Published in final edited form as:

Nat Protoc. 2014 December ; 9(12): 2755–2770. doi:10.1038/nprot.2014.188.

A primary cell model of HIV-1 latency that uses activation through the T cell receptor and return to quiescence to establish latent infection

Michelle Kim^{1,2}, Nina N. Hosmane¹, C. Korin Bullen¹, Adam Capoferri^{1,2}, Hung-Chih Yang³, Janet D. Siliciano¹, and Robert F. Siliciano^{1,2}

Michelle Kim: michellekim@jhmi.edu; Nina N. Hosmane: nhosman1@jhmi.edu; C. Korin Bullen: korin23@gmail.com; Adam Capoferri: capoferri.adam@gmail.com; Hung-Chih Yang: hcyang88@ntu.edu.tw; Janet D. Siliciano: jsilicia@jhmi.edu; Robert F. Siliciano: rsiliciano@jhmi.edu

¹Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

²Howard Hughes Medical Institute, Baltimore, MD 21205, USA

³Department of Microbiology, National Taiwan University College of Medicine, Taipei 10051, Taiwan

Abstract

A mechanistic understanding of HIV-1 latency depends upon a model system that recapitulates the *in vivo* condition of latently infected, resting CD4⁺ T lymphocytes. Latency appears to be established after activated CD4⁺ T cells, the principal targets of HIV-1 infection, become productively infected and survive long enough to return to a resting memory state in which viral expression is inhibited by changes in the cellular environment. This protocol describes an *ex vivo* primary cell system that is generated under conditions that reflect the *in vivo* establishment of latency. Creation of these latency model cells takes 12 weeks and, once established, the cells can be maintained and used for several months. The resulting cell population contains both uninfected and latently infected cells. This primary cell model can be used to perform drug screens, study CTL responses to HIV-1, compare viral alleles, or to expand the *ex vivo* lifespan of cells from HIV-1 infected individuals for extended study.

INTRODUCTION

The major barrier to curing HIV-1 infection is the latent reservoir. This reservoir is predominantly comprised of a small pool of CD4⁺ T lymphocytes (CD4s) that are in a resting memory state and harbor a stably integrated, replication-competent HIV-1 provirus. These latently infected cells are phenotypically indistinguishable from uninfected cells and, consequently, are difficult to target for elimination. Latently infected cells occur at a

Competing Financial Interests

The authors declare that they have no competing financial interests.

Author Contributions

H.C.Y. and R.F.S. conceived and developed the original Bcl-2 model cell system. M.K. modified the protocol and wrote the manuscript. M.K., N.N.H., and C.K.B. performed experiments. All authors reviewed the manuscript.

frequency of ~1 in 10⁶ resting CD4s and have a half-life of approximately 44 months^{1–5}. Thus, the reservoir is extremely stable and infected individuals must receive lifelong treatment with antiretroviral therapy (ART) to inhibit viral propagation. Fortunately, current antiretroviral drug regimens are highly effective and have far fewer side effects compared to earlier drugs used to treat the infection. Although the life expectancy of an HIV-1 positive individual adhering to an effective ART regimen is similar to that of an uninfected healthy person⁶, not all infected individuals have the same adherence or access to necessary treatment. Treatment interruption can lead to viral rebound within weeks, even for infected individuals who have maintained undetectable viral loads⁷. Furthermore, it is not yet known whether treatment with ART that spans decades will cause adverse effects. As a result, there is much effort to find a cure for HIV-1 infection.

The focus of cure research has been on purging the latent reservoir. One approach, called “Shock and Kill”^{8,9}, involves reactivation of viral expression by a latency reversing agent (LRA) and subsequent viral clearance. The expectation is that global reactivation of viral gene expression would result in elimination of the reactivated cells by immune mechanisms or viral cytopathic effects (CPE), while the antiretroviral drug regimen of the treated individual would prevent viral spread. After serial rounds of reactivation, the hope is that the reservoir would ultimately be eradicated.

Development of the protocol and comparison with other methods

Effective reactivation of HIV-1 from latency is predicated on an understanding of how latency is established and maintained. Studying latency using cells from infected individuals is complicated by the extremely low frequency of latently infected cells. The need to obtain sufficient numbers of CD4s from HIV-1 infected individuals limits the breadth and frequency of experiments that can be performed. In addition, variation from patient to patient, as well as variation within the same patient (*e.g.*, drug adherence, drug regimen, *etc.*), can complicate the analysis of certain types of experiments. Therefore, there is a great need for reliable *in vitro* models of HIV latency. In these models, the frequency of latently infected cells may be higher than is seen *in vivo*. This allows examination of a greater number of experimental conditions and permits better measures of the reproducibility or toxicity of a particular treatment. One of the principles guiding cure research is the necessity for finding LRAs that do not induce global T cell activation, which causes clinical toxicity. Cell models are advantageous because potential toxicity, which may be due to cytokine release, can be easily assessed.

Early *in vitro* models were based on immortalized, proliferating T lymphoblast lines such as Jurkat and SupT1 that were infected with HIV-1 or HIV-1/GFP reporter viruses. These cell lines have been informative in identifying potential factors that may contribute to latency such as integration site and epigenetic state^{10,11}. However, transformed cell lines, even those that are derived from CD4s, are critically distinct from latently infected cells present *in vivo* because of their proliferative nature; they do not recapitulate the quiescent state of latently infected, resting CD4s. Another shortcoming of cell lines is their clonal nature, which means the proviral integration site is identical in every cell. Thus, studies of integration site-dependent variability would require other approaches. These cell lines are still a vital tool in

analyses that require vast numbers of cells, *e.g.*, genome-wide studies and proteomics^{12,13}, but they are not optimal for studying the *in vivo* mechanisms of HIV-1 latency in resting memory CD4s.

For this reason, other efforts to establish models for HIV latency have utilized freshly isolated CD4s from healthy donors. These cells are infected *in vitro* with HIV-1 under various conditions, some of which differ from the way in which latency is likely established *in vivo*^{14–19} (for review, see Spina, 2013²⁰). The model developed by Bosque and Planellas involves the infection of primary naïve CD4s that have been polarized by growth factors and antibodies to induce differentiation into central memory cells²¹. Another model created by the O'Doherty lab¹⁵ and developed by the Greene lab¹⁹ also calls for direct infection of resting CD4s by spinoculation. In the primary cell model designed by the Lewin lab, resting CD4s are treated with chemokines to make these typically non-permissive cells permissive to infection. The benefit of these primary cell models is that they all exhibit significant frequencies of cells with integrated HIV-1 DNA and can be used in viral reactivation assays in about one week after infection. A caveat with models involving direct infection of resting CD4s is that the infection is inefficient and not all of the viral genomes may be integrated. On the other hand, the models in which infection is preceded by activation do not allow sufficient time for the cells to return to a fully resting state. Furthermore, many of these primary cell models are limited by the short *ex vivo* lifespan of CD4s.

Here, we describe a primary cell model that more closely approximates the condition of latently infected CD4s in HIV-1 infected individuals. This model solves the problem of the short *ex vivo* lifespan of CD4s by transducing cells with the *B cell Lymphoma 2* gene (*Bcl-2*)¹⁷. Latency can then be established on a time scale that mimics physiological CD4 activation, infection, and return to a resting state.

The process begins with the isolation of CD4s from healthy donors and involves two rounds of lentiviral infection following activation through T cell receptor (TCR) engagement. Isolated CD4s are plated in medium containing antibodies to CD28 on a surface that is pre-coated with antibodies to CD3. Dual binding of the antibodies to CD3 and CD28 initiates intracellular signaling cascades that turn on expression of genes required for cytokine secretion, proliferation and differentiation – all hallmarks of T cell activation. Resting CD4s are relatively non-permissive to infection, and this natural activation produces a cell state that is receptive for HIV-1 infection. In the first round of infection, activated CD4s are transduced with a lentivirus expressing anti-apoptotic *Bcl-2*. The expression level of *Bcl-2* in these cells is similar to that in freshly isolated CD4s¹⁷ (Fig. 1) and extends the lifespan of the cells *ex vivo*. Thus, the cells can be infected with an HIV-1 reporter virus following another round of activation, and then cultured without exogenous cytokines for several weeks to allow a return to a resting state in which HIV-1 latency is established.

Applications and variations of the method

The *Bcl-2* model has been used in screens to search for drugs and drug classes that induce expression of latent HIV-1^{17,22–25}. In this protocol, for the second infection we describe the use of a replication-deficient HIV-1/GFP reporter virus called CM6¹⁷ (Fig. 2) that expresses only Tat, Rev, and GFP. This virus was designed to assess drug effects on activation of the

5'LTR (which functions as the promoter) without inducing viral cytopathic effects (CPE). The *env* gene is disrupted by insertion of a destabilized, enhanced GFP, and all other loci (*gag*, *pol*, *vif*, *vpr*, *vpu*, *nef*) are mutated to eliminate their expression. Using this reporter virus in one such screen, disulfiram was identified as a putative LRA and study of this drug advanced to clinical trials^{22,26}. In addition, the Bcl-2 model cells were used in identification of quinolines as another group of putative LRAs²³. Our lab continues to use these cells to screen drug libraries for potential LRAs or classes of drugs that may stimulate viral expression from the 5'LTR.

While we have utilized CM6 to study drug effects on viral expression, this virus could also be used to look at the effects of over-expression or knockdown of endogenous cellular proteins on viral gene expression; Bcl-2 model cells could be nucleofected with a construct that constitutively expresses a protein of interest, or that expresses shRNA to a protein of interest. The cells could then be easily assayed for viral activation by measuring GFP positivity by flow cytometry.

Both viruses in this protocol are pseudotyped with VSV-G envelope, which has a broad tropism and is associated with high stability in resulting virions; however, WT envelope could be substituted if, for example, a particular co-receptor tropism were being studied.

Since the virus used in the second round of infection can be uniquely engineered, the investigator has the flexibility to examine many facets of viral expression. Also, the second round virus need not be a reporter virus. For example, if one wished to study the differences between alleles of *gag* or *env*, a single, expanded batch of Bcl-2-transduced cells could be infected with a panel of viruses that each differed in one of those loci. The effects of those sequence differences on protein function in virion assembly or entry, respectively, could then be measured. If one were interested in looking at specific points in the life cycle of infection, one could, for instance, generate viruses that had mutations in RT, integrase, or protease. This could also be achieved by using drugs that inhibit these enzymes, but a virus mutationally null in any of these loci would obviate the need to continually add drugs over the course of an assay.

Bcl-2 model cells can also be employed to study “shock and kill” approaches to viral eradication, despite the anti-apoptotic function of Bcl-2. Its overexpression in model cells does not preclude viral CPE, as shown in work by Shan, *et al*²⁷. To examine the death of infected cells following reversal of latency, Shan *et al.* used a second round virus that expressed *vif*, *vpr*, and *env*, which are associated with CPE. These model cells died rapidly after reactivation through TCR engagement despite overexpression of Bcl-2. This study also showed that increased Bcl-2 expression does not render the model cells resistant to HIV-1-specific cytolytic T lymphocyte (CTL) responses. In those experiments, patient and healthy donor CD4s were transduced with Bcl-2 and then infected in the second round with a virus that expressed all HIV-1 genes except *vpr* and *env*; importantly, this virus expressed Gag protein, which is highly immunogenic. CTL-mediated killing by autologous CD8⁺ T cells (CD8s) could then be monitored over the course of a multi-week long coculture assay. Thus, Bcl-2 model cells also provide a system for testing strategies that kill HIV-1 infected cells, as well as strategies that induce HIV-1 gene expression.

Because it is an effective method for extending the *ex vivo* lifespan of primary CD4s, the Bcl-2 model also enables examination of the long-term effects of particular treatments or viruses.

As mentioned earlier, an additional cell sorting step can be inserted in this protocol to enrich the resulting population for latently infected cells. After Step 79, the GFP⁺ cells can be selected by flow cytometric sorting before the subsequent 4–6 week incubation without exogenous cytokines (Step 80). While this enrichment step increases the frequency of latently infected cells overall (from ~2% to ~30%), the final yield of cells is far lower, resulting in the same absolute number of latently infected cells¹⁷. Nonetheless, this population of cells that is enriched for latent infection offers a larger dynamic range and better signal-to-noise ratio for studies of potential LRAs.

Limitations

Due to the cytopathic effects of productive HIV-1 infection and the comparatively low frequency of latent infection, the final yield of latently infected cells in this model is modest. As noted above, an additional cell sorting step immediately after reporter virus infection increases the percentage of latently infected cells, though with a lower absolute number of cells. We are currently developing a modification to the Bcl-2 model using constructs similar to reporters developed by Dahabieh, *et al.*²⁸ and Calvanese, *et al.*²⁹. This modification will increase both the frequency of latent infection and the absolute number of cells produced. The CM6 reporter virus described in this protocol contains GFP in the *env* open reading frame (Fig. 2) and green fluorescence indicates HIV-1 gene expression that only occurs when the HIV-1 5'LTR is activated. To this CM6 HIV-1/GFP reporter virus we are adding a second fluorescent marker, monomeric Kusabira Orange (mKO), which is constitutively expressed. In the final cell sorting (Step 84), selection of cells that are GFP[−] and mKO⁺ will isolate the cells that are latently infected. This will be a pure population since expression of mKO reflects successful infection; in the absence of a second fluorescent marker, the GFP[−] population contains a majority of cells that were never infected.

The total time required to produce one batch of Bcl-2 model cells is 12–14 weeks. One step that can be shortened is the Bcl-2 transduction step; we are developing a Bcl-2 transducing virus that carries the puromycin resistance gene (Pm^R). This virus would ensure that every cell that is transduced also has Pm^R such that, at Step 49, the incubation will be decreased from 3 weeks to 1 week by puromycin resistance selection. This circumvents the need to wait for non-transduced cells to exhaust their natural *ex vivo* lifetime. Another way to expedite the protocol is to freeze expanded Bcl-2 cells, as described in Step 65. The expansion (Steps 57–65) can yield up to 10⁹ Bcl-2 cells; cells that are not used right away can be stored frozen and thawed as needed and used immediately in Part III of the protocol.

Biohazardous materials

HIV-1 is a class 2 human pathogen and infectious HIV-1 should be handled in a biosafety level 2 (BSL-2) facility. The viruses described and produced in this protocol cannot establish a spreading infection; however, we observe guidelines that are in accordance with

BSL-3 practices when working with these viruses. Liquid waste is inactivated by bleach and all waste is autoclaved before disposal.

Experimental design

This protocol to establish the primary Bcl-2 model cells is comprised of three major parts and has convenient pause points after Parts I and II. The following subsections briefly describe each part.

Part I Timing 3 d—Virus production (Steps 1–18). Viruses needed to generate latently infected cells are produced using a three-plasmid packaging system^{30,31}. Two types of viruses are made (Fig. 3): one is the Bcl-2-encoding transducing virus (from pEB-FLV) and the other is an HIV-1/GFP reporter virus (from pCM6) that is not replication-competent. Steps 1–18 must be performed twice to make both viruses. We prepare DNA for transfection using an endotoxin removal kit such as NucleoBond Xtra Maxi EF from Clontech to ensure the highest transfection efficiency. Using Lipofectamine 2000 from Life Technologies according to the manufacturer's instructions, we transfect HEK293T cells with a plasmid mixture. In addition to either pEB-FLV or pCM6, the transfection complexes include a packaging helper plasmid (pC-Help) and a VSV-G envelope pseudotyping plasmid (pVSV-G); pC-Help and pVSV-G are required for viral packaging and single-round infection³¹. After incubating the transfected HEK293T cells for 48 h, we clarify the supernatant (which contains the virus) and pellet the virions by ultracentrifugation over a 20% sucrose cushion. Virus can be produced concomitantly with the start of Part II, or can be produced in advance and stored at -80°C until needed.

Part II Timing 5.5 weeks—Isolation of healthy donor CD4s, Bcl-2 transduction, and expansion (Steps 19–65). CD4s are isolated from 120 ml blood from a healthy donor by Ficoll density gradient centrifugation and negative selection over a magnetic column. CD4s are subsequently activated by TCR engagement and infected with the Bcl-2 transducing virus produced in Part I. Transduced cells are selected by removing dead cells through another Ficoll density gradient centrifugation that is performed on a smaller scale than with the initial whole blood; we call this a “mini Ficoll”. Once isolated, the transduced cells are activated and expanded by incubation with IL-2 for 7–10 days (Fig. 4). After expansion, which can result in an increase of up to 1.5 logs, Bcl-2 cells can be frozen at -80°C and stored for later use.

Part III Timing 5.5 weeks—HIV-1/GFP reporter virus infection and isolation of Bcl-2 model cells (Steps 66–89). Expanded Bcl-2 transduced cells are activated by TCR engagement and infected with the HIV-1/GFP reporter virus produced in Part I. After infection, the cells are incubated without exogenous cytokines for several weeks to allow a return to a resting state. GFP⁺ cells are isolated by flow cytometric sorting following the culturing period (Fig. 5). The spinoculation in this part is nearly identical to Steps 39–55 in Part II. The primary differences are that we are spinoculating Bcl-2 cells instead of CD4s, and that we are using the CM6 HIV-1/GFP reporter virus instead of the EB-FLV Bcl-2 transducing virus. After spinoculation, we let the cells rest for 4–6 weeks in the absence of exogenous cytokines before sorting for GFP negativity at a BSL-2 cell sorting facility.

Before use, we measure the frequency of latently infected cells in a test reactivation using PMA and ionomycin. If the Bcl-2 model cells are not used within four weeks of cell sorting, we perform a mini Ficoll to remove the dead cells before experimental use.

MATERIALS

REAGENTS

- HEK293T cells (ATCC, cat. no. CRL-3216)
- Plasmids for lentiviral production: pEB-FLV¹⁷, pCM6¹⁷, pVSV-G (Clontech), and pC-Help³¹
- CD4⁺ T cells isolated from 120 ml blood from a healthy donor, see Reagent Setup ! **Caution** Study protocols involving human subjects must conform to all institutional and governmental guidelines and ethical regulations. Informed consent must be obtained when working with human subjects. ! **Caution** Human blood may transmit infectious diseases; gloves and protective eyewear should always be worn when working with blood.
- Heparin, cell culture grade, 100 KU (Sigma, cat. no. H3149-100KU)
- Ficoll-Paque PLUS, endotoxin-free (GE Healthcare, cat. no. 17-1440-03)
- Ultrapure, sterile water (e.g., Milli-Q)
- PBS pH 7.4, 10× (Life Technologies, cat. no. 70011-044)
- PBS pH 7.4, 1× (Life Technologies, cat. no. 10010-023)
- Opti-MEM (Life Technologies, cat. no. 31985-070)
- DMEM (Life Technologies, cat. no. 10569-010)
- RPMI 1640 medium, GlutaMAX (Life Technologies, cat. no. 61870-036)
- Trypan blue solution, 0.4% (Life Technologies, cat. no. 15250-061)
- Penicillin-streptomycin (Life Technologies, cat. no. 15140-122)
- Recombinant human IL-2, 22 × 10⁶ U/vial (Novartis, cat. no. NDC0078-0495)
- Human AB serum (Gemini Bio-products, cat. no. 100-112)
- Fetal bovine serum (FBS) (Gemini Bio-products, cat. no. 100-106)
- Heat inactivated fetal bovine serum (HI-FBS) (Life Technologies, cat. no. 10438-026)
- Newborn calf serum, heat inactivated, New Zealand Origin (HI NBCS) (Life Technologies, cat. no. 26010-074)
- Trypsin-EDTA, 0.05% (Life Technologies, cat. no. 25300-054)
- Lipofectamine 2000 (Life Technologies, cat. no. 11668-019)
- T cell growth factor, see Reagent Setup

- Phytohaemagglutinin (PHA) (Fisher, cat. no. R30852801)
- Phorbol 12-myristate 13-acetate (PMA) (Sigma, cat. no. P8139-1MG)
- Ionomycin (Sigma, cat. no. I0634-1MG)
- DMSO (Sigma, cat. no. D2650-5X5ML)
- HEPES, pH 7.2, 1 M (Life Technologies, cat. no. 15630-080)
- Tris-HCl, pH 8.0, 1 M (Quality Biological, cat. no. 351-007-101)
- NaCl, 5 M (Life Technologies, cat. no. AM9760G)
- Dextrose (Fisher, cat. no. D16-500)
- Sucrose (JT Baker, cat. no. JT4072-1)
- NucleoBond Xtra Maxi EF, endotoxin-free plasmid DNA purification kit (Clontech, cat. no. 740424) ! **Critical Step** For transfection, it is preferred to use plasmids that have had endotoxins removed since these can decrease transfection efficiency and cell viability.
- CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega, cat. no. G3582)
- CD4⁺ T cell isolation kit, human (Miltenyi Biotec, cat. no. 130-096-533)
- LS column (Miltenyi Biotec, cat. no. 130-042-401)
- CaviCide surface disinfectant (VWR, cat. no. 89132-866)
- Bleach ! **Caution** Bleach is an irritant and, when undiluted, releases a toxic gas when exposed to sunlight. Avoid contact with eyes, skin, and clothing.

Antibodies

- Anti-human CD3, 1 mg/ml (BD Biosciences, cat. no. 555336)
- Anti-human CD28, 1 mg/ml (BD Biosciences, cat. no. 555725)

Plasticware

- Plate, 6-wells, sterile (Corning, cat. no. 3736)
- Plate, 96-wells, round-bottomed, sterile (Corning, cat. no. 3799)
- T-75 tissue culture flasks, sterile (Corning, cat. no. 353136)
- T-150 tissue culture flasks, sterile (Corning, cat. no. 355001)
- 1.5 ml microfuge tubes, sterile (Axygen, cat. no. MCT-175-C-S)
- 15 ml conical tubes, sterile (Corning, cat. no. 430828)
- 50 ml conical tubes, sterile (Corning, cat. no. 430052)
- Ultracentrifuge tubes, ~30 ml capacity, sterile (Beckman Coulter, cat. no. 355642)
- 60 ml Luer-lok syringes, sterile (BD, cat. no. 136898)

- Sterile basins (Fisher, cat. no. 13681501)

EQUIPMENT

- Tissue culture capabilities
- BSL-2 tissue culture capabilities
- BSL-2 cell sorting facility
- Cell incubator (37 °C, 5% CO₂)
- Sterile serological pipettes
- Centrifuge with rotors and adaptors for 50 ml and 15 ml conical tubes and 96-well plates
- Vacuum-driven 0.45 µm sterile filtration units (Nalgene, cat. no. 169-0045)
- Vacuum-driven 0.2 µm sterile filtration units (Nalgene, cat. no. 569-0020)
- Vacuum-driven bottle-top 0.2 µm sterile filters (Nalgene, cat. no. 595-4520)
- Steriflip 0.2 µm filter unit (Millipore, cat. no. SCGP00525)
- Hemocytometer or other cell counter
- Light microscope
- Mechanical beam balance (VWR, cat. no. 12350-032)
- Beckman Ultracentrifuge (or equivalent)
- Beckman Ultracentrifuge swinging bucket SW 32 Ti rotor (or equivalent)
- 20–200 µl multichannel pipettor
- FACS instrument (at least two lasers)
- MACS MultiStand (Miltenyi Biotec, cat. no. 130-042-303)
- MidiMACS Separator (Miltenyi Biotec, cat. no. 130-042-302)
- Microplate reader (e.g., Molecular Devices VMax kinetic microplate reader, cat. no. 3545829)
- Mr. Frosty cell freezing container (Thermo Scientific, cat. no. 5100-0001)

REAGENT SETUP

DMEM—For culturing 293T cells, supplement DMEM with 10% (vol/vol) HI FBS (Life Technologies) and 1% (vol/vol) penicillin-streptomycin. Store medium at 4 °C and warm up to at least room temperature (18–23 °C) before use. Once a bottle of DMEM has been supplemented with HI FBS and antibiotics, use only as long as the pH indicator dye remains red (usually within 1 to 2 weeks); discard if pH indicator dye has turned purple.

Cytokine-Free medium (CFM)—For culturing CD4s, supplement RPMI with 10% (vol/vol) HI FBS (Life Technologies) and 1% (vol/vol) penicillin-streptomycin. Store medium at

4 °C and warm up to at least room temperature before use. **! Critical Step** If the downstream use of the Bcl-2 model cells is drug screening, omit antibiotics from the medium. Once a bottle of RPMI has been supplemented with HI FBS and antibiotics (if added), use only as long as the pH indicator dye remains red (usually within 1 to 2 weeks); discard if pH indicator dye has turned purple.

RPMI-HS—See Box 1.

FBS—The serum arrives frozen in 500 ml bottles and is stored at –20 °C upon arrival. When needed, one bottle is thawed slowly overnight at 4 °C and heat inactivated for 30 m at 56 °C. The heat inactivated serum is then stored at 4 °C for up to 4 weeks. **! Critical Step** When purchasing FBS from Gemini Bio-products, we test several lots against each other in a cell proliferation assay as described in Box 3. We select the lot that promotes the most cell proliferation and order enough bottles to make media for two or three years.

HI-FBS—The serum arrives frozen in 500 ml bottles and is stored at –20 °C upon arrival. When needed, one bottle is thawed slowly overnight at 4 °C and stored at 4 °C for up to 4 weeks. **! Critical Step** When purchasing HI-FBS from Life Technologies, we use their reserve serum testing program and test several lots against each other in a cell proliferation assay as described in Box 3. We select the lot that promotes the most cell proliferation and order enough bottles to make media for two or three years.

Human AB serum—The serum arrives frozen in 100 ml bottles and is stored at –20 °C upon arrival. When needed, one bottle is thawed slowly overnight at 4 °C and heat inactivated for 30 m at 56 °C. The heat inactivated serum is then stored at 4 °C for up to 4 weeks.

Dextrose, 10% (wt/vol)—Bring 100 g dextrose to 1 L with ultrapure water and dissolve with stirring. Filter sterilize and store at room temperature. 10% dextrose is stable at room temperature for at least one year.

Wash medium (WM)—To make 1 L of WM, start with 848 ml ultrapure sterile water and add 10 ml of 10% dextrose (wt/vol), 100 ml of 10× PBS, pH 7.4, 10 ml pen-strep (10^3 U/ml- 10^3 µg/ml), 12 ml 1 M HEPES, pH 7.2, and 20 ml HI NBCS. Sterile filter and store at 4 °C for up to 4 weeks.

Heparin—Dissolve heparin in 1× PBS pH 7.4 at a final concentration of 1000 U/ml. Filter sterilize through a 0.2 µm filter and aliquot into 2 ml tubes. Store at 4 °C for up to 2 years. These stocks are 100×.

IL-2 stock—Reconstitute with 1.2 ml ultrapure, sterile water and then add 98 ml sterile WM for a final stock concentration of 2.24×10^5 U/ml. Store in 1 ml aliquots at –80 °C for up to 1 year. For the Bcl-2 cell expansion (**Part II**), thaw one vial of the IL-2 stock and add 1.24 ml CFM. This is a 1000× (10^5 U/ml) solution that will be stable for the duration of the expansion; it will be 100 U/ml when diluted 1:1000. **! Critical Step** Whenever we

reconstitute a new bottle of IL-2, we test the new IL-2 against the previous IL-2 in a cell proliferation assay as described in Box 3 to ensure its effectiveness.

PHA—Resuspend 2 mg of the powder in 5 ml WM and then add 8.3 ml more WM for a stock concentration of 150 µg/ml. Store in 1 ml aliquots at −80 °C for up to 6 months. Once thawed, store at 4 °C and use within two weeks.

PMA—Resuspend 1 mg of the powder in 1 ml sterile DMSO for a stock concentration of 1 mg/ml. Store at −20 °C in 50 µl and 5 µl aliquots for up to 6 months. For the T cell growth factor preparation (Box 1), dilute 50 µl of the 1 mg/ml stock with 950 µl WM for a final concentration of 50 µg/ml. For measuring the frequency of latently infected cells (**Part III**), dilute 5 µl of the 1 mg/ml stock with 995 µl RPMI for a final concentration of 5 ng/µl. The working solutions should be prepared just before use.

Ionomycin—Resuspend 1mg in 1.338 ml sterile DMSO for a stock concentration of 1 mM. Store at −20 °C in 5 µl aliquots for up to 6 months. For measuring the frequency of latently infected cells (**Part III**), dilute 5 µl of the 1 mM stock with 45 µl of sterile DMSO for a final concentration of 100 µM. This working solution should be prepared just before use.

T cell growth factor preparation—See Box 1 and Fig. 8.

Cell proliferation assay for T cell growth factor preparation—See Box 2.

Super T Cell medium (STCM)—Add the following to 500 ml RPMI: 55 ml of heat inactivated FBS (Gemini Bio-products), 224 µl IL-2 stock (for a final concentration 100 U/ml), and 5–10 ml T cell growth factor (depending on T cell growth factor preparation – see Box 2). Store at 4 °C for up to 1 month and only as long as the pH indicator dye remains red (usually within 1 to 2 weeks); discard if pH indicator dye has turned purple..

20% Sucrose Buffer—Dissolve 100 g sucrose with 10 ml 1 M Tris-HCl, pH 8.0 and 15 ml 5 M NaCl in a final volume of 500 ml with ultrapure water. Filter sterilize and store at 4 °C. If the buffer lasts several months, pass the remaining solution over a vacuum-driven 0.45 µm sterile filtration unit every month to ensure sterility.

PROCEDURE

Part I: Virus production Total time 3 d

Transfecting HEK293T cells

- 1 Seed 6 × T-150 flasks with $\sim 18 \times 10^6$ HEK293T cells per flask. The final volume of each flask should not exceed 23 ml. When seeding each flask, be sure to swirl the cells thoroughly for an even distribution of cells across the surface. Seeding the flasks at this density typically results in ~60–90% confluency after 24 hours. Incubate the cells overnight (18–24 h) at 37 °C. **! Critical step** Do not use HEK293T cells that have a high passage number or that are growing slowly. The doubling time for healthy HEK293T cells is ~24 h.

- 2 Examine flasks under the microscope. The cells are ready to transfect when they are 80–90% confluent.
- 3 Aliquot 11.5 ml Opti-MEM into each of two 50 ml conical tubes and warm in a 37 °C water bath.
- 4 Prepare the plasmid mixture by adding 36 µg pVSV-G, 72 µg pC-Help, and 72 µg pEB-FLV or pCM6 to one of the 50 ml tubes containing pre-warmed Opti-MEM.
- 5 Prepare the Lipofectamine 2000 mixture by adding 900 µl Lipofectamine 2000 to the other 50 ml tube containing pre-warmed Opti-MEM.
- 6 Add the plasmid mixture to the Lipofectamine 2000 mixture and mix. Incubate the transfection complexes for 5 m at room temperature.
- 7 Add ~4 ml of transfection complexes to each flask. At this point, we move the flasks to the BSL-3 room and incubate cells there. ! **Caution** After transfection, cells and supernatant contain lentiviral particles. While neither of the viruses described in this protocol can establish a spreading infection, we treat all flasks and cells after this point as if they could. It is good practice to use the same precautions when working with any kind of virus.
- 8 Incubate cells for 48 h at 37 °C.

Pelleting virus

- 9 Turn on the ultracentrifuge and pre-chill the vacuum chamber, rotor, and swinging buckets.
- 10 Prepare an ice bucket that will comfortably accommodate 8 × 50 ml conical tubes and place it in the hood. Chill 4 × 50 ml conical tubes and 4 steriflip filter units on ice.
- 11 Divide the supernatant from the 6 flasks over the 4 chilled 50 ml conical tubes and pellet the debris at 335 × *g* for 3 m at 4 °C. When making the CM6 reporter virus, which contains GFP in the *env* locus, the transfection efficiency can be evaluated at this point by running a small aliquot of transfected 293T cells on a FACS instrument and measuring the GFP⁺ population. Typically, 10–40% of the 293T cells are GFP⁺.
- 12 Attach a steriflip unit to each 50 ml conical tube and invert with vacuum to clarify the supernatant.
- 13 Place 6 ultracentrifuge tubes on ice to chill. Divide the clarified supernatant over the 6 ultracentrifuge tubes.
- 14 Using a 2 ml serological pipette, slowly underlayer 2.5 ml of 20% sucrose buffer. ! **Critical Step** Be careful not to disrupt the interface between the sucrose and the supernatant. Eject the sucrose solution using the slowest speed on the pipettor.
- 15 Balance the ultracentrifuge tubes in pairs using a mechanical beam balance.

- 16 Spin at $76,755 \times g$ for 2 h at 4 °C.
- 17 Place the ultracentrifuge tubes on ice and aspirate all the liquid. **! Critical Step** When aspirating, do not go near the bottom of the tube where the virus pellet rests. The virus pellet can be difficult to see, but when virus production is robust, the pellet appears as a small, slightly beige and sticky mass at the base of the tube.
- 18 Resuspend virus by adding 300 μ l CFM to each ultracentrifuge tube. Without creating bubbles, pipette the CFM over the pellet several times. Transfer the virus to 2 $\times$ 2 ml screw-cap microfuge tubes and freeze on a dry ice/ethanol bath. Store at –80 °C. **! Critical Step** We do not titer the virus because we continuously make model cells in our laboratory; we have found that, generally, the virus produced from one T-150 flask is sufficient to transduce/infect $10\text{--}15 \times 10^6$ activated CD4s.

Pause Point Virus can be produced in advance, frozen, and stored at –80 °C for several years. Frozen aliquots of virus should only be thawed once before use in order to ensure maximal infectivity.

Part II: Isolation of healthy donor CD4s, Bcl-2 transduction, and expansion Total time 5.5 w

Isolating and activating CD4s

- 19 For each 120 ml of blood from a healthy donor, prepare 2 $\times$ 6-well plates for activation (See Box 4).
- 20 Prepare 2 $\times$ 60 ml syringes with 600 μ l heparin (for a final concentration of 10 U/ml blood). Also, ensure that the centrifuge for spinning 50 ml conical tubes is at room temperature.
- 21 Aliquot 15 ml Ficoll-Paque PLUS into 4 $\times$ 50 ml conical tubes (one Ficoll tube for every 30 ml of blood).
- 22 Aliquot 10 ml WM into 4 $\times$ 50 ml conical tubes (one WM tube for every Ficoll tube).
- 23 After the blood is drawn, gently invert the syringes a few times to mix the heparin additive to prevent clotting. **Pause Point** The blood in the syringes can be stored up to 30 m in the 37 °C incubator if not proceeding immediately to the next step. If pausing, be sure mix the blood by inversion before layering onto the Ficoll since separation may occur of red blood cells (RBCs) and plasma while the tubes rest in the incubator.
- 24 By touching the tip of the syringe to the inside of the tube, slowly layer 30 ml of blood per tube already filled with 15 ml Ficoll-Paque PLUS. Spin at $400 \times g$ for 25 m at room temperature.
- 25 After spinning, the emergent upper layer is serum and the bottom layer is the Ficoll, granulocytes, and RBCs. At the interface of serum and Ficoll is a white layer, called the buffy coat, which contains PBMCs. Aspirate 15–20 ml serum, but do not disturb the buffy coat. Using a 10 ml pipette, insert the tip directly

into the buffy coat and transfer the cells into the 50 ml conical pre-aliquoted with 10 ml WM in Step 22. Bring the volume of each tube up to 40 ml with WM and pellet the PBMCs at $335 \times g$ for 10 m at 4°C .

- 26 Aspirate the supernatant and add 1 ml of WM. Resuspend thoroughly by pipetting up and down vigorously several times with a P1000 pipette. Pool the PBMCs into one 50 ml conical tube and bring the volume up to 40 ml with WM. Count an aliquot of the cells using a 1:10 dilution with trypan blue. We typically see $150\text{--}350 \times 10^6$ PBMCs isolated from 120 ml blood. **! Critical Step** There should not be many RBCs at this stage; if there is a small quantity, it will be removed by the column selection in Steps 29–33. If there are too many RBCs, the cells should be centrifuged over Ficoll-Paque PLUS for a second time by resuspending the cells in 40 ml of WM, pelleting at $335 \times g$ for 10 m at 4°C , and resuspending in 40 ml WM. Count an aliquot of the cells again using a 1:10 dilution with trypan blue.

To facilitate the subsequent column selection steps, we make a multiplier chart based on the cell count. Table 1 shows an example where 187×10^6 cells are isolated by the end of this step.

- 27 Pellet the PBMCs at $335 \times g$ for 10 m at 4°C .
- 28 Aspirate the supernatant and resuspend in $4 \mu\text{l}$ per 10^6 PBMCs of WM (using the example in Table 1, this would be $748 \mu\text{l}$ WM).
- 29 Add $1 \mu\text{l}$ per 10^6 PBMCs of the Biotin-Antibody cocktail from the Miltenyi CD4⁺ T cell isolation kit (using the example in Table 1, this would be $187 \mu\text{l}$). Mix and incubate for 5 m at 4°C .
- 30 Add $3 \mu\text{l}$ per 10^6 PBMCs of WM (using the example in Table 1, this would be $561 \mu\text{l}$).
- 31 Add $2 \mu\text{l}$ per 10^6 PBMCs of the Anti-Biotin Microbeads from the Miltenyi CD4⁺ T cell isolation kit (using the example in Table 1, this would be $374 \mu\text{l}$). Mix and incubate for 10 m at 4°C .
- 32 While the cells are incubating with the Microbeads, prepare the LS columns. Use one column per 10^8 PBMCs. Clean the MACS MultiStand and MidiMACS Separator and place them in the hood. Open the LS column packages inside the hood and magnetically attach each column to the Separator, taking care to maintain the sterility of the column tip. Prime each column with 3 ml WM and discard the flow-through. Arrange a 50 ml conical tube in ice under each column for cell collection.
- 33 When the Microbead incubation is finished, add WM to bring the final volume of the cells to 3 ml per LS column and apply 3 ml to each column. The flow-through is the eluate containing the CD4s. Apply another 3 ml of WM to each column and pool all the eluates.
- 34 Count the cells by taking a small aliquot and using a 1:4 dilution with trypan blue. Pellet the remaining CD4s at $335 \times g$ for 10 m at 4°C .

- 35 Resuspend the CD4s in STCM at a final concentration of 10^6 cells/ml.
- 36 Add anti-CD28 antibody at a final concentration of 1 μ g/ml.
- 37 Wash the anti-CD3 plates prepared in Step 19 and then gently add the CD4s from Step 36 to the 6-well plates, dividing the volume evenly across all wells.
- 38 Incubate the cells for 3 d at 37 °C to activate.

Spinoculating and selecting Bcl-2 transduced CD4s

- 39 Ensure that a centrifuge for spinning 96-well plates is at room temperature. Check the CD4s in the 6-well plates. If they have activated well, the color of the STCM will change from pink to yellow and the cells will adhere to each other and form small clumps. Under the microscope, the cells will be much larger than unactivated cells.
- 40 Pre-warm ~15 ml STCM to at least room temperature. Pool and count an aliquot of the cells using a 1:2 dilution with trypan blue. Pellet the cells at $335 \times g$ for 10 m at 4 °C and resuspend them in pre-warmed STCM at a final concentration of 5×10^6 cells/ml.
- 41 Thaw the Bcl-2 virus produced in Part I (from **Step 18**) in a 37 °C water bath. Add the virus to the activated CD4s and mix well.
- 42 Transfer the cell/virus mix to a sterile basin and aliquot 100 μ l (0.5×10^6 cells) per well of a 96-well round bottom plate using a multichannel pipettor.
- 43 Spin the plates at $1200 \times g$ for 2 h at room temperature.
- 44 Add 100 μ l of STCM to each well and pipette up and down to resuspend the cells.
- 45 Incubate overnight at 37 °C.
- 46 Transfer the cells to a T-150 flask and supplement the volume up to 120 ml with STCM as follows: the final volume here should be, in milliliters, double the number of millions of cells that were spinoculated. For example, if 50×10^6 cells were spinoculated, the final volume should be 100 ml; if 80×10^6 cells were spinoculated, the final volume should be 160 ml. **! Critical Step** The optimal volume range for a T-150 flask is 30–75 ml. Use as many flasks as necessary to ensure the volume per flask is within this range.
- 47 Incubate the cells for 2 d at 37 °C.
- 48 Count an aliquot of the cells using a 1:2 dilution with trypan blue. Pellet the remaining cells at $335 \times g$ for 10 m at 4 °C and resuspend in CFM in T-150 flasks at a final concentration of 10^6 cells/ml. See **Critical Step** in Step 46.
- 49 Incubate for 3 w at 37 °C. Change the medium after 10 d by pelleting half the flask volume at $335 \times g$ for 10 m at 4 °C, resuspending the cells in fresh CFM, and returning the suspension to the flask.

- 50 Pellet the cells at $335 \times g$ for 10 m at room temperature and pre-warm ~30 ml CFM in a 37 °C incubator or water bath.
- 51 While the cells are spinning, aliquot 4.5 ml Ficoll-Paque PLUS into a 15 ml conical tube for mini Ficoll. One mini Ficoll tube per 10^8 cells (counted in Step 48) is sufficient.
- 52 Resuspend up to 10^8 cells in 9 ml of the pre-warmed CFM and slowly layer over the Ficoll-Paque PLUS. Spin at $335 \times g$ for 10 m at room temperature.
- 53 The cells that are transduced (*i.e.*, alive) will settle at the interface between the CFM and the Ficoll. Carefully remove these cells with a P1000 pipette and transfer to a 50 ml conical tube pre-aliquoted with 10 ml WM. These are the Bcl-2 transduced cells (Bcl-2 cells). We typically get $10\text{--}30 \times 10^6$ Bcl-2 cells if we start with $60\text{--}90 \times 10^6$ CD4s at Step 40.
- 54 Bring the volume up to 40 ml with WM.
- 55 Count the Bcl-2 cells. If not pausing after Step 56, prepare one 6-well plate for activation (see Box 4) for every 30×10^6 Bcl-2 cells.
- 56 Pellet the cells at $335 \times g$ for 10 m at 4 °C and resuspend in STCM at a final concentration of 10^6 cells/ml. **Pause Point** If not proceeding directly to the next step, resuspend the cells in CFM at a final concentration of 10^6 cells/ml and incubate at 37 °C for up to 2 d. When ready to proceed to Step 57, pellet the cells at $335 \times g$ for 10 m at 4 °C and resuspend in STCM at a final concentration of 10^6 cells/ml.

Expanding Bcl-2 transduced CD4s

- 57 Add 1 µg/ml anti-CD28 antibody to the Bcl-2 cells.
- 58 Wash the anti-CD3 plates prepared in Step 55 and then gently add the Bcl-2 cells from Step 57 to the 6-well plates, dividing the volume evenly across all wells.
- 59 Incubate the cells for 3 d at 37 °C to activate.
- 60 Check the CD4s in the 6-well plates. If they have activated well, the color of the STCM will change from pink to yellow and the cells will adhere to each other and form small clumps. Under the microscope, the cells will be much larger than unactivated cells. Pool and count an aliquot of the cells. Pellet the cells at $335 \times g$ for 10 m at 4 °C and resuspend in CFM at a final concentration of 10^6 cells/ml.
- 61 Add IL-2 (10^5 U/ml solution) at a 1:1000 dilution for a final concentration of 100 U/ml.
- 62 Incubate the cells at 37 °C.
- 63 Count the cells every other day. Supplement the volume with CFM to readjust the concentration to 10^6 cells/ml and add IL-2 at a 1:1000 dilution based on the new volume. As the number of cells and volume increase, divide the cells over a

sufficient number of flasks to ensure the volume per flask is within the recommended range (see **Critical Step** in Step 46).

The first few times the cells are counted, they should triple; the next few times, they will only double. When the cells stop doubling, proceed to the next step. Usually, the expansion will take 7–10 d.

- 64** Mini Ficoll the cells as in Steps 50–54.
- 65** Count the Bcl-2 cells. Aliquot and set aside 60×10^6 Bcl-2 cells to use in the next step of this protocol.

Pause Point The expansion can yield anywhere from 0.2×10^9 to 1.5×10^9 Bcl-2 cells, so we freeze whatever cells we are not using right away. To freeze, pellet the cells at $335 \times g$ for 10 m at 4 °C. Resuspend the cells at a final concentration of $5\text{--}30 \times 10^6$ cells/ml in 90% HI-FBS (Life Technologies), 10% DMSO. Aliquot 1 ml in 2 ml screw-cap cryotubes and freeze in a Mr. Frosty cell freezing container at -80 °C according to the manufacturer's instructions. In our experience, aliquots retain at least 50% viability even after 1 year of storage at -80 °C.

Part III: HIV-1 reporter virus infection and isolation of the latent cell-containing population (model cells) Total time 6 w

Spinoculating Bcl-2 cells with HIV-1 reporter virus

- 66** Coat 2×6 -well plates (see Box 4) for activating the Bcl-2 cells.
- 67** Pre-warm ~15 ml STCM to at least room temperature. Pellet 60×10^6 Bcl-2 cells at $335 \times g$ for 10 m at room temperature, and resuspend in pre-warmed STCM at a final concentration of 10^6 cells/ml. Add 60 μ l of anti-CD28 antibody for a final concentration of 1 μ g/ml.
- 68** When the anti-CD3 antibody coating incubation is finished, wash the anti-CD3 plates prepared in Step 67 and then gently add the Bcl-2 cells from Step 67 to the 6-well plates, dividing the volume evenly across all wells.
- 69** Incubate the cells for 3 d at 37 °C to activate.
- 70** Ensure that a centrifuge for spinning 96-well plates is at room temperature. Check the CD4s in the 6-well plates. If they have activated well, the color of the STCM will change from pink to yellow and the cells will adhere to each other and form small clumps. Under the microscope, the cells will be much larger than unactivated cells.
- 71** Pre-warm ~15 ml STCM to at least room temperature. Pool and count the cells using a 1:2 dilution with trypan blue. Pellet the cells at $335 \times g$ for 10 m at 4 °C and resuspend them in pre-warmed STCM at a final concentration of 5×10^6 cells/ml.
- 72** Thaw the HIV-1/GFP reporter virus produced in Part I in a 37 °C water bath. Add the virus to the activated Bcl-2 cells and mix well.

- 73 Transfer the cell/virus mix to a sterile basin and aliquot 100 μ l (0.5×10^6 cells) per well of a 96-well round bottom plate using a multichannel pipettor.
- 74 Spin the plates at $1200 \times g$ for 2 h at room temperature.
- 75 Add 100 μ l of STCM to each well and pipette up and down to resuspend the cells.
- 76 Incubate overnight at 37 °C.
- 77 Transfer the cells to a T-150 flask and supplement the volume with STCM as follows: the final volume here should be, in milliliters, double the millions of cells that were spinoculated. For example, if 50×10^6 cells were spinoculated, the final volume should be 100 ml; if 80×10^6 cells were spinoculated, the final volume should be 160 ml. **! Critical Step** The optimal volume range for a T-150 flask is 30–75 ml. Use as many flasks as necessary to ensure the volume per flask is within this range (see **Critical Step** in Step 46).
- 78 Incubate the cells for 2 d at 37 °C.
- 79 Count an aliquot of the cells using a 1:2 dilution with trypan blue. Pellet the remaining cells at $335 \times g$ for 10 m at 4 °C and resuspend in CFM in T-150 flasks at a final concentration of 10^6 cells/ml. The ideal volume range for a T-150 flask is 30–75 ml. Use as many flasks as necessary to ensure the volume per flask is within this range (see **Critical Step** in Step 46). If desired, the infection efficiency can be evaluated at this point by running a small aliquot of cells on a FACS instrument and measuring the GFP⁺ population. Typically, ~10–20% of the cells are GFP⁺. See Fig. 6 for representative FACS data of this measurement.
- 80 Incubate for 4–6 w at 37 °C. Change the medium every other week by pelleting half the flask volume at $335 \times g$ for 10 m at 4 °C, resuspending the cells in fresh CFM, and returning the suspension to the flask.
- 81 Mini Ficoll the cells as in Steps 50–54 to remove dead cells.
- 82 Count the cells and resuspend in CFM at a final concentration of 10^6 cells/ml.

Isolating the latent cell-containing population (Bcl-2 model cells)

- 83 The parameters of this step will depend on the cell sorting facility used. In the facility we use, we are directed to resuspend our cells in WM at a final concentration of 30×10^6 cells/ml. We sort for a GFP[−] cell population that is comprised of both cells that were not infected and cells that are latently infected. These are the Bcl-2 model cells.
- 84 After the cell sorting, wash the cells once with 40 ml of WM and resuspend at a final concentration of 10^6 cells/ml in CFM. Incubate at 37 °C until used.

Measuring the frequency of latent infection in the Bcl-2 model cells—! Critical Step To determine the percent of latent infection, we compare an aliquot of untreated model

cells to an aliquot that is treated with PMA and ionomycin. We assay the levels of GFP expression with and without drug treatment on a FACS instrument.

- 85** Aliquot 300 μ l (3×10^5 cells) in each of two 1.5 ml microfuge tubes. To one tube, add 3 μ l of PMA (5 ng/ μ l solution) for a final concentration of 50 ng/ml and 3 μ l ionomycin (100 μ M solution) for a final concentration of 1 μ M.
- 86** For both untreated and treated samples, divide 100 μ l over 3 wells of a 96-well round bottom plate.
- 87** Incubate the cells for 24 h at 37 °C.
- 88** Spin the plate at $335 \times g$ for 10 m at 4 °C, carefully aspirate most of the medium, and resuspend the cells in 100 μ l 1 $\times$ PBS.
- 89** Measure the GFP⁺ cells on a FACS instrument. This is the frequency of latently infected cells. See Fig. 7 for representative FACS data.

TIMING

Virus production and initial isolation of CD4s can begin at the same time. We also stockpile virus so that it is not a limiting factor.

Virus Production

Day 1

Step 1, seeding T-150 flasks: 15 m

Day 2

Steps 2–7, 293T transfection: 15 m

Day 4

Steps 9–15, ultracentrifugation preparation: 50 m

Steps 16–18, ultracentrifugation spin and resuspension: 2.5 h

Making model cells

Week 1

Day 1

Steps 19–35, isolation of CD4s: 2.5 h

Steps 36–38, activation of CD4s: 3 d

Day 4

Steps 39–44, spinoculation: 3 h

Steps 45–47, incubation: 3 d

Day 7

Step 48, media transfer: 1 h

Step 49, incubation and selection: 3 w

Day 28

Steps 50–54, mini Ficoll: 1 h

Steps 55–59, activation: 3 d

Day 31

Steps 60–61, media transfer: 30 m

Days 33–41

Steps 62–63, IL-2 and CFM supplement: 15 m every other day; expansion: 9 d total

Day 42

Step 64, mini Ficoll: 1h

Steps 65–69, activation of CD4s: 3 d

Day 45

Steps 70–75, spinoculation: 3 h

Steps 76–78, incubation: 3 d

Day 48

Step 79, media transfer: 1 h

Step 80, incubation: 4–6 w

Day 84

Steps 81–82, mini Ficoll: 1 h

Steps 83–84, cell sorting: variable, 2 h

Steps 85–86, test reactivation: 15 m

Step 87, incubation: 1 d

Day 85

Step 89, latency measurement: 30 m

TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

ANTICIPATED RESULTS

Typically, we obtain $150\text{--}350 \times 10^6$ PBMCs from 120 ml of healthy donor blood after the Ficoll sedimentation spin and $60\text{--}90 \times 10^6$ CD4s after the column separation. After Bcl-2 transduction of 60×10^6 CD4s and the mini Ficoll in Steps 50–55, we consider an outcome of 10×10^6 Bcl-2 cells to be successful and sufficient to continue to the expansion. The amount of expansion that occurs will vary from donor to donor so that even if one batch only generates 10×10^6 Bcl-2 cells, the expansion could still result in 10^9 Bcl-2 cells. Once frozen (see Step 66), most batches will exhibit 60–80% survival upon thawing within the first few months; thus, one batch can provide stock for numerous experiments.

The percent of latently infected cells that are established by this protocol ranges from 0.5 to 3%. Factors that largely influence this are the conditions of the 293T cells used for producing virus (*i.e.*, virus quality) and the donor-to-donor variation of the Bcl-2 cell batches. We isolate the latent cell population 6 weeks after the HIV-1/GFP virus-infected cells are transferred to CFM. Again, due to the aforementioned variation, we have seen batches generate $22\text{--}63 \times 10^6$ Bcl-2 model cells; on average, of the cells that we take to the cell sorting facility, more than two-thirds are GFP⁺.

Acknowledgements

Funding

The authors would like to thank all members of the Siliciano lab, past and present, who have contributed to the development and testing of the Bcl-2 model cells. This work was supported by the Martin Delaney CARE and DARE Collaboratories (NIH grants AI096113 and 1U19AI096109), by an ARCHE Collaborative Research Grant from the Foundation for AIDS Research (amFAR 108165-50-RGRL), by the Johns Hopkins Center for AIDS Research, by NIH grant 43222, and by the Howard Hughes Medical Institute.

REFERENCES

1. Chun TW, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature*. 1997; 387:183–188. [PubMed: 9144289]
2. Chun TW, et al. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat Med*. 1995; 1:1284–1290. [PubMed: 7489410]
3. Wong JK, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science*. 1997; 278:1291–1295. [PubMed: 9360926]
4. Siliciano JD, et al. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4⁺ T cells. *Nat Med*. 2003; 9:727–728. [PubMed: 12754504]
5. Finzi D, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science*. 1997; 278:1295–1300. [PubMed: 9360927]
6. Samji H, et al. Closing the gap: increases in life expectancy among treated HIV-positive individuals in the United States and Canada. *PloS one*. 2013; 8:e81355. [PubMed: 24367482]
7. Davey RT Jr, et al. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. *Proc Natl Acad Sci U S A*. 1999; 96:15109–15114. [PubMed: 10611346]
8. Hamer DH. Can HIV be Cured? Mechanisms of HIV persistence and strategies to combat it. *Current HIV research*. 2004; 2:99–111. [PubMed: 15078175]

9. Archin NM, Margolis DM. Emerging strategies to deplete the HIV reservoir. *Current opinion in infectious diseases*. 2014; 27:29–35. [PubMed: 24296585]
10. Schroder AR, et al. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell*. 2002; 110:521–529. [PubMed: 12202041]
11. Jordan A, Bisgrove D, Verdin E. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *EMBO J*. 2003; 22:1868–1877. [PubMed: 12682019]
12. Gautier VW, et al. In vitro nuclear interactome of the HIV-1 Tat protein. *Retrovirology*. 2009; 6:47. [PubMed: 19454010]
13. Jager S, et al. Global landscape of HIV-human protein complexes. *Nature*. 2012; 481:365–370. [PubMed: 22190034]
14. Spina CA, Guatelli JC, Richman DD. Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. *J Virol*. 1995; 69:2977–2988. [PubMed: 7707524]
15. Swiggard WJ, et al. Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli. *J Virol*. 2005; 79:14179–14188. [PubMed: 16254353]
16. Saleh S, et al. CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4+ T cells to HIV-1 infection: a novel model of HIV-1 latency. *Blood*. 2007; 110:4161–4164. [PubMed: 17881634]
17. Yang HC, et al. Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *J Clin Invest*. 2009; 119:3473–3486. [PubMed: 19805909]
18. Bosque A, Planelles V. Studies of HIV-1 latency in an ex vivo model that uses primary central memory T cells. *Methods*. 2011; 53:54–61. [PubMed: 20970502]
19. Lassen KG, Hebbeler AM, Bhattacharyya D, Lobritz MA, Greene WC. A flexible model of HIV-1 latency permitting evaluation of many primary CD4 T-cell reservoirs. *PloS one*. 2012; 7:e30176. [PubMed: 22291913]
20. Spina CA, et al. An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients. *PLoS pathogens*. 2013; 9:e1003834. [PubMed: 24385908]
21. Bosque A, Planelles V. Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. *Blood*. 2009; 113:58–65. [PubMed: 18849485]
22. Xing S, et al. Disulfiram reactivates latent HIV-1 in a Bcl-2-transduced primary CD4+ T cell model without inducing global T cell activation. *J Virol*. 2011; 85:6060–6064. [PubMed: 21471244]
23. Xing S, et al. Novel structurally related compounds reactivate latent HIV-1 in a bcl-2-transduced primary CD4+ T cell model without inducing global T cell activation. *The Journal of antimicrobial chemotherapy*. 2012; 67:398–403. [PubMed: 22160146]
24. Miller LK, et al. Proteasome inhibitors act as bifunctional antagonists of human immunodeficiency virus type 1 latency and replication. *Retrovirology*. 2013; 10:120. [PubMed: 24156270]
25. Boehm D, et al. BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism. *Cell cycle*. 2013; 12:452–462. [PubMed: 23255218]
26. Spivak AM, et al. A Pilot Study Assessing the Safety and Latency-Reversing Activity of Disulfiram in HIV-1-Infected Adults on Antiretroviral Therapy. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2014; 58:883–890. [PubMed: 24336828]
27. Shan L, et al. Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. *Immunity*. 2012; 36:491–501. [PubMed: 22406268]
28. Dahabieh MS, Ooms M, Simon V, Sadowski I. A doubly fluorescent HIV-1 reporter shows that the majority of integrated HIV-1 is latent shortly after infection. *J Virol*. 2013; 87:4716–4727. [PubMed: 23408629]
29. Calvanese V, Chavez L, Laurent T, Ding S, Verdin E. Dual-color HIV reporters trace a population of latently infected cells and enable their purification. *Virology*. 2013; 446:283–292. [PubMed: 24074592]

30. Naldini L, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996; 272:263–267. [PubMed: 8602510]
31. Mochizuki H, Schwartz JP, Tanaka K, Brady RO, Reiser J. High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J Virol*. 1998; 72:8873–8883. [PubMed: 9765432]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Box 1 | T cell growth factor (TCGF) preparation Timing 1 w + 4 d

About one week before we plan to make TCGF, we order 4 leukopak from the New York Blood Center (phone #718-752-4777, website <http://nybloodcenter.org>). Request that the blood be tested to ensure that it is HIV-1 negative. The following steps can also be scaled down to prepare TCGF from one leukopak. In steps 8–11 below, medium is removed by aspiration from flasks containing PBMCs, which are suspension cells; the PHA/PMA incubation causes most of the cells to become activated and clump together in aggregates that are generally adherent to the surface of the flask. We typically see only losses of ~15% of the cells cumulatively over all of the washes. **Do not** spin down the medium removed in each step to recover cells, as adding recovered cells back to the culture does not improve activity in our experience.

1. Prepare RPMI-HS by adding to each of two 500 ml bottles of RPMI: 5 ml pen-strep and 12.5 ml heat inactivated human serum type AB.
2. Isolate PBMCs by the standard Ficoll procedure (as in Steps 21–27 of the main protocol). On average, one leukopak will yield $\sim 10^9$ PBMCs, though we have seen a range of $0.5\text{--}1.5 \times 10^9$ PBMCs per leukopak. Typically, one can expect to isolate 4×10^9 PBMCs in this step.
3. For all of the isolated PBMCs, resuspend 400 million PBMCs per T-150 tissue culture flask in 70 ml of the RPMI-HS.
4. Incubate overnight at 37 °C.
5. To each T-150 flask containing 70 ml PBMCs + medium, add 933 μ l PHA (150 μ g/ml solution) for a final concentration of 2 μ g/ml and add 7 μ l PMA (50 μ g/ml solution) for a final concentration of 5 ng/ml.
6. Incubate for 4 h at 37 °C.
7. While the flasks are incubating, pre-warm WM and RPMI-HS at 37 °C. Depending on how many PBMCs were isolated in Step B, more RPMI-HS may have to be prepared.
8. Gently move the flasks from the incubator to the tissue culture hood.
9. Slowly tilt the flask so that it is upright (Fig. 8A). Using a 2 ml aspirating pipette, carefully remove the culture supernatant.
10. Gently add 15 ml warmed WM by slowly ejecting the fluid onto the inner top surface of the flask (Fig. 8B), *i.e.*, not onto the surface where the cells are resting. At a tilted angle, slowly rotate the flask 360° once (Fig. 8C). Incubate the flasks for 10 m at 37 °C to let the cells settle again.
11. Repeat Steps I and J twice for a total of three WM washes. These steps are to remove the PHA and PMA.
12. Gently add 70 ml RPMI-HS to each flask by slowly ejecting the fluid onto the inner top surface of the flask (Fig. 8B), *i.e.*, not onto the surface where the cells are resting.

- 13.** Incubate for 40 h at 37 °C.
- 14.** To harvest culture supernatants containing TCGF, transfer the supernatants from the flasks to 50 ml conical tubes and spin at $1455 \times g$ for 10 minutes at 4°C to remove cell debris.
- 15.** Sterilize the supernatants, 500 ml at a time, over vacuum-driven 0.45 µm filtration units.
- 16.** Store in 40 ml aliquots at −20 °C for up to 6 months.
- 17.** To assay the amount of TCGF produced, see Box 2.

Box 2 | Cell proliferation assay to assess TCGF effectiveness Timing 1.5 h + 2–3 d + ~3 h

To assess the ability of the TCGF that was produced in the procedure described in Box 1 to support the growth of T cells, we use a cell proliferation assay. We test each new batch against the previous batch of TCGF; however, for first-time analyses, one can perform a titration series of STCM made with 0 ml, 5 ml, 6 ml, 7 ml, 8 ml, 9 ml, and 10 ml of the TCGF-containing medium made in Box 1. Using a colorimetric assay kit from Promega, we determine the minimum amount of TCGF addition that stimulates maximal cell growth. Each STCM/TCGF condition is tested in triplicate and requires 3×10^5 PBMCs; testing all seven conditions requires $\sim 2.1 \times 10^6$ PBMCs.

1. Obtain 30 ml blood from a healthy donor and isolate PBMCs by the standard Ficoll procedure (as in Steps 21–27 of the main protocol). A typical yield from this amount of blood is $35\text{--}75 \times 10^6$ PBMCs. Resuspend the PBMCs in WM at 10^6 cells/ml.
2. Into each of seven 1.5 ml microfuge tubes (one for each STCM/TCGF condition), aliquot 300 μ l of PMBCs. Pellet the cells at $2095 \times g$ for 1 m and aspirate the WM.
3. Resuspend each tube of PBMCs in 300 μ l of a different STCM/TCGF medium (containing 0 ml, 5 ml, 6 ml, 7 ml, 8 ml, 9 ml, or 10 ml TCGF-containing medium made in Box 1).
4. In triplicate for each STCM/TCGF condition, plate 100 μ l (10^5 PBMCs) per well in a 96-well round-bottom plate.
5. Wrap the plate in aluminum foil and incubate for 2–3 d at 37 °C.
6. Wrap the CellTiter 96 Aqueous One Solution Reagent in foil to protect it from light and thaw for about 1 h at room temperature.
7. Add 20 μ l of the CellTiter 96 Aqueous One Solution Reagent to each well.
8. Incubate the plate for 2–5 h at 37 °C.
9. Read the absorbance of the plate on a microplate reader at 496 nm according to the manual for the Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit. Compare the average absorbance of the replicates for each titer of TCGF. An average absorbance of 1 is optimal; measurements above 1 are considered oversaturating amounts of proliferation.

For subsequent preparations of TCGF, compare the new batch with the previous batch to ensure equivalent quality.

Box 3 | Cell proliferation assay to assess sera or IL-2 effectiveness Timing 1.5 h + 2–3 d + ~3 h

To measure the effectiveness of different lots of sera or ensure the effectiveness of new batches of IL-2, we perform a cell proliferation assay using a colorimetric kit from Promega as described in Box 2. To test sera, we prepare one bottle of STCM (as described in Reagent Setup) for each lot of serum that is to be tested. Likewise, to test IL-2, we prepare one bottle of STCM (as described in Reagent Setup) with the new, freshly reconstituted IL-2 and one bottle with the previous IL-2. We test sera lots or IL-2 batches in triplicate and measure the ability of the serum or IL-2 to stimulate cell growth. Testing each bottle of STCM requires 3×10^5 PBMCs.

1. Obtain 30 ml blood from a healthy donor and isolate PBMCs by the standard Ficoll procedure (as in Steps 21–27 of the main protocol). A typical yield from this amount of blood is $35\text{--}75 \times 10^6$ PBMCs. Resuspend the PBMCs in WM at 10^6 cells/ml.
2. Into 1.5 ml microfuge tubes (one for each bottle of STCM medium), aliquot 300 μ l of PMBCs. Pellet the cells at $2095 \times g$ for 1 m and aspirate the WM.
3. Resuspend each tube of PBMCs in 300 μ l from each bottle of STCM being tested (made with different lots of sera, or made with new or old IL-2).
4. In triplicate for each bottle of STCM, plate 100 μ l (10^5 PBMCs) per well in a 96-well round-bottom plate.
5. Wrap the plate in aluminum foil and incubate for 2–3 d at 37 °C.
6. Wrap the CellTiter 96 AQueous One Solution Reagent in foil to protect it from light and thaw for about 1 h at room temperature.
7. Add 20 μ l of the CellTiter 96 AQueous One Solution Reagent to each well.
8. Incubate the plate for 2–5 h at 37 °C.
9. Read the absorbance of the plate on a microplate reader at 496 nm according to the manual for the Promega CellTiter 96 AQueous One Solution Cell Proliferation Assay kit. Compare the average absorbance of the replicates for each bottle of STCM. An average absorbance of 1 is optimal.
10. We select the serum lot that promotes the most cell proliferation; every IL-2 batch we have tested has been at least as effective as the previous batch and is usually more effective.

Box 4 | Preparing 6-well plates for activation of CD4s Timing 95 m

We activate CD4s using T cell receptor (TCR) engagement which we believe is the *ex vivo* method that most closely resembles *in vivo* activation. The average yield of CD4s from 120 ml of blood is $50\text{--}90 \times 10^6$ cells. To activate these cells, we prepare one 6-well plate for every 30×10^6 cells.

1. Mix 120 μl anti-CD3 antibody in 12 ml of $1\times$ PBS for a final concentration of 10 $\mu\text{g/ml}$. Aliquot 1 ml of this solution per well. Be sure that the whole bottom surface of the well is covered.
2. Incubate for at least 90 m at 37 °C. **Pause Point** The 6-well plates can be coated the day before they are needed; after the incubation for 90 m at 37 °C, seal the plates with parafilm and store them overnight at 4 °C.
3. Just before plating the isolated CD4s, wash the wells to remove excess, unbound anti-CD3 antibody. To do this, aspirate the antibody-PBS solution from each well. Wash each well twice with 5 ml of $1\times$ PBS. **! Critical Step** Be careful not to wash the bound antibody off the surface; use a wide-bore pipette such as a 25 ml pipette and slowly add the $1\times$ PBS by touching the tip of the pipette to the side of well. **! Critical Step** Do not let the wells dry at all; it is better to wash one plate at a time.
4. Aspirate the $1\times$ PBS. When adding the CD4s, use the same slow and gentle technique with a wide-bore pipette as in Step C.

Figure 1. Representative results of intracellular Bcl-2 expression levels

Freshly isolated CD4s (blue) and Bcl-2 model cells (purple) were stained with a FITC-conjugated antibody. Cells were also stained with a FITC-conjugated isotype control antibody (gray). 20,000–40,000 cells were measured for each sample.

Figure 2. Structure of the CM6 HIV-1/GFP reporter virus

The second round HIV-1 reporter virus described in this protocol expresses only Tat, Rev, and GFP.

PART I: Virus production **Figure 3. Part I. Schematic of virus production**

Two types of viruses are produced in HEK293T cells using a three-plasmid packaging system. After a few days of incubation, supernatant is ultracentrifuged over a sucrose cushion to concentrate virions.

PART II: Bcl-2 transduction and expansion **Figure 4. Part II. Schematic of Bcl-2 transduction and expansion**

CD4s are isolated by column separation and activated by TCR engagement prior to infection with the Bcl-2 transducing virus produced in Part I. The cells are cultured for a few weeks to allow the untransduced cells to expire. The surviving cells, *i.e.*, the Bcl-2 transduced cells, are isolated by Ficoll density centrifugation. The Bcl-2 cells are then activated by TCR engagement and expanded by incubation with IL-2.

PART III: HIV-1/GFP reporter virus infection and isolation of Bcl-2 model cells

Figure 5. Part III. Schematic of HIV-1/GFP reporter virus infection and isolation of Bcl-2 model cells

Expanded Bcl-2 cells are activated by TCR engagement prior to infection with the HIV-1/GFP reporter virus produced in Part I. Cells are then cultured for several weeks without exogenous cytokines to allow a return to a resting state. By the end of this incubation period, there will be a heterogeneous population: cells that were never infected by the reporter virus (A), cells that have died (B), cells that are actively infected (C), and cells that are latently infected (D). Isolation of GFP⁻ cells by flow cytometry yields a population containing uninfected cells and latently infected cells.

Figure 6. Representative FACS measurement of HIV-1/GFP reporter virus infection efficiency
As described in **Step 79**, FACS measurement of the infection efficiency of Bcl-2 cells spinoculated with the HIV-1/GFP reporter virus in **Steps 70–79**. 60,000 cells were measured.

Figure 7. Representative FACS measurement of frequency of latent infection in Bcl-2 model cells

As described in **Step 89**, FACS measurement of the frequency of latently infected Bcl-2 model cells in the final GFP⁻ population sorted by flow cytometry in **Steps 83–84**. Two aliquots of Bcl-2 model cells are untreated (left panels) or treated with PMA/Ionomycin (right panels) for 24 hours and then assessed by flow cytometry. Live cells are gated based on forward and side scatter (top panels) and GFP positivity is measured using the FITC channel (lower panels). 50,000 cells were measured for each sample.

Figure 8. TCGF preparation

As described in Box 1. (A) In Step I, slowly tilt the flasks containing PBMCs from the leukopaks to the upright position before removing the culture supernatant by aspiration. (B) When adding fresh WM (Step J) or RPMI-HS (Step L) to the flasks, the liquid should be slowly added along the surface opposite the cells. (C) After adding WM to the flasks, wash the cells by gently and slowly tilting the flask 360° and then returning the flask to the flat position.

Table 1

Example of CD4 column isolation multiplier chart

Multiplier	Volume	Reagent
1×	187 μ l	Biotin-Antibody cocktail from the Miltenyi CD4 ⁺ T cell isolation kit
2×	374 μ l	Anti-Biotin Microbeads from the Miltenyi CD4 ⁺ T cell isolation kit
3×	561 μ l	WM
4×	748 μ l	WM

Table 2

Troubleshooting table

Step	Problem	Possible reason	Solution
17	No visible virus pellet	Inefficient transfection	Make sure the 293T cells are the right confluency at the time of transfection Use 293T cells that have a low passage number
26	Residual RBCs	Inadequate Ficoll spin	Load 30 ml of the pooled PBMCs on 15 ml of Ficoll-Paque PLUS for another sedimentation spin
34	Low yield of CD4s	Inadequate Ficoll spin Inaccurate PBMC count in Step 26 Donor variability	Make sure the centrifuge is at least 18 °C Undercounting cells results in insufficient antibody in Steps 29 and 31. Select a different donor – even some healthy donors have comparatively low numbers of CD4s
39, 60, 71	Poor activation of CD4s	Old antibodies Bad STCM	Use fresh antibodies or coat with anti-CD3 antibody for a longer period of time Check an alternate batch of TCGF
53–55, 83	No transduction/poor infection	Any of the above reasons	We have found that the quality of the virus is the most important factor in successful transduction/infection
63–66	Weak expansion	Bad STCM or IL-2	Check an alternate batch of TCGF and replace IL-2 with a fresh aliquot