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Biosafety Guidelines for Sorting of Unfixed Cells†

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The International Society of Analytical Cytology (ISAC) Biohazard Working Group presents guidelines for sorting of unfixed cells, including known biohazardous samples, using jet-in-air, deflected-droplet cell sorters. There is a risk that personnel operating these instruments could become exposed to droplets and aerosols containing biological agents present in the samples. The following guidelines can aid in the prevention of exposures of laboratory personnel to pathogens contained in the sort samples. The document provides biosafety recommendations for sample handling, operator training and protection, laboratory facility design, and instrument setup and maintenance. In addition, it describes in detail methods for assessment of instrument aerosol containment. Recommendations provided here may also help laboratories to obtain institutional and/or regulatory agency approval for sorting of unfixed and known biohazardous samples. Cytometry 28:99–117, 1997. © 1997 Wiley-Liss, Inc.

Key terms: flow cytometry; biohazard; occupational health; safety; cell sorting; aerosol containment

INTRODUCTION

At the Congress of the International Society for Analytical Cytology (ISAC) held in October 1994 in Lake Placid, New York, a workshop was held to discuss the hazards associated with the sorting of infected biological specimens. It was becoming apparent that the hazards of sorting HIV-infected cells, in particular, were not well known, even though such sorting was becoming more prevalent. Also, many other types of unfixed cells were being sorted with unknown consequences. Following the Congress it was suggested to the President of ISAC that the Society should become involved in this issue. To this end, Francesco Mauro, then President of the Society, asked Phillip Dean, then Secretary of the Society, to establish an international Biohazards Working Group with the ultimate goal of publishing guidelines for sorting of unfixed samples. The results of the efforts of the Working Group is this document, Biosafety Guidelines for Sorting of Unfixed Cells, which was approved by the ISAC Council in January 1997 for publication as official recommendations of the Society.

Purpose

The purpose of this document is to provide written recommendations for handling and sorting of potentially biohazardous specimens and methods to assess the risk of

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exposure of laboratory personnel to biological aerosols that may be produced by deflected-droplet fluorescence-activated cell sorters. There is a possibility that operators of cell sorters could become infected with biological agents present in the specimens they are sorting. The following guidelines can aid in preventing exposures of laboratory personnel to infectious agents from sorting of unfixed cells. The recommendations in this document focus on sorting of unfixed samples. However, it is important to note that other flow cytometric applications, e.g., phenotypic analysis, calcium flux evaluations, apoptosis measurements of unfixed cells, when performed using jet-in-air flow cytometers, can also expose operators to potentially hazardous aerosols. Therefore, the recommendations set forth here apply as appropriate whenever unfixed samples are run through a jet-in-air flow cytometer.

Biohazard potential of unfixed cells. Biologic specimens can harbor known and unknown pathogens that may be transmitted through droplets and aerosols that are generated either through laboratory manipulations (5,16) or the sorting process (20). Samples may also consist of genetically manipulated cells, which contain genomic sequences of potentially infectious organisms. Although most 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 particles (see Appendix 1, Table 1). For instance, commonly known bloodborne infectious agents, e.g., the Human Immunodeficiency Viruses (HIV-1, -2) and Hepatitis viruses, are primarily transmitted through inoculation. However, infection through aerosolization of virus particles has at least been documented for Hepatitis B (1). Biological particles of 0.1 μm to 60 μm sizes (i.e., aerosols) are considered important in the spread of infectious diseases (23,17,26). During inhalation, particles $\leq$3 μm are deposited into the lung of the exposed individual, particles in the size range from 3–7 μm into the tracheal area and pharynx, and larger particles mainly into the nasal passages (2,3). Droplets that fall out of suspension in air will fall onto surfaces and can be transmitted by exposure to broken skin or mucous membrane, or by ingestion. Both HIV and HBV have been shown to be transmitted through mucous membrane exposure. Therefore, it is important that all laboratory personnel, and in particular the sorter operator, be protected from exposure to aerosols, droplets and accidental splashes.

Creation of droplets and aerosols during the sorting process. In general, cell sorters produce droplets in the size range of 40–200 μm and microdroplets (satellite droplets, 3–7 μm) during their normal operation. Usually, droplets larger than 80 μm will fall out of the air rapidly; smaller droplets may be aerosolized, particularly when they are elevated by air currents. Cell sorters create secondary aerosols of various droplet sizes when the undeflected center stream and the side streams splash into receptacles (20,3). During failure operation of the cell sorter, e.g., a clogged sort nozzle or air in the fluidic lines, aerosol production may increase substantially. For instance, a partially clogged nozzle will cause deflection in the fluid streams that are then likely to strike an obstacle and create considerable amounts of aerosols. Usually, sorter generated aerosols are contained within the sorting chamber when the door is properly closed and the interlock is engaged. Because of the potential health risk to sorter operators if aerosols escape into the room, it is strongly recommended that aerosol containment of a cell sorter be verified. Refer below for recommendations for the assessment of aerosol containment.

International Society for Analytical Cytology (ISAC) Biohazard Working Group

In recognizing the need for establishing guidelines for sorting of unfixed and potentially biohazardous specimens, ISAC in Fall 1994, under the presidency of Francesco Mauro and the chairmanship of Phillip Dean, established the international ISAC Biohazards Working Group with the goal of publishing a set of guidelines for sorting of unfixed cells including known biohazardous samples. The following is the list of members: Phillip N. Dean, Chair; Ingrid Schmid, Guidelines Coordinator; Janis V. Giorgi; George Janossy; Annalisa Kunkl; Peter A. Lopez; Janet K.A. Nicholson; Stephen Perfetto; and Larry Seamer.

Review of the Guidelines

The guidelines and document revisions are subject to approval of the ISAC Council. They will be reviewed at a meeting of the ISAC Biohazard Working Group during each Congress of the International Society for Analytical Cytology. The recommendations set forth here may be updated in the interim whenever critical new information becomes available.

LEVEL OF CONTAINMENT

"Containment" refers to safe methods for managing infectious agents in the laboratory. The four biosafety containment levels (BSL1, BSL2, BSL3, and BSL4) consider the laboratory facilities, laboratory practices and techniques, and the equipment used (27). The level of containment depends on the potentially infectious agents present in the samples to be sorted. BSL1 containment is used for work with agents that are not known to cause disease in humans. BSL2 practices, safety equipment, and facilities are used for pathogens that are known to cause disease in humans but can be easily contained and are not usually transmitted by aerosols. This guideline is designed for viable cell sorting of specimens that harbor agents which are contained by BSL2, including HIV and HBV. BSL3 containment is applicable to agents that cause serious and potentially lethal disease as a result of exposure by the inhalation route. BSL4 practices, safety equipment, and facilities are used 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 be 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. Cell sorters do not generally 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 the cell sorter may be breached during a sort exposing the operator to potentially biohazardous aerosols. Therefore, to compensate for the fact that aerosols cannot be contained within a biosafety cabinet during cell sorting, it is recommended that BSL2 containment be combined with BSL3 work practices.

Considering the potential for exposure to aerosols during cell sorting, it is the responsibility of the investigator who wishes to sort such unfixed cells to determine the appropriate biosafety level (BSL) and, in conjunction with the flow cytometer operator, review all the appropriate safety procedures for the particular pathogen. Such a review should also take into account the presence of medication intake, compromised immunity and general health of the cell sorter operator (28). An extensive list of biological agents and their associated BSL is found in US HHS Publication: Biosafety in Microbiological and Biomedical Laboratories, 3rd Edition, 1993 (27).

RECOMMENDATIONS FOR CONTAINMENT CONTROLS

Universal Precautions

Guidelines for specimen handling are available from U.S. Centers for Disease Control (7,8) and are also published in the document M29-T by the National Committee for Clinical Laboratory Standards (NCCLS) (21). All laboratory personnel who handle human cells are required to follow procedures as outlined in the document US HHS, Occupational Exposure to Bloodborne Pathogens, 1991 (22). Each laboratory should 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. Appropriate safety practices should be selected by 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 specimens as if infected is recommended. If samples are fixed, appropriate, and reliable methods must be used to inactivate potentially biohazardous agents. These 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.

Cell Sorter Operator-Specific Precautions

The protection of operators from infection and biohazard exposure during sorting of unfixed cells is of critical importance. The following recommendations also apply to others who may be present in the room during the sort, e.g., other scientists involved in the experiment.

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.

Protective clothing.

Laboratory coats. The sorter operator should wear a disposable, wrap-around, solid front, long sleeved laboratory coat.

Gloves. Examination gloves are required whenever manipulating unfixed specimens. For added safety, double-gloving may be preferred. When the outer gloves come in contact with potentially biohazardous material, they must be discarded, and 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, it is recommended that gloves be changed often.

Face protection for eyes and nose. It is recommended that the sorter operator wear a respiratory protective device, a High Efficiency Particulate Air (HEPA) N-95 (NIOSH certified) air-purifying, particulate respirator (Safeware, Inc., Largo, MD, or equivalent), covering nose and mouth, and safety glasses with side shields. For added splash protection, a full face shield can be placed over the respirator and the eye glasses.

Specimen handling. As much as possible, 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 specimen centrifugation, use sealed vessels or safety carriers.

Use of "sharps" avoided. Avoid the use of needles, glass pipets, glass transfer pipets, or glass containers whenever possible for handling or transferring any biological material and use suitable replacements. Dispose of any "sharps" using a leakproof, puncture-resistant container.

No mouth pipetting. No mouth pipetting is allowed. Manual pipetting devices must be used.

Work area clean-up. Discard all contaminated materials, e.g., sample and collection tubes, pipets, pipet tips, gloves, and 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 (4). 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 (19,24,6,12).

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 NCCLS document (M29-T2) (21) or the biosafety office of your institution.

Accidental exposure. It is recommended that all laboratory personnel including sorter operators provide when they start their employment, a serum sample 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 written records of the medical evaluation, surveillance, and treatment must be maintained. Guidelines for postexposure chemo-prophylaxis against HIV infection are available from CDC (10).

Environmental Controls

The cell sorter should be located in a separate room where no other laboratory activity is performed. Ideally, cell sorting of unfixed samples should be performed in a BSL3-type room which requires a ducted air ventilation system, water resistant interior surfaces (walls, floor, and ceiling), laboratory furniture that can be easily cleaned and decontaminated, sealed windows, and a sink that can be operated without hands. For further details refer to HHS Publication Biosafety in Microbiological and Biomedical Laboratories, 3rd Edition, 1993 (27). 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.

Room air flow. Proper air flow in the room requires negative pressure and no less than ten changes of air per hour. Air flow should be directed away from the sorter operator.

Personnel access. Access to the sorting room should be limited 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.

Instrument Considerations

Proper operation of the cell sorter, including the sorting mechanism. Follow all the manufacturer's recommendations for instrument operation and maintenance carefully.

Routinely perform monthly leak checks on the fluidic lines of the cell sorter. To do this, gain access to the fluidic lines. Carefully check for wet areas, which indicate leaks in the tubing. Inspect tubing for cracks and signs of stress, particularly around the fittings and where tubing passes through pinch valves. Also inspect sheath lines and waste lines. Replace any leaking tubing.

Test 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 test sort, correct the problem before attempting a potentially biohazardous sort.

After each sort, the instrument should be decontaminated with a disinfecting agent, taking into account the biohazard under study. Suitable agents include 70% ethanol, appropriately diluted antimicrobial soap/disinfectant, and a 1/100–1/10 volume dilution of 0.71 M sodium hypochlorite (undiluted household bleach). Refer to Appendix 1, Table 3 for summary information on disinfectants and their application. Wipe off all surfaces inside the sort chamber, the sample introduction port and holder. Run the disinfectant through the instrument for at least 10 min. Always follow with distilled water to completely remove the disinfectant, because some disinfecting agents are corrosive.

Sample introduction system. Most cell sorters have a sample introduction system that is pressurized once a tube is placed onto the sample introduction port. Each time a sample tube is put on the instrument, check the tube's 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 introduction line back-drips, creating a potential biohazard through spraying of droplets. To avoid splashing of sample droplets allow the back-drip to go into a tube until the sample is flushed out of its introduction line. The catch tray or trough should be decontaminated regularly.

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 should be either removed by filtration (e.g., through a nylon mesh) prior to sorting and/or prevented from reaching the sort nozzle by attaching an in-line filter onto the sample introduction line. Sort nozzles should be cleaned frequently between sorts to prevent build-up of cellular debris. Even during sorting of properly prepared samples, cells can aggregate inside the sort nozzle at the intersecting surface of the sample injection fluid with the sheath fluid. Accumulated cells at this intersection eventually break free and may partially clog the nozzle tip. When this occurs, clamp the sample introduction line, turn off the high voltage, and put the fluidic control into the off position. The sort chamber door must remain closed until aerosol has been cleared from the chamber. A 3 min delay will be sufficient for aerosol

clearance on most cell sorters. 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 sort sample, open the door and take out your collection vials. Cap all tubes. Backflush through the nozzle tip using a syringe until the clog is cleared. 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.

Access to the sort chamber. A standard safety feature adopted by most manufacturers of cell sorters includes an interlocking sort chamber door and an enclosed sample collection chamber. However, because these barriers are not sealed, it is strongly recommended that testing of aerosol containment as described below and in Appendix 3 be performed before a potentially biohazardous sort is attempted. Visual verification of gaps between the instrument and the doors can be performed with bottled smoke. Repeat the smoke test periodically, e.g., every 1 to 3 months, to check for leaks.

Control of effluent and removal systems for aerosols from the sort chamber. The undeflected center stream should be collected into a receptacle connected with a waste evacuation system to reduce aerosol production during cell sorting. An efficient vacuum containment system on the sorter (see below) 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, it is strongly recommended that containment on the cell sorter be verified in regular sorting mode and in instrument failure mode simulating a clog as described below and in Appendix 3 before a potentially biohazardous sort is attempted. If aerosol containment is incomplete, the standard biosafety features of the cell sorter should be modified such that no escape of aerosol can be detected using the T4 bacteriophage/settle plate method as described below and in Appendix 3. For added safety, escape of droplet nuclei can be tested during a T4 bacteriophage sort using an Andersen air sampler as described in detail in Appendix 3.

Stream view video camera. Use of a sort stream viewing video camera is advisable as it will prevent the sorter operator from coming close to the area of the instrument that poses the greatest potential biohazard.

Special considerations for high-speed sorters. Sorting cells at higher than normal speed is performed on instruments that generally operate with higher fluidic system pressure and increased droplet frequency formation as compared to regular cell sorters. Both features lead to an increased hazard potential for the sorter operator during sorting of unfixed cells. Generation of more and smaller droplets and higher stream velocity can result in increased aerosol formation. High system pressure can cause rapid release of large quantities of potentially biohazardous liquid and aerosol into the instrument in the event of an accident, e.g., a break in a fluid line. Therefore, the precautions set forth below are recommended.

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 an unmodified, regular cell sorting instrument, because the fluidic lines, valves, and fittings cannot reliably withstand the higher pressure.

Aerosol containment on the cell sorter should be complete in regular sorting mode and in failure mode as tested by both the T4 bacteriophage/settle plate and the active air sampling method, as described below and in Appendix 3 before any viable and potentially biohazardous cell sorting is performed.

Carefully inspect and test all the instrument functions including the sorting mechanism and correct all problems each time before attempting a viable sort. Also, check for leaks and loose fittings on the fluidic lines of the cell sorter prior to each sort.

Only operators trained and experienced in high-speed cell sorting should perform viable and potentially biohazardous sorts. Ideally, the operator should have at least 6 months prior experience of high-speed sorting of fixed samples.

RECOMMENDATIONS FOR OPTIONAL CONTAINMENT CONTROLS

The recommendations set forth in this document are for cell sorting of samples that harbor biological agents that are contained by BSL2 practices, safety equipment, and facilities. For cell sorting of agents which require BSL3 containment or radioactive agents, additional precautions are needed. BSL3 level containment requires a BSL3 room, specialized laboratory clothing, and a respirator to completely protect the operator from exposure to the agents under study. Requirements may also include a shower facility when leaving the room, and wipe tests and monitoring with a hand-held Geiger counter for radioactive agents. Since the greatest potential of exposure or infection from these agents is from aerosolization (e.g., *M. Tuberculosis*) (9) and they are known to cause substantial morbidity and mortality, the need to perform such viable or radioactive sorting experiments should be reviewed on a case by case basis with the biohazard safety office and the infectious disease professional of the institution, as detailed below. Cell fixation and alternate cell separation technologies, e.g., magnetic beads or coated flasks, that can be performed in biosafety cabinets should be used whenever possible.

Installation of custom-designed biosafety features on cell sorters is recommended but generally not critical unless aerosol containment is incomplete. The following is a list of custom modifications that may aid in obtaining aerosol containment or provide an added level of safety.

Installation of an auxilliary vacuum pump to increase the negative pressure in the sort sample collection drawer can improve the removal of sort generated aerosols on instruments that do not have this biosafety feature. Care

needs to be taken so that no air turbulences are created that will affect the stability of the sort streams. The effluent vacuum lines must be connected to 1) a cartridge-type HEPA filter for removal of airborne particles and 2) a filter flask of appropriate size, e.g., 250 to 4,000 ml, containing a disinfectant appropriate for the biohazard under study. Usually, the flask will be filled approximately half-full with concentrated household bleach (0.71 M sodium hypochlorite). For each sorting experiment the flask should be emptied and refilled with fresh, concentrated bleach.

Attachment of a grounded wire to the sort sample collection vials will disperse the build-up of electrostatic charges that can lead to droplet spraying. Use of sample collection tubes coated with a conductive film, e.g., generated by coating the tube interior with medium containing 10% to 20% serum, can improve dispersion of electrostatic charges.

Installation of a droplet-containment module that eliminates the back-drip from the sample introduction line will prevent droplet splashes. Alternatively, installation of a plastic shield around the sample introduction port can block droplet spraying from the sample back-drip.

Installation of a timed interlock for the sort chamber door will prevent the sorter operator from opening the door for a pre-set time, determined experimentally, after the sort has been stopped to allow aerosol clearance from the sort chamber.

TRAINING AND EXPERIENCE

Minimum Experience of Sorting Cells

Only experienced flow cytometry operators should perform potentially biohazardous sorts. The operator should have previous laboratory experience and a minimum of 2 years of experience in flow cytometry. Ideally, this should include training in performing sorting on deflected-droplet cell sorters using fixed control material of the same type that will contain the known biohazard, e.g., peripheral blood mononuclear cell preparations.

Experience With Potential Pathogens

Previous working experience with potentially biohazardous specimens is strongly recommended. Knowledge of the characteristics of the infectious organism present in the specimens to be sorted will help the sorter operator to formulate safe working practices.

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. An example of a research protocol requesting the sorting of unfixed, known biohazardous samples is found in Appendix 2.

ASSESSMENT OF AEROSOL CONTAINMENT

Methods

Standard methods for the assessment of aerosol containment on deflected-droplet cell sorters using aerosolized bacteriophage and a detection system of bacterial lawns have been described (20,18,3,11,13,14). The protocol outlined in Appendix 3 has been published elsewhere (15), and is described in more detail with additional explanations of the rationale by Schmid et al. (25). Before attempting to sort any potentially biohazardous specimens, it is necessary to verify that no aerosols produced during the regular sorting process and during instrument failure modes escape into the room. Refer to Appendix 3 for a description of the T4 bacteriophage sorting technique combined with *E. coli* settle plates and for a description of using an air sampler for testing room air during a T4 bacteriophage sort. If aerosol containment is incomplete, the cell sorter should be modified such that no aerosols are detected outside the sort chamber. Contact the manufacturer of the cell sorter for instructions and information on necessary instrument modifications. If viable cell sorting is performed on a regular basis, it is recommended that testing for aerosol containment be repeated every 1 to 3 months. Testing should 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 replacement of the sort chamber door. If sorting of unfixed samples is performed infrequently (less than once every 1–3 months), aerosol containment testing should be done each time before attempting to sort unfixed and potentially biohazardous specimens.

Proposed Methods

Although the actual aerosol containment test using T4 bacteriophage on the flow cytometer is relatively simple, the preparation of the biological materials needed for the test is time consuming and can be difficult. Therefore, efforts are underway to develop simpler methods. Other proposed methods for testing aerosol escape on cell sorters include fluorescent detection of beads, labeled cells, and dyes. These techniques will be included in these guidelines when their sensitivity and limitations have been demonstrated and formally evaluated.

CONCLUSIONS

An increasing number of laboratories operate cell sorters, conduct experiments to study viral infections, in particular HIV, and need to isolate viable human cells, e.g., stem cells, for clinical research or patient treatment. As applications of flow cytometry are being utilized in the study of potentially biohazardous materials, the prevention of exposure of laboratory personnel to this material is of great importance. Although up to this time, no infection due to sorting of unfixed material has been documented, the 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 prompt cell sorter manufacturers to modify or design instruments specifically to prevent release of hazardous materials and work with their users to create a safe laboratory environment.

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APPENDIX 1

TABLE 1.
Infectious Agents Associated With Laboratory-Acquired Infections due to Manipulation of Biological Samples[a]

Agent	Source of infection	Species	Route of infection	Biosafety level practices, safety equipment, and facilities
Hepatitis B, C, D virus	Blood, cerebrospinal fluid, urine, tissues	Human, naturally or experimentally infected primates	Inoculation, exposure of mucosal membranes to aerosols, broken skin	BSL2 BSL3 in case of aerosol production, large quantities or high concentrations
Herpes virus simiae (B virus)	Primary cultures (Rh monkey kidney cells)	Macaque or human	Inoculation, ? aerosol inhalation minimal	BSL3 BSL4 for large quantities or high concentrations
Herpes Simplex 1,2 varicella virus	Ubiquitous	Opportunistic pathogen in immunocompromised host	Direct contact or aerosol inhalation	BSL2
Cytomegalovirus Epstein-Barr virus (EBV)	Blood, tissues, EBV-transformed lines	Human	Inoculation, exposure of mucosal membranes to aerosols, broken skin	BSL2
Herpes 6, 7 virus	Blood, bronchoalveolar lavage	Human	Risk not known	BSL2
Influenza virus	Bronchoalveolar lavage, respiratory tissues	Human, naturally or experimentally infected animals	Aerosol inhalation	BSL2
Lymphocytic chorio-meningitis virus	Blood, cell cultures, nasopharynx secretions, bronchoalveolar lavage, tissues	Nude mice, SCID mice, naturally infected macaques or marmosets, possibly man	Inoculation, exposure of mucosal membranes to aerosols, broken skin well documented transmission by aerosol inhalation	BSL2 BSL3 in case of aerosol production, large quantities or high concentrations
Pox viruses genetically engineered recombinant vaccinia virus	Lesion fluid, tissues, respiratory secretions, bronchoalveolar lavage	Infected volunteers or animals	Inoculation, exposure of mucosal membranes to aerosols, broken skin	BSL2 practices by vaccinated personnel
Human immunodeficiency virus (HIV-1, 2)	Blood, body fluids, tissues	Human	Inoculation, exposure of mucosal membranes to aerosols (containing concentrated virus), broken skin	BSL2 BSL3 in case of aerosol production, large quantities or high concentrations
Simian immunodeficiency virus (SIV)	Blood, body fluids, tissues	Macaque	Inoculation	BSL2 BSL3 in case of aerosol production, large quantities or high concentrations
HTLV-1, 2 virus	Blood, body fluids, tissues	Human	Inoculation, exposure of mucosal membranes to aerosols (containing concentrated virus), broken skin	BSL2 BSL3 in case of aerosol production, large quantities or high concentrations
Rickettsia prowazekii	Infected tissues	Naturally infected non-human primates	Inoculation, aerosol inhalation	BSL2 BSL3 for tissue cultures of infected cells
Mycobacterium atypicum	Bronchoalveolar lavage, lesion tissues	Human	Inoculation, direct skin contact, aerosol inhalation	BSL2
Mycobacterium tuberculosis	Gastric lavage, cerebrospinal fluid, pleural fluid, urine	Human, naturally infected primates	Aerosol inhalation	BSL3
Neisseria gonorrhoeae	Sinovial fluid, urine, cerebrospinal fluid	Human	Inoculation, direct skin contact	BSL2
Neisseria meningitidis	Pharyngeal exudates, bronchoalveolar lavage, cerebrospinal fluid, blood	Human	Inoculation, direct skin contact, aerosol inhalation	BSL2 BSL3 in case of aerosol production or high concentrations
Salmonella Salmonella typhi	Blood	Human	Inoculation, direct skin contact	BSL2 BSL3 for large quantities
Brucella	Blood, cerebrospinal fluid, tissues	Human, experimentally infected animals, sheep	Inoculation, direct skin contact	BSL2 BSL3 for tissue cultures of infected cells
Treponema pallidum	Lesion fluid	Humans with primary and secondary syphilis	Inoculation, direct skin contact, aerosol inhalation	BSL2
Toxoplasma Trypanosoma Leishmania Plasmodium	Blood	Humans or experimentally infected animals	Inoculation, aerosol inhalation	BSL2
Blastomyces Coccidioides Histoplasma	Tissues	Infected animals	Inoculation, aerosol inhalation	BSL2 BSL3 for cultures containing Coccidioides, Histoplasma

[a]This table was adapted from US HHS Publication: Biosafety in Microbiological and Biomedical Laboratories, 3rd Edition, 1993.

TABLE 2.
Summary of Laboratory Practices, Equipment, and Facilities Associated With Biosafety Levels[a]

Biosafety levels	BSL1	BSL2	BSL2 using BSL3 practices
A. Hazard levels	Low risk	Low to moderate	Moderate to high
B. Standard microbiological practices			
1. Public access while experiments are in progress	Not recommended	Controlled	Not permitted
2. Decontamination	Daily and upon spills	Daily and upon spills	Daily, upon finished work with infectious material, and spills
3. Infectious waste decontamination	Before disposal	Before disposal	Before disposal[b]
4. Pipetting	Mechanical devices	Mechanical devices	Mechanical devices
5. Eating, drinking, smoking and application of cosmetics	Not permitted at any time	Not permitted at any time	Not permitted at any time
6. Handwashing facilities	Required	Required	Required
7. Minimization of aerosol production	Recommended	Recommended	Recommended
8. Laboratory coats	Recommended (front button coats), not worn outside the laboratory	Required (front button coats), not worn outside the laboratory	Wrap-around disposable clothing required for all workers with potential exposure to infectious agents
C. Special practices			
1. Autoclave on-site facility	Not required	Must be available within the building	Must be available within the building
2. Insect/rodent control program	Required	Required	Required
3. Bench top work	Permitted	Permitted	Permitted in some circumstances
4. Transport of infectious material or waste materials for processing (i.e., decontamination) away from the laboratory	Durable leakproof container	Durable leakproof container	Durable leakproof container
5. Animals not involved with laboratory experiments	Not permitted	Not permitted	Not permitted
D. Containment equipment			
1. Biological safety cabinets or other physical containment system	Recommended for all aerosol generating processes	Required for all aerosol generating processes	Required for all work with infectious agents
2. Other physical containment	Recommended that equipment be decontaminated immediately after use	Appropriate physical containment devices are used when procedures with a high potential for creating infectious aerosols are being conducted[c]	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 aerosol exposure[d]
3. Freezers/refrigerators	Recommended that biohazard sign be posted	Biohazard sign must be posted	All agents must be stored in separate, closed, labeled, containers
4. Biosafety cabinet certification	Annually recommended	Annually	Annually
5. HEPA-filtered vacuum lines	Recommended	Recommended	Recommended
6. Biosafety cabinet decontamination	Recommended after each use	Required after each use	Required after each use
7. Personal protective equipment (i.e., laboratory coats, gloves, etc.)	Laboratory coats recommended; gloves are worn when skin contact with infectious material is unavoidable	Required—gloves should be worn when skin contact with infectious material is unavoidable	Required—appropriate combinations of special protective clothing, masks, gloves, respirators, etc. are used for all activities with infectious materials that pose a threat of aerosol exposure[e]
E. Laboratory facilities			
1. Ventilation	Negative pressure	Negative pressure	Negative pressure
2. Posted hazard sign	Recommended	Required	Required
3. Laboratory separated from the general public	No	Yes, while experiments are in progress	Yes, while experiments are in progress
F. Training			
1. Technical training	Recommended	Required	Required
2. Medical surveillance (i.e., baseline serology)	Recommended	Required when appropriate	Required when appropriate

[a]This table was adapted from "Working with Biohazardous Materials," Facilities Safety Procedure 360.01, Lawrence Livermore National Laboratory (1992).

[b]Infectious waste must be placed in a marked, closed, leak proof container and must be under direct control of the responsible laboratory worker(s) until it is placed in a locked disposal area or autoclaved (waste is not permitted to be left in laboratory overnight).

[c]These procedures include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, and opening containers of infectious materials whose internal pressures may be different from ambient pressures.

[d]These procedures include manipulation of cultures and of clinical or environmental material that may be a source of infectious aerosols.

[e]Required with aerosol generating equipment, manipulation of high concentrations or large volumes of infectious materials; activity involving all clinical specimens, body fluids, and tissues from humans or from infected animals.

TABLE 3.
Summary of Practical Applications of Chemical Disinfectants for Decontamination[a]

Chemical name	Quaternary ammonium compounds	Phenolic compounds	Chlorine compounds	Iodophor	Alcohols		Aldehydes	
					Ethyl	Isopropyl	Form-(Formalin)	Glutar-
Practical requirements								
Use dilution	0.1–2%	1–5%	1/100 dilution of 0.71 M sodium hypo-chlorite, ~500 ppm[b]	0.0075%–10%, ~25–1600 ppm[b]	70–85%	70–85%	0.2–8%	2%
Contact time to lipovirus	10 min	10 min	10 min	10 min	10 min	10 min	10 min	10 min
Broad spectrum	Not effective	Not effective	30 min	30 min	Not effective	Not effective	30 min	30 min
Inactivates								
Vegetative bacteria	√	√	√	√	√	√	√	√
Lipovirus	√	√	√	√	√	√	√	√
Non-lipid viruses		c	√	√	c	c	√	√
Bacterial spores			√	√			√	√
Physical characteristics								
Type	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
Stability[d]	√	√		√	√	√	√	√
Corrosive		√	√	√				
Flammable					√	√		
Residue		√	√	√			√	√
Organic material inactivated[e]	√		√	√				
Skin irritant	√	√	√	√			√	√
Eye irritant	√	√	√	√	√	√	√	√
Respiratory irritant			√				√	
Toxic[f]	√	√	√	√	√	√	√	√
Potential application								
Surfaces[g]	√[h]	√	√	√	√	√	√	√[h]
Instrument surfaces and parts			√		√	√		
Flow cytometer fluid lines			√		√			

[a]This table was adapted from "Biohazardous Operations," Facilities Safety Procedure 360, Addendum 1, Lawrence Livermore National Laboratory (1995).
[b]Available halogen.
[c]Variable results depending on the virus.
[d]Shelf life of greater than 1 week when protected from light and air.
[e]Prior to decontamination cleaning with lipophilic detergent/disinfectant necessary.
[f]By skin or mouth or both—refer to the manufacturer's literature and/or the Merck index.
[g]Work surfaces, dirty glassware, decontamination of fixed or portable equipment surfaces.
[h]Usually compatible with optics, but consider interference from residues and effects on associated materials such as mounting adhesives.

APPENDIX 2

University of California, Los Angeles

IA #90-006-3

REGISTRATION DOCUMENT
for
Research Involving Infectious Agents

Principal Investigator of Project (P.I.) Janis V. Giorgi, Ph.D.

UCLA Status of P.I. (circle): (Faculty) Post-doc Graduate Student Other

Exact Title of Project Core Facility for Sorting Cells from HIV-infected People

Department Medicine/JCCC Division Clinical Immunology & Allergy/ Core Flow Laboratory

Mailing Address Medicine/CIC, Factor 12-939 Telephone (310) 206-8217

Actual Work Location HIV sorter is located in Factor 12-637

Identify below the class designation of the etiologic agents to be used in the proposed project.

1) [X] Class III Agent — APPROVAL from Institutional Biosafety Committee and the Biosafety Officer required before initiation of the experiments.

2) [ ] Class II Agent — REGISTRATION with Biosafety Officer required for some of these agents (see Classification List, Appendix A, *The Use of Infectious Agents: Policies and Practices*)

3) [ ] Project requires USDA and/or USPHS permit(s) (attach copy)

Funding to which it will be submitted and/or other funding source if (if any) NIH and CFAR

Contract and Grant Number (if known) AI-72631, AI-28697, AI-32883

Name of P.I. of Main Grant (if different from P.I. of project) ________

Actual Starting Date of Research 6/01/90 Proposed Ending Date of Research indefinite

Type of Submission (circle): New (Continuation) Renewal Addendum Other

- -

For Office Use Only

IA #: 90-006-3

DATE REGISTERED/APPROVED Nov. 12 1992 (11.12.92 to 11.11.93)

DATE FOR RE-SUBMISSION September 11, 1993

Registered (Class II* Agent) ________ Biosafety Officer

Approved (Class III Agent) ________ Chair, IBC

________ Biosafety Officer

If a Class III agent is to be used in the proposed project, please provide the information requested in Roman numerals I to VII below in sufficient detail to assist the Institutional Biosafety Committee and the Biosafety Officer in their review. If you require more space than is available, prepare the information on a separate piece of paper. Forward the designated copies to the IBC, 6-956 Factor Building, Campus, Telephone 58714, and to the Biosafety Officer, Research & Occupational Safety, 601 Westwood Plaza, Campus, Telephone 53793.

If a Class II agent that requires registration is to be used in the proposed project, complete Roman numerals I to VII below and forward this document to the Biosafety Officer only.

I. Agent Human Immunodeficiency Virus (HIV), Class III
(Name and Class)

II. Strain ____________

III. Source Leukocytes from HIV-infected individuals and cell lines infected with HIV

IV. Brief description of the proposed project:
Continuing approval is sought for a Core facility to sort leukocytes from HIV-infected individuals and also to sort cell lines infected with HIV. A laser-based flow cytometer is used to sort the HIV-infected material. Up to 100ml of peripheral blood will be processed at a time. Leukocytes (10^8 maximum) will be treated with monoclonal antibodies to allow their separation by the cell sorter into subpopulations. These cell subpopulations will then be studied for their immunological function or to determine the amount of viral nucleic acid or proteins.

V. Methods of storage and location of stored strain(s):
Virus will not be stored as such. Leukocytes from HIV-infected individuals may be cryopreserved by standard methods and stored in liquid nitrogen tanks in thelaboratory located in Factor 12-243.

VI. Is a Class II vertical laminar flow biological safety cabinet available? Yes, two are available

Please identify the location Factor 12-243

VII. Medical Surveillance and Protection:

A. Vaccination of personnel No vaccine available
Date(s)

B. Base-line serum drawn Sera on all laboratory personnel has been drawn

C. Room/location where sera are stored -70°C freezer located in Factor 12-243

I understand that safety in activities involving etiologic agents depends on the individual conducting them and that motivation and good judgment are the key essentials to protection of health and environment.. I am familiar with and agree to abide by the provisions of the current UCLA Policies and Practices and other specific NIH instructions pertaining to the proposed project. I further agree to comply, when applicable, with all federal requirements pertaining to shipment and transfer of etiologic agents.

10/28/92 Janis V. Giorgi Associate Professor of Medicine
Date Signature and Title of Principal Investigator

____________ ____________
Date Signature of Faculty Sponsor (when applicable) and Title

APPENDIX 3

Testing of Aerosol Containment on Cell Sorters 1) Bacteriophage and *E. Coli* Settle Plates 2) Testing Room Air With an Active Air Sampling Method

Introduction: A laboratory protocol is provided for testing of aerosol containment which was expanded from previous publications (1, 2, 3) and will be described in further detail in a chapter in Current Protocols in Cytometry (4). This protocol, written by Ingrid Schmid, was developed in the laboratory of Dr. Giorgi at UCLA and incorporates work performed by Lance E. Hultin and Dr. John Ferbas.

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 then strike 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.

Procedures: 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 cell 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. Most of the protocol involves the settle plate method; the method for active air sampling is described in section IV.

Test evaluation: 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 except for the positive control plate. Otherwise, the instrument has to be modified to achieve aerosol containment. Use the attached form for test result evaluation and reporting.

Frequency: Repeat every 1 to 3 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 Erlenmyer 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 2,000g 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 $>1 \times 10^9$ 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 using an adequate container to prevent boil-over); then, approximately 20 ml are pipetted into each of 23 100 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 some plates are damaged during handling); 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

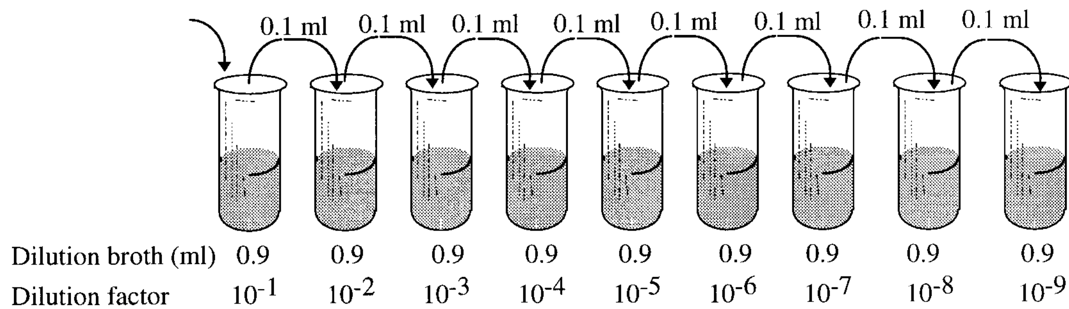
During this procedure, the bacteriophage stock is titrated, the throughput of bacteriophage per unit time on the flow cytometer is determined, and the viability of the bacteriophage stock after it is run through the instrument is assessed.

A) Titration of the Bacteriophage Stock:

SCHEMATIC FOR TITRATION OF BACTERIOPHAGE STOCK:

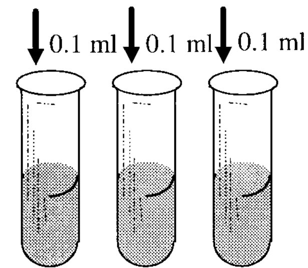
1. Prepare serial dilutions

0.1 ml of bacteriophage stock



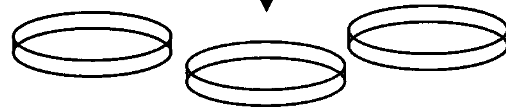
2. Inoculate top agar

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 in section I.C)c), 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:

Dilution	Number of plaques
10^{-7}	TNTC[a]
10^{-8}	40[b]
10^{-9}	1

[a]Too numerous to be counted.
[b]Select a Petri dish with an intermediate number of plaques (usually between 10 and 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.

e.g., 40 plaques $\times 10^{10} = 4 \times 10^{11}$ 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 g

- Run the sample through the instrument under normal sorting conditions for 10 min, and re-weigh.

e.g., 0.6 g

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

e.g., 1.0 g − 0.6 g = 0.4 g or 400 μl
400 μl/10 min = 40 μl/min

- Calculate the expected number of bacteriophage flowing through the instrument, e.g., where the concentration of bacteriophage is 4×10^{11} PFU/ml.

40 μl/min $\times 4 \times 10^{11}$ PFU/1000 μl = 1.6×10^{10} PFU/min

b) Measurement of the *actual* throughput.
Here, the number of bacteriophage flowing through the instrument is measured directly from the sheath stream as it exits the nozzle tip.

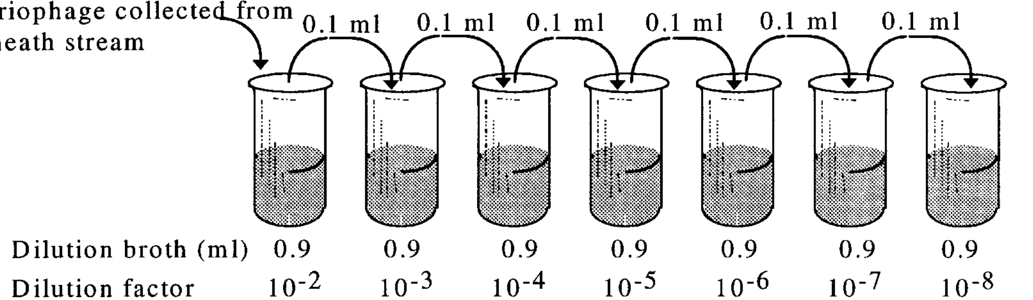
- Collect the sheath stream for exactly 1 min while running bacteriophage through the instrument into a tube that has been weighed empty. Re-weigh it to determine the volume collected in 1 min, e.g., 2.35 ml.
- Immediately transfer 0.1 ml of the sample to 0.9 ml of dilution broth. This is important because 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

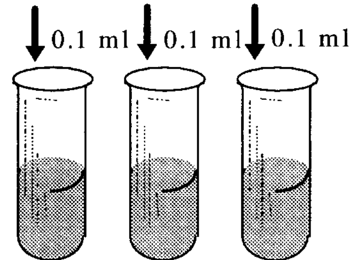
SCHEMATIC FOR TITRATION OF BACTERIOPHAGE IN SHEATH FLUID:

1. Prepare serial dilutions

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

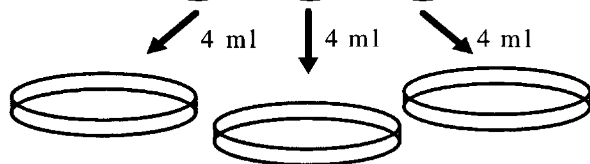


2. Inoculate top agar



Transfer 0.1ml 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:

Dilution	Number of plaques
10^{-6}	40[a]
10^{-7}	1
10^{-8}	0

[a]To calculate the actual throughput, select a petri dish with an intermediate number of plaques (usually between 10 and 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 $\times$ 10^7/0.1 ml) $\times$ 2.35 ml = 9.4 $\times$ 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, six 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 ~1,000 sort decisions per sec each left and right. Approximately 3.5 ml of bacteriophage suspension are needed.
- Run the good mode for 2 h. Replace the collection vials when they are full. Wait for ~3 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°C overnight for plaque development.
- Next, the bad mode is performed with six new petri dishes with confluent *E. 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 μm (droplet nuclei) is performed in combination with the T4/*E. 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. coli* lawn with the lid off into the sampling stage. Collect room air for 10 min. This dish will serve as negative control.
- Place two *E. 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 min of the good mode containment test.
- Place two *E. 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 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.

References

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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 10^9 PFU/ML)

Plate ID	Dilution	# of plaques	Dilution factor	Vol on plate	PFU/ml
A1	10^{-7}				
A2	10^{-8}				
A3	10^{-9}				

Does the T4 phage stock meet minimal concentration requirement (10^9 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 TROUGH CELL SORTER (MINIMUM 10^7 PFU/MIN)

Collect sheath stream for 1 min

Volume collected (ml): ________ml

Plate ID	Dilution	# of plaques	Dilution factor	Vol on plate	Sheath rate	PFU/min
B1	10^{-6}					
B2	10^{-7}					
B3	10^{-8}					

Does the T4 phage stock meet optimal established criteria for flow rate through flow cytometer (>10^7 PFU/min)? YES ☐ NO ☐

V. AEROSOL CONTAINMENT IN GOOD SORTING MODE

Plate ID	Plate location	# of plaques	Time
C1	inside right		2 h
C2	inside left		"
C3	door right		"
C4	door left		"
C5	x adjust		"
C6	top of machine		"

INSTRUMENT SETUP

Sheath pressure	psi
Drop frequency	KHz
Sort rate	decision/s
Droplets sorted	
Sample flow rate	µl/min
Door closed	
Vacuum on	
Nozzle tip	µ m
Auxiliary vacuum	
Drop drive amplitude	volts

Is aerosol contained in good sorting mode? YES ☐ NO ☐

VI. AEROSOL CONTAINMENT IN BAD SORTING MODE

Plate ID	Plate location	# of plaques	Time
D1	inside right		15 min
D2	inside left		"
D3	door right		"
D4	door left		"
D5	x adjust		"
D6	top of machine		"

Is aerosol contained in bad sorting mode? YES ☐ NO ☐

REMARKS: ____Signed: