

Measuring Containment of Viable Infectious Cell Sorting in High-Velocity Cell Sorters

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Background: With the advent of high-speed sorters, aerosols are a considerable safety concern when sorting viable infectious materials. We describe a four-part safety procedure for validating the containment.

Methods: This procedure includes aerosol containment, physical barriers, environmental controls, and personal protection. The Aerosol Management System (AMS) produces a negative pressure within the sort chamber, where aerosols are forced through a HEPA filter. Physical barriers include the manufacturer's standard plastic shield and panels. The flow cytometer was contained within a BSL-3 laboratory for maximum environmental control, and the operator was protected by a respiratory system. Containment was measured by using highly fluorescent Glo-Germ particles under the same conditions as the cell sort.

Results: Escaping aerosols were vacuumed for 10 min onto a glass slide and examined. With the AMS active and the cytometer producing the maximum aerosols possible, Glo-Germ particles remained within the sort chamber. Measurements taken directly outside the door averaged fewer than one particle per slide, and those taken at 2 ft away and on top of the sorter were completely negative.

Conclusions: With this monitoring system in place, aerosols can be efficiently measured, thus reducing the risk to the operator while sorting viable infectious cells. Cytometry Part A 52A:122-130, 2003.

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Key terms: flow cytometry; high-speed sorting; biosafety; biohazard

With the advent of high-speed sorters and the demand for infectious cell sorting increasing, containment of infectious aerosols and an effective means for measuring that containment is crucial. We developed a sensitive, reproducible, and efficient method for determining aerosol containment, which can be performed before each infectious sort procedure. In addition, we provide a complete safety protocol with guidelines to minimize operator risk of exposure.

The measurement of aerosol containment began with T4 bacteriophage sorting onto *Escherichia coli*-sensitive plates. Plaques formed as a result of natural infection and could be enumerated. Hence, the larger the plaque count, the poorer the aerosol containment (1,2). Several instrument "failure" modes were tested, but this system was found to be cumbersome and non-reproducible. These tests took several days to perform and often were performed at 2- to 3-month intervals. In addition, laboratories involved in sorting viable cells for cultures were faced with the possibility of *E. coli* contamination. In light of these drawbacks, highly fluorescent particles were used as a substitution for T4 bacteriophage sorting and collected on microscopic slides (3,4). These particles, called Glo-Germ, were developed to mimic hospital infection for studying the possible spread of bacteria within a hospital setting. Under a Woods lamp (using ultraviolet light),

these particles are extremely fluorescent and are ideal for this application. In the flow cytometer these particles were sorted under "failure" modes and detected on microscope slides. Thus, loss of containment was correlated with the increased number of particles per slide.

MATERIALS AND METHODS

Particle Preparation and Sort Conditions

Glo-Germ particles (5- μ m melamine copolymer resin beads in a 5-ml volume of ethanol) were washed in 100% ethanol (900g) for 10 min and washed again for the same

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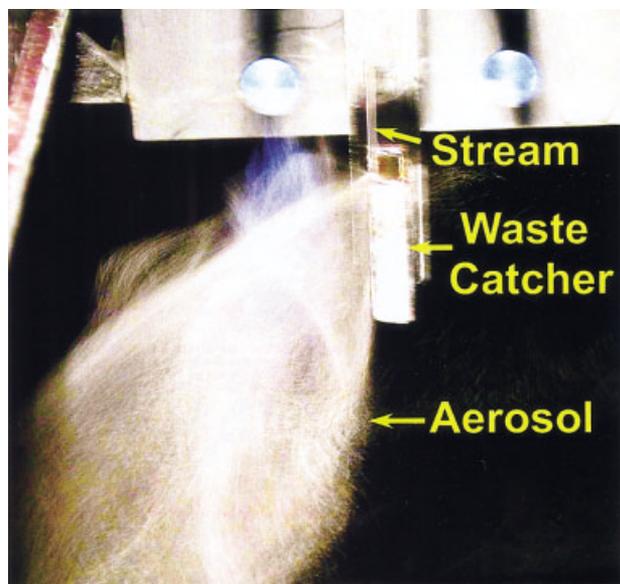


FIG. 1. Aerosol produced after deflection from the waste catcher. This figure shows the amount and position of aerosols produced after deflecting from the side of the waste catcher. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

time and speed in suspension medium (10% fetal calf serum, 1% Tween 20, and 1 mg/ml of sodium azide in phosphate buffered saline). The particles were resuspended in 100 ml of suspension medium and stored in an opaque glass container at 4°C for up to 1 year. Particles were filtered through a 100- μ m filter, and this stock was diluted with phosphate buffered saline (~1:20) before sorting to achieve an acquisition rate of 20,000 particles per second. Two modes of sorting were used, the standard sort mode and the aerosol sort mode. In the aerosol mode, the waste stream carrying all the particles was deflected to the edge of the waste catcher by using the voltage balance adjustment to create a large aerosol (Fig. 1). This simulated a worst-case sort stream failure or blocked tip. The particle rate was set to 20,000 particles per second at a sheath pressure of 25 psi. In the standard mode, all settings remained the same, except the waste stream was returned to normal alignment and 1,000 particles per second were sorted left and right into 15-ml conical tubes.

Vacuum Containment and Collection Systems

The Aerosol Management System (AMS; Becton Dickinson, Mountain View, CA.) was installed on a standard FACSDiVa. This system consists of six vacuum ports inside the sort chamber and is operated by a vacuum pump at 2–3 standard cubic feet per minute (SCFM). This system is self-contained, and aerosols collected by this system are pushed into a povidone-iodine trap before entering a 0.2- μ m HEPA filter. Approximately 2.2 air exchanges per minute are removed from the chamber without distortion of the sort streams (Fig. 2). After each sort, the vacuum

ports are cleaned with 70% ethanol and distilled water (dH₂O) to maintain optimal vacuum. After sorting infectious specimens, the flow cytometer is disinfected by running a 10% β -dyne solution through the sample tubing for 5 min, followed by 1-min intervals of 0.1 N NaOH, Coulter Clenz (Coulter Corp., Miami, FL) and dH₂O. The AeroTech concentrator (Aerotech Laboratories, Phoenix, AZ) was used to collect and concentrate aerosols onto a glass slide taped to a Petri dish (15 \times 100 mm) placed inside this device. A constant vacuum pressure (45 LPM) was applied and measured by an adjustable inline Matheson flow meter (Fig. 3). This device and meter were connected to an independent vacuum source (in-house) separate from the AMS. For this device to operate correctly, it must be cleaned regularly by sonication for 5 min in a water bath followed by one wash with a mild detergent and one wash with dH₂O.

Personal Protection

The personal protection system was purchased from ProVison (DePuy, Chesapeake Surgical, Sterling, VA.) and exceeds requirements of the Centers for Disease Control (CDC) and Occupational Safety and Health Administration (OSHA) for protection from infectious aerosols larger than or equal to 0.1 μ m size. This system consists of a full-body suit containing a 0.1- μ m HEPA filter, which is designed to fit onto a helmet. A battery-operated blower in the helmet moves room air constantly through this filter and down to an opening at the bottom of the suit (Fig. 4). This suit, combined with gloves and boots, is used for all viable infectious sort procedures.

Particle Measurement

A variety of experimental sorting conditions were tested with the standard or aerosol sort mode of operations and running the AMS vacuum at normal or compromised levels, as described in the Results. In all cases a Petri dish containing a clean glass slide (taped on the frosted end to the Petri dish to prevent sliding) was placed in the AeroTech device. When the device draws in heavy aerosols, a pattern from the sheath solution (sterile phosphate buffered saline with 25 μ g/ml of gentamicin) forms. This characteristic pattern is developed when these aerosols are pulled through the holes in the cover of the AeroTech system (Fig. 5). Under the sort conditions as described above, Glo-Germ particles are gated on the light scatter gate and a histogram of fluorescein isothiocyanate versus phycoerythrin parameters (Fig. 6A). After collection all slides were analyzed for particles by using a standard fluorescent microscope equipped with a 450/490-nm excitation filter and a 10 \times objective. Bright green particles indicate a positive test, and all particles were enumerated from the entire slide (Fig. 6B).

RESULTS

Table 1 shows the sensitivity of the AeroTech collection system. Under no containment vacuum pressure and with the front shield open, particles are effectively collected into this device. As the vacuum is dropped from 90 to 45

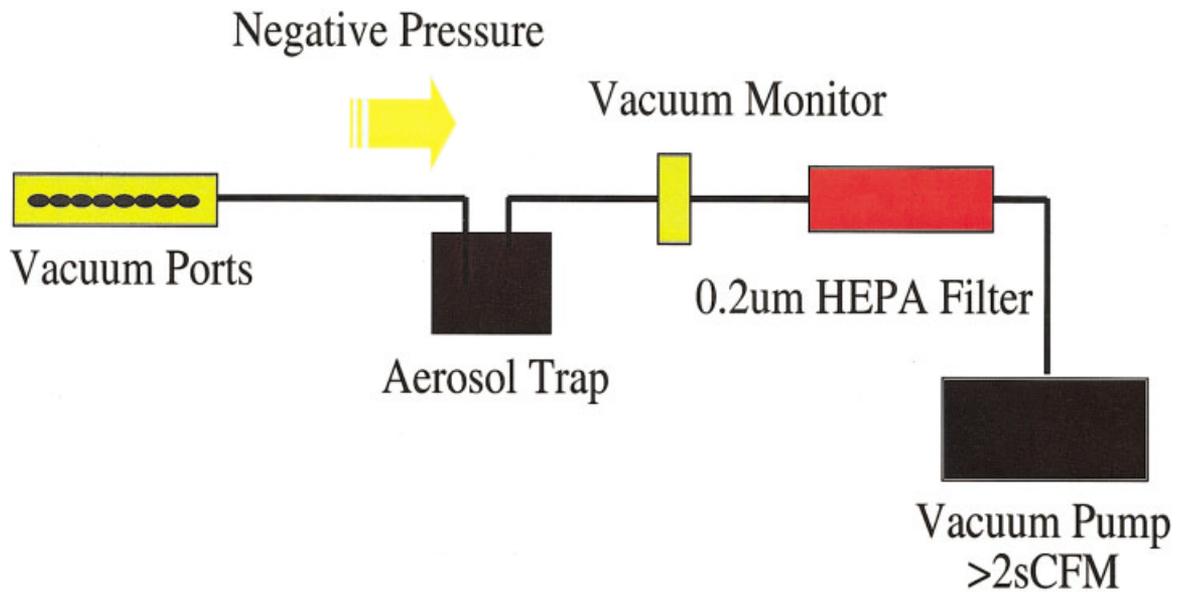


FIG. 2. Vacuum flow of the Aerosol Management System. This system consists of six vacuum ports, a povidone-iodine trap, a vacuum monitor, a 0.2- μm HEPA filter, and a vacuum pump (>2 SCFM).



FIG. 3. AeroTech collection device. This picture shows the device in separate parts and assembled (inset). The AeroTech collection system consists of the base plate, the middle holed plate, and a funnel plate. The base plate holds a Petri dish, where a glass slide can be placed to detect the Glo-Germ particles. The middle plate is placed on top of the base and contains 400 precision holes, which produce a characteristic pattern if large amounts of aerosols are present. The top plate contains a funnel, where aerosols are forced down through the holes and onto the glass slide.

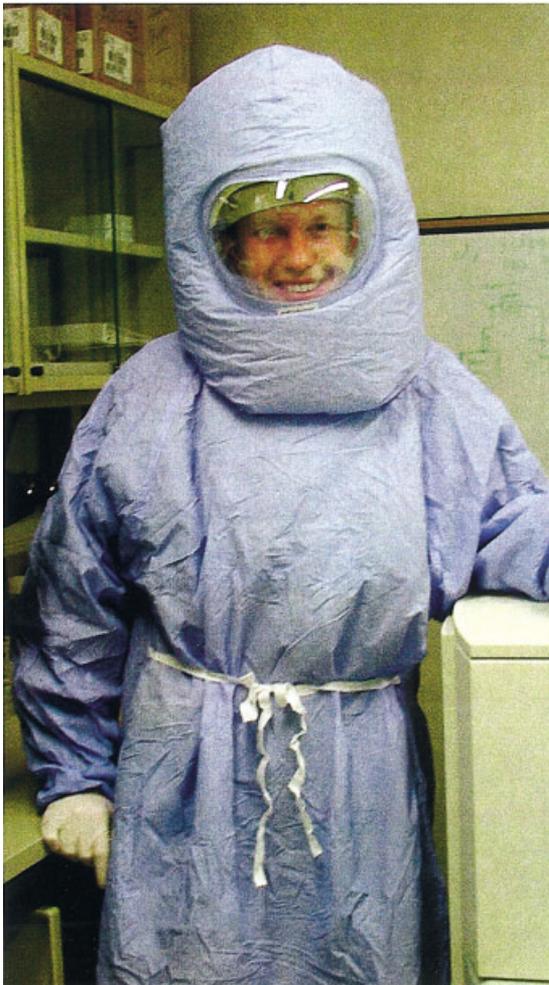
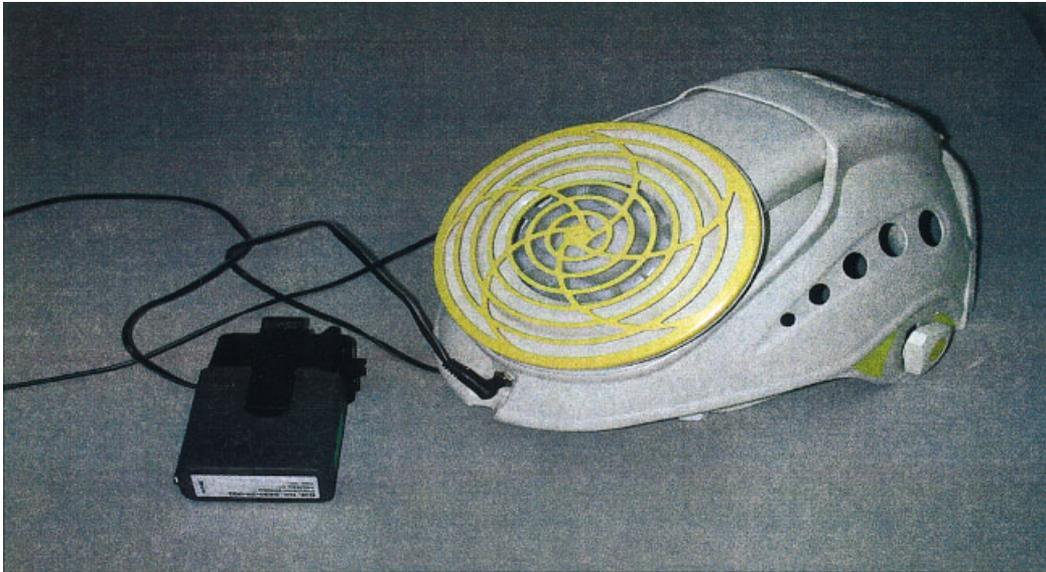


FIG. 4. The ProVison Personal Protection System. These photographs show the ProVison Personal Protection System and its mechanical helmet. The helmet consists of a battery-powered blower, which constantly moves room air through a 0.1- μm HEPA filter in the top of the suit. The respiratory suit as shown is a full-body suit with complete face protection and, when worn with gloves and boots, protects hands and feet from contamination during a viable infectious sort procedure.

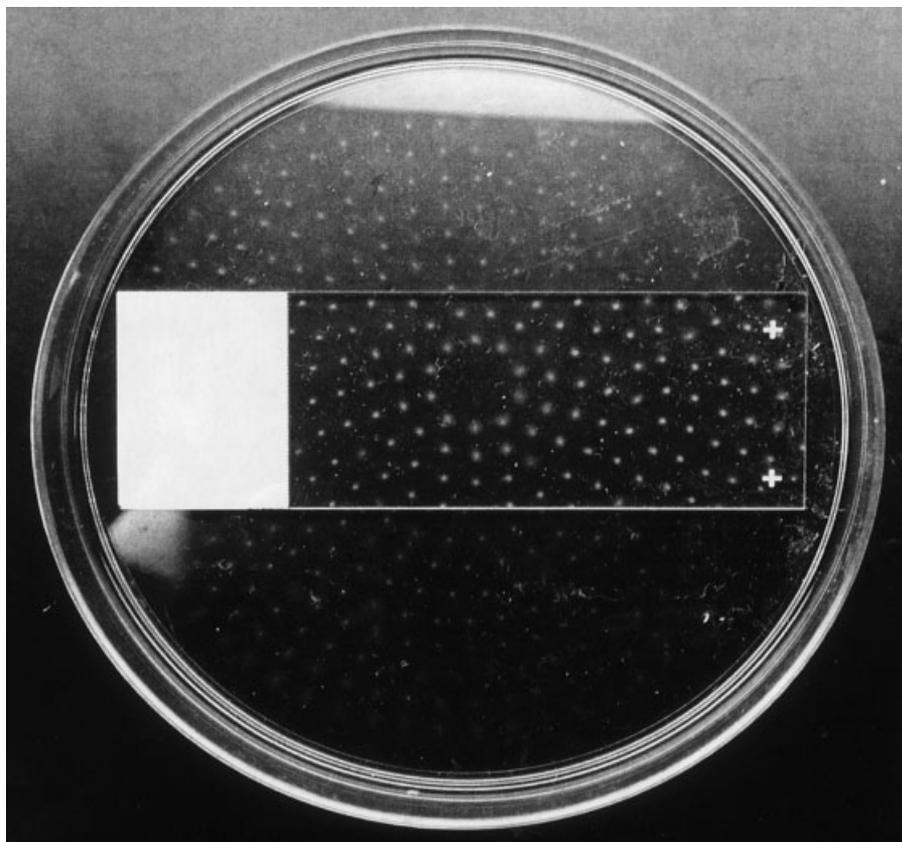


FIG. 5. Glo-Germ particles collected onto a glass slide after 10 min of exposure to heavy aerosols at a vacuum pressure of 45 LPM. Due to the positioning of the glass slide in the AeroTech collection system, approximately 130 dots are formed as a result of phosphate buffered saline sprayed through the holes in the cover plate. These salt dots provide a reference for the location of the Glo-Germ particle deposits. Under normal testing conditions with full containment, there should be no aerosols; hence, no spots will be visible on slide. The entire slide is scanned for these particles under fluorescent light with a 10 \times objective.

LPM, approximately half as many particles are collected. To determine the highest vacuum that could be applied to the AeroTech system without creating false positives, we tested the system under full aerosol conditions with the front shield closed and the containment vacuum on. With the AeroTech vacuum approaching the same level as the AMS (\sim 90 LPM), we were able to pull out 270 Glo-Germ particles from around the edges of the front shield. However, as we decreased the vacuum of the AeroTech device to 45 LPM, only one particle was detected after 10 min of sampling. Thus, to avoid competing with the vacuum of the AMS and to avoid pulling Glo-Germ particles from the sort chamber as false positives, a vacuum pressure of 45 LPM was selected as the standard level in all experiments. Under these conditions and without containment (Table 2, condition a), sensitivity of detection was measured in front of the sort chamber, on top of the sort chamber, and 2 ft from the sort chamber (Fig. 7). After 10 min of aerosol collection, 60 particles were collected 2 ft from the sort chamber, 50 particles on top of the cytometer, and 6,500 particles in front of the sort chamber.

While still simulating maximal aerosol generation (e.g., after a sample clog) and with containment active (AMS on) and the front shield closed, fewer than one particle per slide was detected in front of the sort shield within 30 min. No particles were detected at 2 ft from the sort chamber or on top of the cytometer (Table 2, condition b).

After changing the sort mode to the standard sort condition, which represents the majority of sort time and perhaps the majority of aerosol production, no particles were detected at any location (Table 2, condition c). If the sort chamber vacuum pressure was compromised (cut in half), simulating a failure or clogged HEPA filter, an increase in particles outside the sort chamber was detected (Table 3). This observation indicates the importance of a monitor for the vacuum on the containment system and shows the degree of sensitivity of the detection system. After sorting in the aerosol mode, particles were detected up to 30 s after stopping the sort by placing the sample in standby. However, when sorting in the standard mode, no particles were detected at any time after stopping the sort (Table 4). Hence, when removing a sample clog from the jet tip or at the end of a sort, the operator must wait at least 30 s for aerosol removal before entering the sort chamber.

DISCUSSION

The safe handling of human blood-borne pathogens is well documented in the OSHA guidelines (Fed. Reg. 29 CFR 1910.1030.33) and in the 1992 and 1997 revised CDC guidelines (5,6). However, these documents do not address cell sorting of viable infectious samples. Procedures for biosafety as a complete method for sorting unfixed cells were introduced in 1997 and 1999 (7,8). These documents do outline safety considerations and proposed

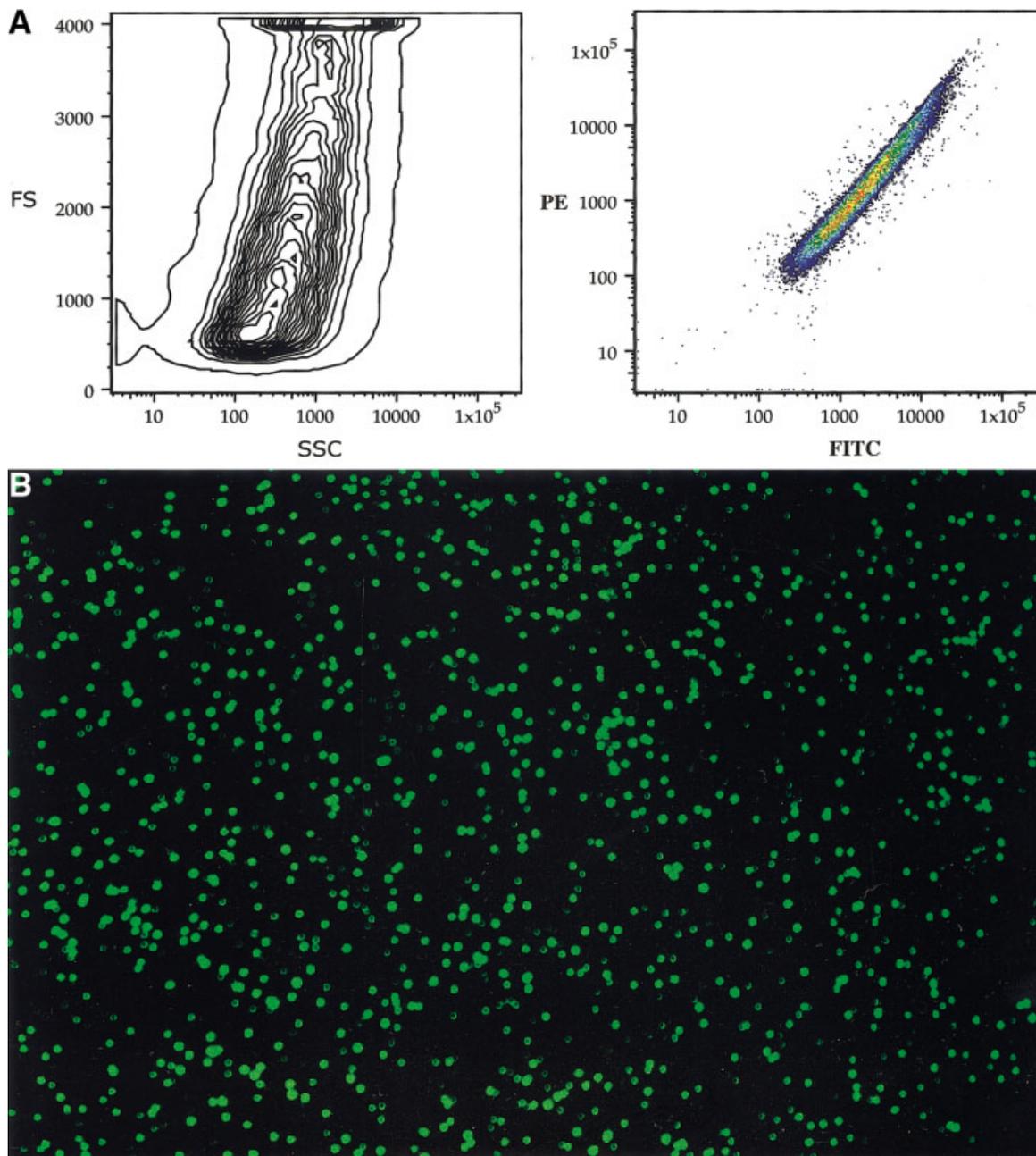


FIG. 6. Glo-Germ histograms and FL microscopic view. **A:** Representative histograms show the intensity of Glo-Germ particles and the variety of sizes as measured by the light scatter pattern. **B:** The intensity of fluorescence allows quick location and identification when scanning a microscopic slide contaminated with Glo-Germ particles. The particle size variation increases the availability of carrying small particles in the form of aerosols as compared with larger particles. FITC, fluorescein isothiocyanate; FS, forward scatter; PE, phycoerythrin; SSC, side scatter.

bacteriophage detection for measuring containment. However, these procedures are cumbersome, time consuming, and not reproducible. For these reasons, they are not done routinely each time an infectious sort is performed. With the detailed procedure presented in this report, we have introduced a reproducible, efficient, and safe procedure to validate aerosol containment, thus ensuring containment for each day of viable, infectious sorts.

This system demonstrated a greater than four-log reduction in aerosols with the use of the AMS. In addition, we used a reliable personnel protection system to ensure the additional safety we believe is required when using high-velocity cell sorters to sort potential infectious cell samples.

Under maximum aerosol conditions with containment on and the sort chamber closed, very few particles (<1/

Table 1
*Sensitivity of the AeroTech System Under no Containment**

Period	90 LPM	45 LPM
1 min	3,900	420
3 min	7,150	2,500
10 min	14,300	7,300
30 min	71,000	22,450

*This table shows the number of particles that escape the sort chamber when different vacuum pressures are applied to the AeroTech system. Increased vacuum increases the particle count, but vacuum pressures closer to the containment system (>2 SCFM) produce false positives by competing with the containing vacuum.

slide after 10 min) escape the chamber as measured directly in front of the sort chamber shield. Because the AeroTech device is so effective in collecting these particles, the detection of these particles may represent the competing vacuum and not loss of containment. However, to produce aerosols large enough to detect outside the sort chamber, the sort stream would need to be deflected for an extended period. This would only represent a brief occurrence if the sort were monitored routinely. Hence, monitoring the sort by use of the Accudrop camera or a similar device should be included in the operational procedure to maintain effective containment. In addition, if particles should escape during this situation, the personnel respiratory system is designed to maintain operator safety. Even when sorting under standard conditions in which normal amounts of aerosols would be produced at the greatest amount of sort time, no particles were detected at any location. This observation suggested that the AMS effectively contains particles within the sort chamber and, hence, reduces the risk of exposure to the operator.

Based on the present data, measurement of containment should be performed routinely for all cell sorters involved in infectious cell sorting. Given our testing conditions (20,000 particles/s, containment vacuum > 2 SCFM, AeroTech vacuum at 45 LPM, in aerosol mode), we recommend testing three locations outside the sort chamber for at least 10 min before live infectious sorting. Because the greatest risk of generating aerosols is during a sample clog condition, we recommend testing where the maximum number of aerosols can be generated. This test condition, as described in Materials and Methods, produces high aerosols and simulates the failure mode of operation. Even with sound containment, the vacuum generated by the AeroTech device may compete slightly with the vacuum inside the sort chamber. This is especially true for samples taken directly in front of the chamber door. Due to the sensitivity of active air sampling, we recommend a tolerance of no more than three particles on any one slide outside the sort chamber to be considered safe for infectious cell sorting. It is important that the AeroTech device is connected to a separate and measurable vacuum system and should be tested frequently under no containment conditions to determine effective collection of aerosols. As demonstrated, the vacuum pressure of the containment system must be constantly monitored, and the HEPA filter should be maintained to the same degree as a laboratory laminar flow hood.

We strongly recommend that, when sorting infectious samples in a high-velocity cell sorter, the operator wear a protective biosafety respiratory suit that protects against aerosols larger than 0.1 μm . Although BSL-3 level protection may not be required for all infectious sorting, we recommended that high-velocity cell sorters engaged in infectious sorting be located within such a facility.

Successful oral transmission of human immunodeficiency virus type 1 is well documented in children who

Table 2
*Measurement of Containment Under a Variety of Test Conditions**

Period	Condition ^a			Condition ^b			Condition ^c		
	A	B	C	A	B	C	A	B	C
1 min	390	15	15	0	0	0	0	0	0
3 min	1,950	23	20	0	0	0	0	0	0
10 min	6,500	50	60	0.25	0	0	0	0	0
30 min	20,850	132	144	0	0	0	0	0	0
60 min	NT	NT	NT	NT	NT	NT	0	0	0

*This table shows the measurement of containment as a result of sorting particles in the aerosol or standard sort mode. Briefly, the aerosol mode simulated a sample clog in which particles were allowed to deflect from the edge of the waste catcher. In the standard mode, the waste stream was returned to normal alignment position, thereby producing smaller amounts of aerosols. A, front of sort chamber; B, top of sort chamber; C, 2 ft from the sort chamber (see Fig. 7); NT, not tested.

^aAeroTech system under no containment and in the aerosol mode (45 LPM, average $n = 5$). This condition shows the number of particles that escaped when the containment was off and the front shield was opened. Even at 2 ft from the sort chamber, particles were collected, with most particles found in the front of the chamber.

^bTolerance of particles under containment and in the aerosol mode (45 LPM, average $n = 5$). This condition shows the number of particles that escape when the containment system was active and the front sort shield was closed. After 10 min, very few particles were detected in front of the chamber, whereas none were detected at the top of the cytometer or at 2 ft from the sort chamber.

^cTolerance of particles under containment and in the standard sort mode (45 LPM, $n = 5$). This condition shows the number of particles that escaped when the sort stream was correctly adjusted to the waste catcher and both sides were sorting particles. No particles were detected at any location outside the sorting chamber.

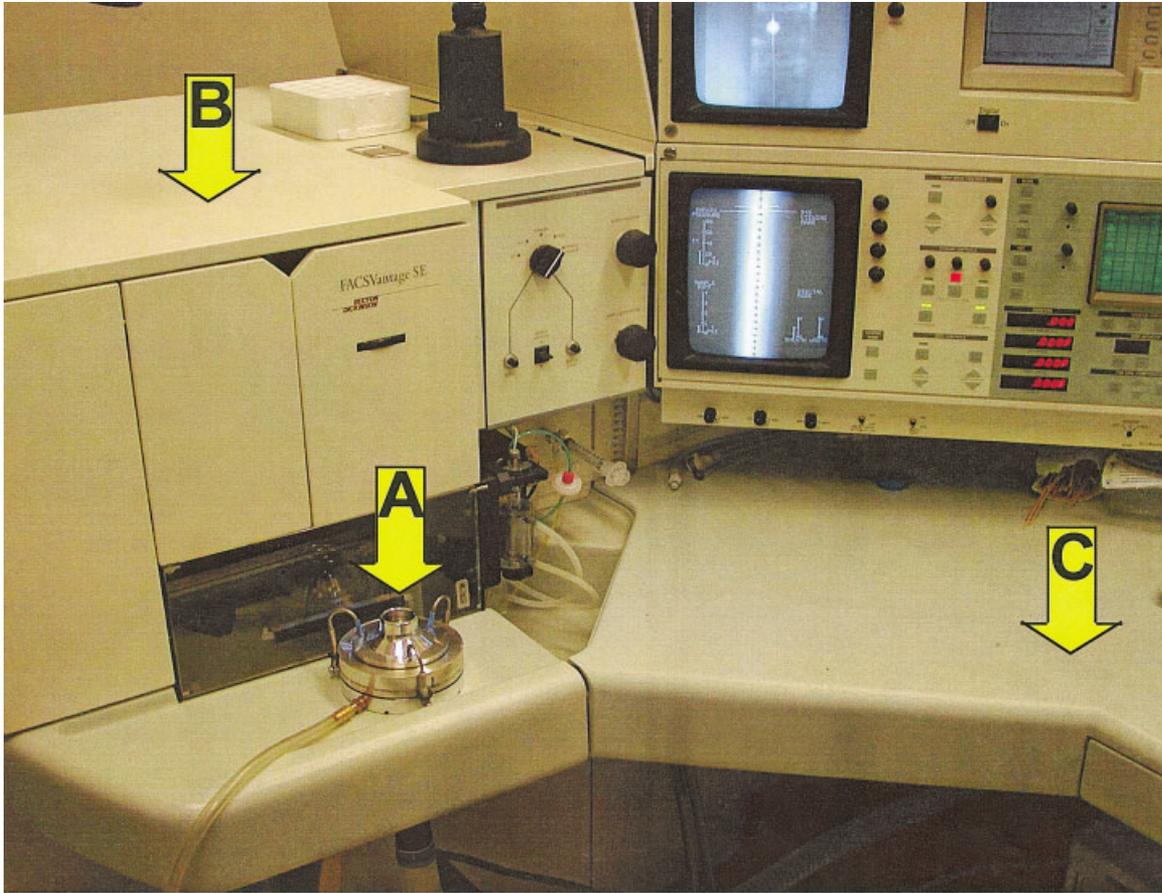


FIG. 7. Collection locations relative to the sort chamber. Arrows show the positions of the AeroTech collection device as described in Table 2 (A, front of sort chamber; B, top of sort chamber; C, 2 ft from the sort chamber).

become infected postnatally through breast milk. In addition, animal studies have shown that viremia and acquired immunodeficiency syndrome develop after nontraumatic oral exposure to several strains of simian immunodeficiency virus. These studies have shown the risks of transmission during oral exposure to highly infectious strains of simian immunodeficiency virus. However, these doses were delivered by syringe and were not aerosols. In fact, no documented animal cases of infection have been reported with aerosol delivery systems (9). The difficulty of infection under these conditions is thought to be due to

Table 3
*Aerosol Detection After Compromising the Aerosol Containment System Sort Chamber**

Period	Containment vacuum		
	>2 SCFM	1 SCFM	Off
10 min	0	140	338

*This table shows the effect on particle containment when the vacuum pressure on the aerosol containment system is compromised. Data show a dramatic increase in particles escaping the chamber with decreasing vacuum pressure.

the fragility of Lentiviruses. Potentially, infectious samples could contain other viruses (hepatitis B or simian herpes simplex) or bacteria (*Mycobacterium tuberculosis*), which are known to transmit through aerosols and infect via inhalation (10-18).

Table 4
*Aerosol Detection After Opening the Sort Chamber**

Standby time	Sort mode	
	Standard	Aerosol
0 s	0	12
15 s	0	5
30 s	0	1.5
60 s	0	0
120 s	0	0

*Five particles per slide were found at 45 LPM with the shield open after sort was stopped and the containment vacuum on. This table shows the amount of particles detected after the sort is stopped and the sorter control is placed in standby mode. When sorting normally, no particles were detected once the sorter was placed in standby mode. However, if the sorter is placed in the aerosol mode, which simulates maximum production of aerosols, particles can be detected up to 30 s but not after 1 min.

Although no data to date have shown that flow cytometer operators are infected when sorting infectious materials, the advent of high-velocity sorters certainly has increased the risk of these procedures. It cannot be overemphasized that personal protection is the most important component to this procedure, and measurement of instrument containment should be required before any infectious cell sort. It is our hope that these procedures will improve the overall safety of the operators involved in infectious cell sorting, and instrument manufacturers should use these methods to improve containment of these valuable instruments in science.

LITERATURE CITED

- Merrill JT. Evaluation of selected aerosol-control measures on flow sorters. *Cytometry* 1981;1:342-345.
- Ferbas J, Chadwick KR, Logar A, Patterson AE, Gilpin RW, Margolick JB. Assessment of aerosol containment on the ELITE flow cytometer. *Cytometry* 1995;22:45-47.
- Sorensen TU, Gram GJ, Nielsen SD, Hansen JE. Safe sorting of GFP-transduced live cells for subsequent culture using a modified FACS vantage. *Cytometry* 1999;37:284-290.
- Oberyszyn AS, Robertson FM. Novel rapid method for visualization of extent and location of aerosol contamination during high-speed sorting of potentially biohazardous samples. *Cytometry* 2001;43:217-222.
- Guidelines for the performance of CD4⁺ T-cell determinations in persons with human immunodeficiency virus infection. *MMWR Recomm Rep* 1992;41:1-17.
- 1997 Revised guidelines for performing CD4⁺ T-cell determinations in persons infected with human immunodeficiency virus (HIV). Centers for Disease Control and Prevention. *MMWR Recomm Rep* 1997;46:1-29.
- Schmid I, Dean PN. Introduction to the biosafety guidelines for sorting of unfixed cells. *Cytometry* 1997;28:97-98.
- Schmid I, Kunkl A, Nicholson JK. Biosafety considerations for flow cytometric analysis of human immunodeficiency virus-infected samples. *Cytometry* 1999;38:195-200.
- Ruprecht RMBT, Liska V, Ray NB, Martin LN, Murphey-Corb M, Rizvi TA, Bernacky BJKM, McClure HM, Andersen J. Oral transmission of primate lentiviruses. *J Infect Dis* 1999;179:S408-412.
- Scott RM, Snitbhan R, Bancroft WH, Alter HJ, Tingpalapong M. Experimental transmission of hepatitis B virus by semen and saliva. *J Infect Dis* 1980;142:67-71.
- Jacobson JT, Orlob RB, Clayton JL. Infections acquired in clinical laboratories in Utah. *J Clin Microbiol* 1985;21:486-489.
- Pearse J. Infection control. Nursing management of a patient with hepatitis A and B. *Nurs RSA* 1992;7:28-29.
- Miller RL. Characteristics of blood-containing aerosols generated by common powered dental instruments. *Am Indian Hyg Assoc J* 1995;56:670-676.
- Holmes GP, Chapman LE, Stewart JA, Straus SE, Hilliard JK, Davenport DS. Guidelines for the prevention and treatment of B-virus infections in exposed persons. The B Virus Working Group. *Clin Infect Dis* 1995;20:421-439.
- Bermudez LE, Goodman J. Mycobacterium tuberculosis invades and replicates within type II alveolar cells. *Infect Immun* 1996;64:1400-1406.
- Moore AV, Kirk SM, Callister SM, Mazurek GH, Schell RF. Safe determination of susceptibility of *Mycobacterium tuberculosis* to antimycobacterial agents by flow cytometry. *J Clin Microbiol* 1999;37:479-483.
- Lever MS, Williams A, Bennett AM. Survival of mycobacterial species in aerosols generated from artificial saliva. *Lett Appl Microbiol* 2000;31:238-241.
- Nunez R, Ackermann M, Saeki Y, Chiocca A, Fraefel C. Flow cytometric assessment of transduction efficiency and cytotoxicity of herpes simplex virus type 1-based amplicon vectors. *Cytometry* 2001;44:93-99.