General Guidelines for Experimenting with HIV

Performing experiments using human immunodeficiency virus (HIV)-infected materials represents a potential biological hazard for the investigator. An intensive training program for laboratory personnel must always precede the actual execution of research involving manipulation of HIV. In addition, appropriate sterile tissue culture techniques are absolute prerequisites for producing meaningful experiments and for achieving safe working conditions. In most circumstances more than one investigator uses the same HIV research facility; therefore, careful training in biosafety is mandatory not only for self-protection, but for the safety of other investigators as well. Basic Protocol 1 establishes a general framework for working safely with HIV and the Basic Protocol 2 describes the proper storage of HIV stocks and test supernatants.

GENERAL GUIDELINES FOR WORKING SAFELY WITH HIV

This protocol describes the organization of a biosafety level 3 containment laboratory for HIV research. CDC-NIH guidelines recommend that concentration of HIV or SIV be conducted in biosafety level 3 (BSL3) facilities. For activities involving viral culture, acceptable safety standards may be met with the use of biosafety level 3 work practices conducted in a secure, self-contained biosafety level 2 facility. Precautions to ensure worker safety are detailed, including the requirements for personal protective equipment, decontamination of work surfaces and infectious waste.

Safety in the immunology laboratory is also discussed in the introduction to Chapter 7.

Materials

- 70% ethanol
- Bleach
- Virucidal disinfectants
- Biohazard door sign
- Biohazard labels
- Biosafety cabinet
- Tabletop centrifuge and microcentrifuge equipped with aerosol-containment devices
- 4°C refrigerator
- −20°C and −80°C freezers
- Sink equipped with foot pedal
- Cryogenic storage unit: mechanical −140°C freezer or liquid nitrogen cryogenic storage unit set up for vapor storage only
- Cryogenic gloves
- Personal protective equipment: Tyvek suits or surgical gowns (legs must be covered), shoe covers, face shields, disposable sleeve covers, gloves (non-powdered latex and/or nitrile, with a long cuff)
- Biohazard bags with temperature-sensitive labels
- Medical pathological waste (MPW) disposable containers for incineration

Set up the HIV laboratory

1. Separate the BSL3 laboratory from public corridors with two doors to ensure a secure, self-contained laboratory. Post a sign on the outer door, which includes the universal biohazard symbol, the biosafety level, a list of the infectious agents in use, entry requirements, and contact information for the principal investigator(s), laboratory
manager, and safety personnel. Limit access to investigators who have completed safety training and demonstrated competence in BSL3 procedures. Provide maintenance and service personnel with appropriate safety information and supervise access to the facility. The HIV laboratory should contain an autoclave and all equipment necessary for HIV experimentation including a biosafety cabinet, −20° and −80°C freezers, tabletop centrifuges, a 4°C refrigerator, and a cryogenic storage unit.

Ensure that windows are closed and sealed. Unidirectional air flow into the laboratory should be maintained by a ducted exhaust system, and the BSL3 space should be under negative air pressure in relation to public corridors and adjoining laboratories. There should be a pressure indicator at the entrance and alarms inside the facility to alert BSL3 laboratory staff of problems with the ventilation controls. Test the pressure differential and alarms annually.

2. Designate a containment laboratory anteroom in the space between the door to the public corridor and the door to the biosafety level 3 laboratory. Keep personal protective equipment in this area. Equip a foot pedal-operated handwashing sink, and an eyewash station within the containment laboratory.

Use appropriate precautions in entering the HIV laboratory

3. Put on personal protective equipment: a Tyvek suit or long gown (legs must be covered), shoe covers, and face shield. Use gloves with long cuffs or disposable sleeve covers to eliminate any exposed skin between the glove and the gown sleeve (two pairs of gloves are required for handling infectious cultures).

NOTE: Gloves should be selected carefully. Low-allergen, non-powdered latex gloves reduce staff sensitization to latex allergens; nitrile gloves are also an excellent option. Criteria for glove selection include: staff allergies or sensitivities, proper fit, and chemical permeability.

Facial shields, or masks and eye protection, should be worn when working with or transporting infectious materials.

4. Enter the HIV laboratory from the anteroom area. Bring needed materials from the anteroom into the laboratory.

Media, reagents, cell cultures, and other materials may move into the laboratory but are not removed unless autoclaved for disposal or properly packaged to be shipped to another BSL3 laboratory, in compliance with the International Air Transport Authority and Department of Transportation regulations. Standardized procedures must be developed for fixation or disinfection of any samples removed from the facility for further analysis (e.g., cells fixed in glutaraldehyde removed from the containment facility for electron microscopy). A review of conditions for HIV inactivation has been published by Sattar (1991).

Careful review of the use of sharps is mandated for BSL3. Needles, razor blades, glass Pasteur pipets, and glass culture vessels are not allowed in the BSL3 laboratory. Viral purification procedures, even of proviral DNA, must never involve the use of sharps. Consult a biosafety professional for advice on alternative procedures and safety products. When appropriate, safety needle products should be used for the injection of animals. In the laboratory, examples of safer substitutes would include the use of disposable plastic slides with counting grids and attached cover slips (KOVA, VWR) instead of glass hemacytometers, and disposable tissue grinders (10-ml and 50-ml size, Kendall) instead of glass Dounce homogenizers.

NOTE: Pipet tips must be ejected into a needle bucket or other closed container for autoclaving. Pipet tips may cause puncture injuries when discarded directly into biohazard bags.
Carry out stringent decontamination procedures inside the HIV laboratory

5. Use biosafety cabinets for all manipulations of infectious materials. Carefully decontaminate the work surface before and after each use with 70% ethanol (an effective, non-corrosive agent for routine decontamination of surfaces that are not visibly contaminated); a registered hospital disinfectant with virucidal properties is required after manipulation of clinical or animal samples.

Decontaminate spills with a freshly prepared 10% bleach solution. Post spill procedures and have available a spill kit consisting of bleach, paper towels, heavy utility gloves, and long forceps. Spill response is part of the initial training and annual re-training of laboratory staff. The following spill advice should be carefully reviewed:

i. If a small spill occurs within the biosafety cabinet, immediately cover the spill with paper towels and decontaminate with 10% bleach.

ii. If a spill occurs outside of the biosafety cabinet (e.g., viral culture dropped on the floor), leave the laboratory immediately and report the spill according to established procedures. Post a “spill” sign on the laboratory door to warn others to stay out of the laboratory. After 30 min, staff with appropriate personal protective equipment should enter, cover the spill with paper towels, and then gently add 10% bleach to the spill.

NOTE: Respiratory protection is required for spills with additional risk factors; consult the biosafety professional for specific advice. If clothing or shoes become contaminated, the items should be removed immediately for decontamination and should not be worn out of the facility. A scrub suit and boots or disposable footwear should be in the anteroom supply inventory in preparation for this type of emergency.

iii. As a general rule for cleaning up radioactive/biohazard spills, first inactivate the biohazards with disinfectant, then proceed with radioactive decontamination. Consult a radiation safety professional for specific procedures appropriate to the radioisotopes in use in the facility.

iv. To prevent hazardous spills, verify that o-rings in centrifuge rotors and buckets are intact before use. Remember that 37°C or 4°C rooms are sealed for temperature control and do not have the exhaust ventilation required for work with infectious materials.

6. Discard pipets, plates, and flasks within the biosafety cabinet to enhance containment by minimizing the number of times the arms are drawn through the protective curtain of air at the front of the biosafety cabinet. Discard pipets and other disposables into a 12 × 24-in. biohazard bag located inside the biosafety cabinet. Support the biohazard bag with a small plastic container; fold the excess length of the bag down over the sides of the container. Consult the biosafety professional to determine whether pipets must be decontaminated with 10% bleach before disposing into the bag. Aspirate culture fluid from plates and flasks prior to disposal to prevent leakage. Close the small biohazard bag before removing it from the biosafety cabinet, then place it inside another biohazard bag (serological pipets that are double-bagged in this manner, minimizes biohazard bag ruptures).

NOTE: Pipet tips can protrude through biohazard bags and cause exposure incidents. Tips should be ejected into needle disposal containers, or other closed containers before decontamination and disposal.

7. Set up vacuum aspiration systems with a liquid disinfectant trap containing 10% bleach and an overflow flask. Use a hydrophobic 0.45-µm in-line vent filter to protect the user from aerosols drawn through a free-standing pump or prevent discharge into the central vacuum system. Do not use glass pasteur pipets for aspiration in a BSL3 laboratory; use plastic aspiration pipets (Falcon). Aspirate culture fluid from plates to reduce the liquid volume in biohazard bags and the potential for infectious leaks and spills. Aspirate additional bleach through the tubing to decontaminate the vacuum aspiration set-up after use.
8. Change the outer pair of gloves frequently throughout the course of the experiment. If the outer glove becomes wet, remove both pairs of gloves immediately, wash hands with soap and water, and put on new gloves.

   Gloves must remain dry; a wet glove allows “wicking” of liquid through the protective barrier.

Decontaminate used materials and exercise care when leaving the laboratory
9. At the conclusion of the experiment, remove all waste from the biosafety cabinet and wipe the work surface with disinfectant. Incubate, properly store, or dispose of infectious materials in biohazard bags.

   NOTE: Radiation safety surveys should be completed at the conclusion of a radioactive experiment. The Radiation Safety Officer should be consulted about the use of radioisotopes in BSL3 research. There may be specific requirements for decontamination of the biohazards in the waste.

10. Place the infectious waste in biohazard bags in the autoclave for decontamination. The time required for effective sterilization varies due to the type of autoclave (gravity versus vacuum cycle) and the type of load (liquid versus solid). Consult a biosafety professional for assistance in setting up effective autoclaving protocols and validating conditions with B. stereothermophilus spores. After autoclaving, place the waste bags in medical pathological waste (MPW) containers for disposal by an MPW vendor.

   Ideally, the autoclave should be arranged for pass-through operations, whereby contaminated material is loaded into the autoclave within the HIV laboratory and the unloading and final disposition in MPW boxes is accomplished outside the HIV laboratory.

11. Exit the HIV laboratory through the anteroom. Dispose of gloves, suits, shoe covers, and other safety gear in the designated container by the exit door, or in the anteroom, of the BSL3 laboratory. Always wash hands thoroughly with soap and water after glove removal.

12. For emergency situations: In case of percutaneous or skin exposures to infected material, immediately wash the affected area with soap and water. For contact with eyes, nose, or mouth, flush with copious amounts of water. Remove any contaminated clothing, wash hands, and report immediately to the Occupational Health Services or other appropriate institutional health service office for evaluation. The CDC has published detailed US Public Health recommendations for post-exposure prophylaxis, and advises that medical follow-up should occur within hours of an exposure incident (CDC, 2001). Report the details of an incident to the principal investigator and to the biosafety professional. In some cases, procedures may be changed or equipment provided to prevent a re-occurrence. It is also important to report “near-miss” incidents in order to facilitate the continual review and update of safety procedures.

13. Post procedures for contacting emergency assistance clearly in the BSL3 laboratory, the anteroom, and other parts of the laboratory. Include contact information for the following:

   Emergency medical assistance in the event of an exposure incident, illness, or injury
   Spill response
   Biosafety and radiation safety professional to request assistance or report problems
   Fire and other emergency reporting information
   Equipment malfunctions
**PRESERVATION OF VIRAL STOCKS AND INFECTED CELLS**

HIV stocks are highly sensitive to degradation during storage at room temperature, although reverse transcriptase activity or p24 antigen levels may remain relatively stable. Viral stocks are relatively stable for 6 to 12 months at −70°C, although a decrease in titer is observable even under these conditions. Storage in liquid nitrogen (−120°C) allows the most stable preservation of HIV stocks, but this may pose a biosafety hazard, as cryovials may accidentally explode when suddenly exposed to room temperature. It is therefore best to limit or avoid liquid nitrogen storage of HIV-bearing cell lines. Indeed, special biosafety procedures described in Basic Protocol 1 (use of plastic facial shield, gowns, and double gloving) should be observed during the initial thawing of vials containing HIV that were frozen in liquid nitrogen. The warning label on plastic cryogenic vials—to be used in vapor storage only—should not be ignored at BSL3. Mechanical freezers that use liquid nitrogen vapor as a back-up are now commercially available for storage at −140°C. If liquid nitrogen cell storage systems must be used, they should be purchased with automatic filling cycles and a “vapor platform.” The vapor platform is a metal plate supported by legs which penetrate the liquid phase of the nitrogen. The leg height is determined by the manufacturer to maintain a minimum temperature of −120°C in the vapor phase storage. The liquid level of the unit must be adjusted to the platform height, and maintained by an automatic fill system. Inventory racks sized to fit on top of the platform allow only vapor phase storage of infected cells or virus.

Supernatants collected from infected cultures (e.g., to be tested for reverse transcriptase activity or p24 antigen production; **UNIT 12.2 & 12.5**) may be stored at 4°C or in a manual defrost −20°C freezer for several days without a substantial decrease in detectable viral levels. However, storage at 4°C is discouraged, as it may result in a biological hazard due to the possibility of accidental fluid spills. Furthermore, small amounts (e.g., volumes in 96-well plates) stored either at 4°C or −20°C longer than 24 hr may be subject to significant evaporation, resulting in artifactual concentration of viral proteins.

In general, storage of stocks and supernatants in the BL-3-contained −70°C freezer is the recommended procedure for all the experimental conditions mentioned. HIV-infected supernatants and cells may be frozen using the same procedures as for the equivalent uninfected materials (**APPENDIX 3**).

**COMMENTARY**

**Background Information**

AIDS results from the destruction of the human immune system, caused by long-term additive affects of human immunodeficiency virus type 1 (HIV-1) replication. Science has made significant contributions to understanding the pathology of the disease since its initial description (Gottlieb et al., 1981), including identification of HIV-1 as the etiological agent and subsequent development of blood tests to detect its presence (reviewed in Gallo, 2002), and development of anti-viral chemotherapeutic reagents to suppress HIV-1 replication and slow disease progression (Pomerantz and Horn, 2003). Despite these seminal advances, the global impact of the pandemic is staggering. By 2003, >60 million people had been infected with HIV-1, with over one-third of the cases resulting in death (Fauci, 2003). Continued understanding of the basic virology, pathology, and immunology of HIV-1 infection is paramount to effective management of this global health dilemma.

**Virology**

HIV-1 is a retrovirus belonging to the Lentivirus genus of Retroviridae. All replication-competent retroviruses carry gag, pol, and env genes that encode for virion structural proteins, replication enzymes, and surface envelope glycoproteins, respectively. HIV-1 carries six additional “regulatory” genes: tat, rev, vif, vpr, vpu, and nef. Whereas vif, vpr, vpu, and nef are dispensable for replication under select tissue culture conditions, the protein products of these genes play important roles in disease progression in vivo (Freed and Martin, 2001). HIV-1 is closely related to an endemic simian immu-
nodeficiency virus of the Pan troglodytes troglodytes subspecies of chimpanzees, and the zoonotic transmission of an archetype lentivirus from chimpanzees to humans likely played a key role in establishing HIV-1 in humans (Gao et al., 1999).

Infection begins with HIV-1 entry into a susceptible target cell, and tropism is determined by the expression of two different types of cell surface receptors: CD4 and a chemokine receptor, typically CCR5 or CXCR4 (Berger et al., 1999). Viral tropism for CCR5 or CXCR4 mainly resides in the V3 loop of the surface gp120 envelope glycoprotein, and viruses with affinity for CXCR4 and CCR5 are referred to as X4 and R5 strains, respectively (Berger et al., 1998). Following entry and a poorly defined “uncoating” step, HIV-1 RNA is converted into double-stranded DNA by the viral enzyme reverse transcriptase (RT) and its associated RNase H activity, and the linear form of viral cDNA is then integrated into a host cell chromosome by the viral enzyme integrase. Integration is essential for HIV-1 replication (Brown, 1997) and it is this step that separates retroviruses from all other animal viruses as only retroviruses require stable association of their genetic material with that of the host to productively replicate. This trait of irreversible integration makes it exceedingly difficult to completely eradicate HIV-1 from the human body once an initial infection is established (Blankson et al., 2002). Following integration, HIV-1 genes are expressed using host cell transcription and translation machineries, and the resulting RNA and virion proteins coalesce and bud through the plasma membrane to form HIV-1 particles. HIV-1 protease (PR) functions concomitantly with or shortly after budding to convert immature virions to infectious particles. Combination chemotherapy based on drugs that target RT and PR, referred to as highly active antiretroviral therapy (HAART), has significantly reduced death rates due to AIDS in regions of the world where the drugs are readily available (Pomerantz and Horn, 2003). The recent addition of an entry inhibitor that targets the initial fusion of HIV-1 with the cell membrane has since expanded the arsenal of HAART weapons (Pierson and Doms, 2003; Pomerantz and Horn, 2003).

Pathology

AIDS is hallmarked by a reduction in the number of circulating CD4+ T-lymphocytes, and key prognostic indicators of the disease state are the CD4+ T-cell count and plasma viral load as assessed by sensitive RT-PCR for HIV-1 RNA (Mellors et al., 1997). The disease course is described in three broad terms: primary or acute infection, clinical latency, and late-stage disease or AIDS. Acute infection is marked by a sharp increase in viral load and concomitant decrease in CD4+ T-cell counts. R5 strains preferentially transmit during acute infection and predominate at most disease stages (Zhu et al., 1993). Approximately 1% of caucasians are homozygous for a mutation that renders the CCR5 protein nonfunctional, and such clinical “non-progressors” resist infection despite repeated exposure to HIV-1 (Pierson and Doms, 2003). These findings highlighted CCR5 as a therapeutic target for intervention. Since drugs that target CCR5 could, in theory, select for outgrowth of X4 strains, the long-term therapeutic value of targeting CCR5 is currently unclear (Pierson and Doms, 2003).

Clinical latency can vary widely from patient to patient, but relatively long periods of ≥10 years are not uncommon. The concentration of plasma virus within a given patient remains relatively constant over long periods (Piatak et al., 1993). Whereas, only a relatively small number (<1%) of plasma T-cells are infected even during late-stage disease, a significantly higher (five- to ten-fold) fraction of CD4+ T-cells are infected in lymphoid tissue (Pantaleo et al., 1993). Despite the fact that the latency phase is relatively asymptomatic, an intense battle between HIV-1 and CD4+ T cells persists. The average life span of productively infected cells is only 2.2 days and the average life of circulating virus is only ~72 hr; nevertheless, ~1.03 × 10¹⁰ virions are produced daily (Perelson et al., 1996). This indicates that the steady-state level of circulating virus during latency results from robust HIV-1 replication and a pool of rapidly turning over infected cells. Drops in CD4+ T-cells to counts below ~200/μl indicates severe malfunction of cell-mediated immunity such that normally non-threatening ubiquitous microorganisms like Pneumocystis carinii become life threatening. The clinical definition of AIDS, in large part, centers on this low CD4+ T-cell count (Centers for Disease Control and Prevention, 1992).

Immunology

HIV-1 encounters dendritic cells (DCs) soon after entering the body and virus in association with DCs is likely carried to paracortical regions of lymphoid organs where it is transmitted to CD4+ T-cells (Steinman et al., 2003). Activated CD4+ T-cells are preferred targets for
HIV-1 and massive numbers of virions trapped within the germinal center follicular DC network present a continuous source of virus for de novo infection (Heath et al., 1995).

The sharp decrease in circulating virus after acute infection is heralded by the appearance of HIV-specific cellular and humoral immune responses (Borrow et al., 1994; Koup et al., 1994). Whereas cell culture techniques indicate responses (Borrow et al., 1994; Koup et al., 1994), whereas cell culture techniques indicate presence of virus, the more sensitive HLA-peptide tetramer technique indicates this frequency as high as 10% (Hoffenbach et al., 1989; Ogg et al., 1998). HIV-1 infection is also associated with increases in proinflammatory cytokines such as TNFα, IL-1β, and IL-6 (Poli and Fauci, 1995).

In addition to CD4+ T-cells, HIV-1 readily infects cells of the monococyte/macrophage lineage. Since HIV-1 replication in these cells is relatively noncytopathic, macrophages may represent one population of relatively long-lived chronically-infected cells (Perelson et al., 1997). However, a much longer-lived chronically-infected cell type originates from a small fraction (<0.05%) of productively infected CD4+ T-cells that escape cell death and revert to a quiescent state of post-integration latency (Blankson, 2002). The half-life of this latently infected pool of “memory” T-cells, estimated at −44 months, presents the most formidable challenge to complete eradication of HIV-1 by HAART. Immunology-based therapies, e.g., administration of IL-2 concomitant with HAART, may help to stimulate this otherwise “invisible” pool of chronically infected cells and aid in their eradication (Chun et al., 1999; Letvin and Walker, 2003).

Although infection can elicit antibodies that neutralize HIV-1 infectivity, the virus rapidly evolves to evade this challenge (Richman et al., 2003; Wei et al., 2003). An effective AIDS vaccine will likely require elicitation of both neutralizing antibody and CD8+ T-cell responses. A plethora of immunogens, which range from purified viral proteins to HIV-expressing DNA vectors, are in clinical trials to assess efficacies as vaccine candidates (McMichael and Hanke, 2003). Results of the first phase 3 clinical trial were reported in 2003, but the gp120 envelope glycoprotein-based immunogen unfortunately failed to elicit protection. This may have been due to the fact that the antigen used for vaccination was a monomeric gp120 protein whereas the actual virion contains a trimeric gp120. Although it is currently unclear if a future vaccine candidate will elicit sterilizing immunity against HIV-1 infection, it is hoped that advances in basic immunology may yield clues in how to obtain this much sought-after goal.

**Method of transmission**

HIV is transmitted by sexual contact with an infected person, or percutaneous exposures, most commonly by sharing needles or syringes during drug abuse. Infants born to HIV-infected women may become infected during the intrapartum or perinatal period, or through breastfeeding.

In the occupational setting, the CDC has reported 3 documented seroconversions in HIV research laboratory staff and 54 among healthcare workers. The type of occupational exposures that resulted in infection were as follows: 48 percutaneous (punctures/cut injuries), 2 mucocutaneous, 2 both percutaneous and mucocutaneous, and 2 with an unknown route of exposure. Of these documented seroconversions, 49 resulted from exposures to HIV-infected blood; 3 to concentrated virus in a research laboratory, 1 to visibly bloody fluid, and 4 to an unspecified fluid. An additional 138 cases of HIV infection occurred among healthcare workers who reported no risk factors for HIV infection except occupational exposures, however, seroconversion after an exposure incident was not documented in this group.

In 1992, two workers in different laboratories seroconverted to SIV. One exposure occurred from a needlestick; the other laboratory worker recalled no specific exposure incident but handled macaque SIV-infected blood specimens without gloves. This worker had dermatitis on the forearms and hands. Occupational infections with simian foamy viruses have also occurred, but have not resulted in disease or sexual transmission.

In the event of a suspected exposure, the laboratory worker must seek immediate medical assistance both for immediate treatment and for prophylactic anti-retroviral therapy.

**Critical Parameters**

The biosafety level 3 work practices described in this unit are specifically designed to minimize the risk of exposures to HIV, and to contain all work with this virus within a clearly defined, contained space. The CDC recommends that nonhuman primates or other animals infected with HIV or SIV be housed in animal biosafety level 2 (ABSL2) facilities using ABSL2 special practices and contain-
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12.1.8


Koup, R.A., Safrit, J.T., Cao, Y., Andrews, C.A., McLeod, G., Borkowsky, W., Farthing, C., and

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**Key References**


Centers for Disease Control and Prevention, 2001. See above. Available online at http://www.cdc.gov/mmwr/PDF/rr/rr5011.pdf, this article contains the latest CDC guidelines for post-exposure prophylaxis against HIV.