) Bacto nutrient broth (Difco 0003-02-05), 8.0 g
  
  b) sodium chloride, 5.0 g
  
  c) glucose, 1.0 g

  Adjust the pH to 7.2 - 7.4 with 1N NaOH before autoclaving for 15 min, 121°C.

- **Dilution broth**
  
  a) Bacto tryptone (Difco 0123-15-5), 10.0 g
  
  b) sodium chloride, 5.0 g

  Adjust the pH to 7.2 - 7.4 with 1N NaOH before autoclaving for 15 min, 121°C.

- **Top agar**
  
  a) minimal agar Davis (Difco 0544-17-4), 6.5 g
  
  b) Bacto tryptone, 13.0 g
  
  c) sodium chloride, 8.0 g
  
  d) sodium citrate (dihydrate), 2.0 g
  
  e) glucose, 3.0 g

  Adjust the pH to 7.2 - 7.4 with 1N NaOH before autoclaving for 15 min, 121°C.

- **Bottom agar**
a) minimal agar (Davis), 10.0 g
b) Bacto tryptone, 13.0 g
c) sodium chloride, 8.0 g
d) sodium citrate (dihydrate), 2.0 g
e) glucose, 1.3 g

Adjust the pH to 7.2 - 7.4 with 1N NaOH before autoclaving for 15 min, 121°C.

B) T4 bacteriophage stock preparation:

- Rehydrate one vial of ATCC 11303 E. coli with 0.3-0.4 ml of nutrient broth and mix well. Transfer 0.1 ml of this suspension to a sterile 25 ml Erlenmeyer flask containing 10 ml of nutrient broth. Put a streak onto nutrient agar slants for short term storage (6 months). The rest of the E. coli suspension can be frozen at -20°C for long term storage.
- Incubate the flask on an orbital shaker (~150 rpm) at 37°C overnight. Also incubate the slants overnight at 37°C.
- Subculture 0.5 ml of the broth culture into sterile 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth. Incubate the flasks on an orbital shaker at 37°C until the culture enters log phase, or becomes slightly turbid. Refrigerate the slants for short term storage at 4°C (6 months).
- Rehydrate the lyophilized T4 bacteriophage (ATCC 11303-B4) with 0.5 ml of nutrient broth. Mix well and inoculate 0.1 ml of phage suspension into the log phase cultures of E. coli prepared as described above. Refrigerate the remaining bacteriophage suspension for short term storage at 4°C (6 months).
- Incubate the E. coli/T4 bacteriophage cultures at 37°C until lysis of E. coli is complete as indicated by clearing of the turbid suspension. This usually takes about 12 h, however, the cultures may be incubated overnight if they are started in the afternoon.
• Transfer the broth from the flasks with sterile 10 ml pipets into sterile 50 ml chloroform-resistant centrifuge tubes. Add 12 drops of chloroform to each tube and shake vigorously. Remove cellular debris by centrifugation at 2000 x g for 20 min.

• This bacteriophage stock solution can be refrigerated in a tube with minimal airspace indefinitely. The titer should be periodically determined to document that the titer has not decreased. The expected titer is > 1 x 10^9 plaque forming units (PFU)/ml and is determined by serial dilution as described below.

• The bacteriophage may be re-expanded, as needed, by inoculating a log phase E. coli culture by repeating this procedure, except that the bacteriophage is passed from the stock solution.

C) Preparation of bacterial lawns

a) About 1 week prior to the aerosolization experiment:

Prepare agar plates.

• First, the bottom agar is liquified (this can be done conveniently in a microwave oven, use an adequate container to prevent boil-over), then, approximately 20 ml are pipetted into each of twentythree 100 mm diameter petri dishes and allowed to solidify (23 plates are prepared because 6 are needed for titration of phage, 12 for the verification of aerosol containment on the flow cytometer, and 5 extra in case of an accident); six more are needed if a test with the air sampler is to be performed. The preparation of these plates (as well as the other procedures in this protocol) may be performed on the benchtop, provided that good aseptic technique is followed. After adding the liquified agar to the plates, leave the lid ajar until the plate cools.

• Once the agar has cooled and solidified, store the plates upside down. The plates may also be placed in a plastic bag to prevent dehydration and stored in a refrigerator or cold room for up to 6 months. The plates should be visually inspected for contamination so that new plates may be prepared prior to the experiment, if needed.

b) 18-24 h prior to the aerosolization experiment:

Set up a broth culture of E. coli.
• Using an inoculating loop (or sterile plastic pipet) transfer some *E. coli* from the agar slant to 100 ml of nutrient broth in a 250 ml sterile Erlenmeyer flask.

• Grow the *E. coli* at 37° C overnight on an orbital shaker. Use a sterile gauze pad to plug the Erlenmeyer flask.

c) **The day of the experiment:**

Combine *E. coli* with liquified “top agar” for the preparation of the plates that are used in the actual aerosol containment experiment on the flow cytometer.

• Heat ~110 ml top agar in a microwave and allow it to cool to between 40°-50° C. Monitor its temperature by placing an ethanol-sterilized thermometer into the liquified agar.

• Add ~2 ml of the *E. coli* broth culture to the top agar, swirl gently, and add 4 ml of this culture to 15 petri dishes (12 plus 3 extra) prepared with bottom agar (see above). Six more dishes are needed if room air is tested with the air sampler.

• Once all the plates are completed and the top agar has solidified, move the plates to a 37° C incubator or warm room to allow the *E. coli* to grow. Keep the plates level at all times so that the top agar remains intact.

• Allow the *E. coli* to grow in these plates for 1-2 h before using them in the aerosolization experiment. The plates should have a slightly opaque appearance after the 1-2 h incubation because the *E. coli* will begin to form a confluent lawn.

II. **PREPARATION FOR THE AEROSOLIZATION EXPERIMENT -**

-TITRATION OF THE T4 BACTERIOPHAGE

During this procedure, the bacteriophage stock is titrated, the throughput of bacteriophage per unit time on the flow cytometer is determined, and the viability of the bacteriophage stock after it is run through the instrument is assessed.

**A) Titration of the bacteriophage stock:**
**SCHEMATIC FOR TITRATION OF BACTERIOPHAGE STOCK:**

1. **Prepare serial dilutions**

0.1 ml of bacteriophage stock

- 0.1 ml 0.1 ml 0.1 ml 0.1 ml 0.1 ml 0.1 ml 0.1 ml

<table>
<thead>
<tr>
<th>Dilution broth (ml)</th>
<th>0.9</th>
<th>0.9</th>
<th>0.9</th>
<th>0.9</th>
<th>0.9</th>
<th>0.9</th>
<th>0.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution factor</td>
<td>$10^{-1}$</td>
<td>$10^{-2}$</td>
<td>$10^{-3}$</td>
<td>$10^{-4}$</td>
<td>$10^{-5}$</td>
<td>$10^{-6}$</td>
<td>$10^{-7}$</td>
</tr>
</tbody>
</table>

2. **Inoculate top agar**

Transfer 0.1 ml of the $10^{-7}$, $10^{-8}$, and $10^{-9}$ dilutions to the 3 tubes that contain 4 ml of top agar combined with *E. coli* prepared as described on page 7 under c), 2nd paragraph, except that the mixture is placed into a tube first.

3. **Pour petri dishes**

Pour the entire contents of these tubes onto petri dishes previously prepared with bottom agar.

- Once the 3 plates are inoculated and the top agar has solidified, move the plates to a 37° C incubator or warm room to allow the *E. coli* to grow overnight. The following day, plaques will appear on the bacterial lawns. By counting the plaques, the actual plaque forming units per ml of bacteriophage stock can be determined. Note that the lower dilutions are discarded in the above schematic because they will show confluent lysis of the bacterial lawns. However, if the titer of the bacteriophage stock is lower, some or all of these dilutions may have to be put onto plates to achieve appropriate numbers of plaques.
Example of results:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Number of plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-7}$</td>
<td>TNTC^</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>40 *</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>1</td>
</tr>
</tbody>
</table>

^ Too numerous to be counted

* Select a petri dish with an intermediate number of plaques (usually between 10-100) and correct the value by the final dilution factor. Note that the final dilution factor in this example actually reflects a dilution factor of $10^{10}$ (not $10^8$), because initially only 0.1 ml of the phage suspension was taken, then serially diluted, and finally 0.1 ml was added to the top agar.

\[
\text{e.g., 40 plaques } \times 10^{10} = 4 \times 10^{11} \text{ PFU/ml}
\]

B) Determination of bacteriophage throughput on the flow cytometer:

Bacteriophage throughput on the cell sorter is determined in order to insure that an adequate number of viable, infectious organisms are present within the sorting aerosol. For assessment of throughput, it is necessary to know the sample flow rate per unit time. The expected and actual throughput is measured in order to validate an inordinate number of bacteriophage are not destroyed during passage through the instrument.

a) Calculation of the expected throughput:

- Record the weight of exactly 1 ml of bacteriophage suspension in a tube that will be used for the sorting experiment.

\[e.g., 1.0 \text{ g}\]
• Run the sample through the instrument under normal sorting conditions for 10 min, and re-weigh.

\[ \text{e.g., } 0.6g \]

• Determine the sample throughput, where 1 mg = 1 µl.

\[ \text{e.g., } 1.0g - 0.6g = 0.4g \text{ or } 400 \mu l \]

\[ 400\mu l/10\text{ min} = 40\mu l/min \]

• Calculate the expected number of bacteriophage flowing through the instrument, e.g., where the concentration of bacteriophage is \( 4 \times 10^{11} \) PFU/ml.

\[ 40\mu l/min \times 4 \times 10^{11} \text{ PFU/1000}\mu l = 1.6 \times 10^{10} \text{ PFU/min} \]

b) Measurement of the \textit{actual} throughput.

Here, the number of bacteriophage flowing through the instrument is measured directly from the sheath stream as it exits the nozzle tip.

• Collect the sheath stream for exactly 1 min while running bacteriophage through the instrument into a tube that has been weighed empty. Re-weigh it to determine the volume collected in 1 min, e.g., 2.35 ml.

• Immediately transfer 0.1 ml of the sample to 0.9 ml of dilution broth. This is important because the bacteriophage in the sample needs to be titered to determine throughput, and bacteriophage may lose infectivity in many sheath fluids as a function of time. This sample can be titered immediately as described below. Alternatively, it may be held in the dilution broth at ambient temperature until the aerosolization experiment is completed.
C) Titration of bacteriophage run through the flow cytometer

SCHEMATIC FOR TITRATION OF BACTERIOPHAGE IN SHEATH FLUID:

1. Prepare serial dilutions

   0.1 ml of 1:10 diluted bacteriophage collected from the sheath stream

   Dilution broth (ml) 0.9 0.9 0.9 0.9 0.9 0.9 0.9
   Dilution factor 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}

2. Inoculate top agar

   Transfer 0.1 ml of the 10^{-6}, 10^{-7} and 10^{-8} dilutions to 3 tubes with 4 ml of liquified top agar combined with E. coli as described for bacteriophage stock

3. Pour petri dishes

   Pour the entire contents of these tubes onto petri dishes previously prepared with bottom agar.

- Once the 3 plates are inoculated and the top agar has solidified, move the plates to a 37° C incubator or warm room to allow the E. coli to grow overnight. The following day, plaques will appear on the bacterial lawns. By counting the plaques, the actual plaque forming units per unit time (actual throughput) can be determined. Note that the lower dilutions are discarded in the above schematic because they usually will show confluent lysis of the bacterial lawns. However, if the titer of the recovered bacteriophage suspension is lower, some or all of these dilutions may have to be put onto plates to get intermediate numbers of plaques.
Example of results:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Number of plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-6}</td>
<td>40 *</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>1</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>0</td>
</tr>
</tbody>
</table>

*To calculate the actual throughput, select a petri dish with an intermediate number of plaques (usually between 10-100) and correct the value by the final dilution factor. Note that the final dilution factor in this example actually reflects a dilution factor of 10^7 (not 10^6) because 0.1 ml of the sample was added to the top agar. Then, the total number of phages collected in 1 min is calculated by multiplying the result with the volume in the tube.

e.g., (40 plaques x 10^7/0.1 ml) x 2.35 ml = 9.4 x 10^9 PFU/min

III. TESTING THE FLOW CYTOMETER FOR AEROSOL CONTAINMENT USING SETTLE PLATES

Testing the flow cytometer for aerosol containment is performed in two parts. First, bacteriophage is run through the instrument for 2 h using instrument settings that approximate the maximum sorting rate that could be used during cell sorting. This condition is referred to as the “good” mode. Once completed, the sorter is adjusted to produce maximal fanning of the sort streams in order to produce as much aerosol as possible and run for 15 min. This condition is called “bad” mode.

- For the good mode, 6 petri dishes with confluent *E. coli* lawns are used. Two are placed within the sorting chamber, near the sorting streams. Two are placed immediately outside of the sorting chamber door, and the remaining two plates are placed somewhere near the instrument in the room. It may be necessary to jerry-rig a level platform for the dishes that are placed inside of the sorting chamber.

- After removing the lids from the plates, and closing the sorting chamber door, initiate the mock sort. Bacteriophage are run through the instrument and sorting streams are generated according to the
instrument-specific software with $\sim 1000$ sort decisions per sec each left and right. Approximately $3.5 \text{ ml}$ of bacteriophage suspension are needed.

- Run the good mode for $2 \text{ h}$. Replace the collection vials when they are full. Wait for $\sim 3 \text{ min}$ for aerosol clearance before opening the door. Place lids onto the petri dishes that are placed outside the door to avoid false positive plaques on the dishes outside the door.

- When the good mode is completed, replace the lids on each plate and incubate the plates at $37^\circ \text{C}$ overnight for plaque development.

- Next, the bad mode is performed with 6 new petri dishes with confluent $E \text{ coli}$ lawns. The bad mode is run in the same manner as the good mode, except that the sorter is adjusted to produce sort stream fanning for the duration of 15 min (the bad mode is created by turning off the droplet drive).

### IV. TESTING ROOM AIR FOR ESCAPE OF DROPLET NUCLEI USING AN ACTIVE AIR SAMPLING METHOD

Testing the cell sorter for aerosol containment of droplets $= 5 \mu \text{m}$ (droplet nuclei) is performed in combination with the T4/$E \text{ coli}$ settle plate test.

- Before T4 bacteriophage are run through the cell sorter, set up the N6 Andersen single-stage air sampler (Graseby-Andersen, GA, or equivalent) and place one petri dish with a confluent $E. \text{ coli}$ lawn with the lid off into the sampling stage. Collect room air for $10 \text{ min}$. This dish will serve as negative control.

- Place two $E. \text{ coli}$ plates each into a sampling stage. Place one stage close to the cell sorter, e.g., where the operator is sitting, and the second one approximately 1 meter away from the instrument. Collect room air during the last $10 \text{ min}$ of the good mode containment test.

- Place two $E. \text{ coli}$ plates each into a sampling stage. Place one stage close to the cell sorter and the second one approximately 1 meter away. Collect room air during the last $10 \text{ min}$ of the bad mode containment test.
• Also, sample room air for 10 min during the bad mode containment test with the sort chamber door open. This plate will serve as positive control.

• After the completion of each test, replace the lids on all the petri dishes and incubate them overnight at 37°C for plaque development.

V. TROUBLESHOOTING

• E. coli lawns are not apparent after overnight incubation. When E. coli are added to top agar in excess of 50°C, the bacterium may be killed. Monitor the temperature of the top agar prior to adding the E. coli.

• The liquified agar solidifies too fast to prepare all the petri dishes. Maintain the liquid state indefinitely in a 50°C water bath.

• Plaques are not observed in the sample collected from the sheath stream. Bacteriophage may be incompatible with the sheath fluid that was used (e.g., PBS is an incompatible, HBSS is an acceptable sheath fluid). Note also that sheath fluids containing anti-bacterial agents, e.g., gentamycin, are not advised for these experiments. For verification, plate lower dilutions of the sheath stream on the E. coli lawns (as described in the above schematic). The measured titer should be >10^7 PFU/min. If the titer is much lower, suboptimal conditions may have been used.

• Plates placed inside of the sorting chamber do not develop plaques, but rather have large and uneven areas of complete lysis. The plates placed within the sorting chamber are used to measure aerosols from the sorting streams, and not the sorting streams themselves. If the plates are placed too near to the sorting streams, the plates will get wet and will be inoculated with sheath fluid rather than aerosols.
**T4 BACTERIOPHAGE SORT FOR TESTING OF AEROSOL CONTAINMENT**

I. **CALCULATION OF SAMPLE RATE (µl/min)**

Volume fed to machine (µl): - 
Feeding time (min): 10 min

Sample rate (µl/min): ________

Does flow rate meet established criteria (30-40µl/min)?

YES □ NO □

II. **TITRATION OF T4 BACTERIOPHAGE STOCK (MINIMUM 10⁹ PFU/ML)**

<table>
<thead>
<tr>
<th>Plate ID</th>
<th>Dilution</th>
<th># of plaques</th>
<th>Dilution factor</th>
<th>Vol on plate</th>
<th>PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>10⁸</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>10⁹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Does the T4 phage stock meet minimal concentration requirement (10⁹ PFU/ML)?

YES □ NO □

III. **CALCULATION OF EXPECTED T4 FLOWING/MIN THROUGH CELL SORTER**

Sample rate: µl/min T4 stock conc: PFU/ml Expected T4 throughput: PFU/min

IV. **T4 FLOWING/MIN THROUGH CELL SORTER (MINIMUM 10⁷ PFU/MIN)**

Collect sheath stream for 1 min

Volume collected (ml): _______ ml

<table>
<thead>
<tr>
<th>Plate ID</th>
<th>Dilution</th>
<th># of plaques</th>
<th>Dilution factor</th>
<th>Vol on plate</th>
<th>Sheath rate</th>
<th>PFU/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>10⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>10⁸</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Does the T4 phage stock meet optimal established criteria for flow rate through flow cytometer (>10⁷ PFU/min)?

YES □ NO □

V. **AEROSOL CONTAINMENT IN GOOD SORTING MODE**

<table>
<thead>
<tr>
<th>Plate ID</th>
<th>Plate location</th>
<th># of plaques</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>inside right</td>
<td>2 h</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>inside left</td>
<td>“</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>door right</td>
<td>“</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>door left</td>
<td>“</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>x adjust</td>
<td>“</td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>top of machine</td>
<td>“</td>
<td></td>
</tr>
</tbody>
</table>

Is aerosol contained in good sorting mode?

YES □ NO □

VI. **AEROSOL CONTAINMENT IN BAD SORTING MODE**

<table>
<thead>
<tr>
<th>Plate ID</th>
<th>Plate location</th>
<th># of plaques</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>inside right</td>
<td>15 min</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>inside left</td>
<td>“</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>door right</td>
<td>“</td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>door left</td>
<td>“</td>
<td></td>
</tr>
<tr>
<td>D5</td>
<td>x adjust</td>
<td>“</td>
<td></td>
</tr>
<tr>
<td>D6</td>
<td>top of machine</td>
<td>“</td>
<td></td>
</tr>
</tbody>
</table>

Is aerosol contained in bad sorting mode?

YES □ NO □

Remarks: ________________________________________

Signed: ________________________________
3) TESTING AEROSOL CONTAINMENT USING GLO GERM™ PARTICLES AND A MICROBIAL PARTICLE SAMPLER

Testing is performed before each biohazardous sort. The instrument is placed in failure mode to simulate conditions such as a partially blocked sort nozzle or air in the fluid system, which result in a marked increase of aerosol production. Aerosol containment results are recorded as PASS / FAIL before each sort.

Materials and Methods

2. Vacuum source, e.g., provided by the institution or a vacuum pump capable of drawing 45L/min of air


   a. Wash Glo Germ™ particles in 5 ml of ethanol at a concentration ranging from 200-400 x 10⁶ particles per ml twice with 100% ethanol by centrifugation at 900 x g for 10 minutes. If Glo Germ™ particles are obtained as powder re-suspend in 5 ml of 100% ethanol before continuing.
   b. Re-suspend in 100 ml of buffer (10% fetal calf serum + 1%Tween 20 + 1mg/ml sodium azide in PBS). Note that particles can be stored in an opaque glass container at 4°C for up to one year.
   c. Filter particles through a 100 μm nylon mesh filter to create a stock suspension. Dilute with PBS (~1:20) before sorting to achieve the appropriate acquisition rate for aerosol testing, e.g., 20,000 to 50,000 particles per second.
II. QUANTITATIVE MEASUREMENT OF CONTAINMENT

A. Aerosol Containment System (ACS)

The following protocol is designed to measure the effectiveness of an ACS on a high-pressure cell sorting flow cytometer. This protocol utilizes fluorescent particle containing aerosols, which can be trapped in a vacuum device and measured in a fluorescent microscope. In the example shown here, a BD FACSARia™ flow cytometer is described, but this protocol can be adapted to any high-speed sorter. (For additional information see the website, [http://www.niaid.nih.gov/vrc/labs_flow.htm](http://www.niaid.nih.gov/vrc/labs_flow.htm)).

- The ACS must be on and functioning according to the manufacturer guidelines. Figure 1 shows an example of the aerosol flow and the locations of the vacuum gauge and monitor on a typical ACS. Using this system, the vacuum monitor is set to 20% and the vacuum gauge typically reads between 1.0 and 1.5 inches of H₂O. If it is outside of this range, the HEPA filter is replaced.

- The sheath waste tank must contain enough sodium hypochlorite to provide a final concentration of 10% when filled (1L bleach to a final 10L waste collected).

- Most flow cytometers, which employ high-speed cell sorting have a digital camera system focused of the sort stream (the Accudrop™ camera is shown in [Figure 1 - Aerosol Containment System](#)).
This camera system is used to monitor the sort stream and alerts the operator to potential sort stream disruption, which can lead to increased aerosols. Figure 2A shows the normal operating sort stream in four-way sorting mode. If the stream is disrupted the droplets form a smear in this display. In this situation, the operator can correct the sort stream and reduce aerosol contamination. Furthermore, some instruments are equipped with a droplet breakoff monitoring technology (the lines in figure 2B), which can detect stream drifts due to possible clogs and automatically shuts down the stream. It is highly recommended that both of these devices are employed during all sorting operations.

![Figure 2 - Sort Stream (A) and Accudrop Camera (B) Displays](image)

**A. Measurement of Containment**

- The ACS is tested under simulated worse case failure mode (active mode). In this mode the instrument is set to 70 psi and 50,000 particles/second, with the stream hitting the waste catcher shield to create excessive aerosol. This is accomplished by covering the waste catcher with a disposable rubber shield (see Figure 3, “standby” refers to normal stream collection; “active” refers to the failure mode).
A glass slide is placed into the AeroTech concentrator and the parts are assembled as shown figure 4. The vacuum pressure to the AeroTech concentrator is adjusted to 55 LPM (liters per minute). The main sort chamber is closed and the AeroTech device is placed directly on top of this chamber door as shown in figure 5.
The ACS is turned on according to manufacturer’s instruction.

Place GloGerm™ particles into the sample station and adjust either the particle concentration or the flow rate to achieve a particles rate of 50,000 particles per second. Note: When creating aerosols, which could contain GloGerm™ particles, it is recommended that the operator wear a 0.2µm particle mask.

Begin acquiring GloGerm™ particles and allow collection for 5 minutes. (see an example of a typical bead histogram in Figure 6). After this time, multiple areas can be measured by moving the AeroTech device to another location while continuing the particle deflection.

Turn off vacuum to the AeroTech concentrator and remove slide. Put in a fresh slide and continue collecting aerosol with the ACS turned off for another 5
minutes. This will act as the positive control slide. Stop sample acquisition and remove shield on waste catcher.

- Examine glass slides for bright green fluorescence using a fluorescent microscope equipped with a FITC filter at 520-640 nm (see Figure 7). When a large amount of aerosol is collected (i.e. positive control slide) the Glo-Germ particles will form a pattern as directed by the holes found in the plate cover.

![Figure 7 - Glo Germ™ Particles](image)

- Scan the entire slide using a 10X objective and count all GloGerm™ particles. The positive control slide can be used as a reference if the slide reader needs help to distinguish between fluorescent debris and actual GloGerm™ particles. Record all data.

B. Acceptable Tolerance

All tolerance ranges as outlined by the manufacturer of the ACS must be strictly followed. Listed below are the acceptable tolerances for the measurement of containment using the GloGerm™ protocol.
- **Particles outside** = Zero tolerance, no particles on entire slide. Any positive result must be investigated, resolved and the instrument retested before proceeding with sorting potentially infectious samples.

- **Particles inside (positive control)** = Greater than 100 per slide.

References:


