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Multi-parametric characterization of rare HIV-infected cells using an RNA-flow FISH technique

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SUMMARY

HIV cure efforts are hampered by limited characterization of the cells supporting HIV replication *in vivo* and inadequate methods for quantifying the latent viral reservoir in individuals receiving antiretroviral therapy. We describe a protocol for flow cytometric identification of viral reservoirs, based on concurrent detection of cellular HIV *gag-pol* mRNA by *in situ* RNA hybridization combined with antibody staining for HIV Gag protein. By simultaneously detecting both HIV RNA and protein, the CD4 T cells harbouring translation-competent virus can be identified. The HIV^{RNA/Gag} method is 1,000-fold more sensitive than Gag protein staining alone, with a detection limit of 0.5–1 *gag-pol* mRNA+/Gag protein+ infected cells per million CD4 T cells. Uniquely, the HIV^{RNA/Gag} assay also allows parallel phenotyping of viral reservoirs, including reactivated latent reservoirs in clinical samples. The assay takes 2 days, and requires antibody labelling for surface and intra-cellular markers, followed by mRNA labelling and multiple signal amplification steps.

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AUTHOR CONTRIBUTIONS

A.E.B, F.P. and D.E.K conceived and developed the HIV^{RNA/Gag} assay, with input from A.F. and N.C.; A.E.B., J.N., R.F., J.R., N.B., M.M. and N.A. modified the protocol, designed specific experiments and provided reagents; J.P.R. obtained IRB approval and recruited participants to provide primary samples; D.E.K. provided supervision; A.E.B. and D.E.K. wrote the manuscript and all authors approved the final version.

CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

Keywords

RNA Fluorescence in situ hybridization (RNA FISH); HIV; CD4 T cells; translation-competent reservoir; flow cytometry

INTRODUCTION

In spite of the tremendous success of anti-retroviral therapy (ART) in controlling HIV replication and limiting progression to AIDS, current drug regimens do not lead to cure. No current scalable treatment can eradicate the virus from a HIV-infected person¹, or generate protective HIV-specific immunity². The major barrier to a HIV cure is the latent viral reservoir, a cell population primarily consisting of resting memory CD4 T lymphocytes that contain a stably integrated copy of the DNA provirus. HIV is able to rebound from this reservoir, usually within days or weeks, when a patient discontinues therapy; therefore long-term adherence to ART is required. Although modern regimens are generally well tolerated, the long term effects of ART remain unknown; individuals on suppressive ART remain at increased risk for a range of non-AIDS defining events^{3, 4}. Therefore, the requirement for a life-long treatment, particularly in the context of limited access to ART and social stigma, remains a key issue driving the need for a cure.

Different strategies, alone or in combination, are currently under investigation to achieve HIV cure⁵, such as preferential killing of viral reservoir cells; repopulation of the immune system by genetically engineered, infection-resistant cells; induction of deep latency; or generation of effective anti-HIV responses by therapeutic vaccines. A major strategy proposed for HIV cure is the “shock and kill”⁶ approach, whereby latency-reversing agents (LRAs) “shock” the latent viral reservoir into reactivating. Cells containing reactivated HIV would either be killed by the pathogenic effects of the virus itself or targeted by the host’s immune system, which may have to be vaccine-adjuvanted. Although this strategy has already been tested in limited clinical trials⁷, the relative ability of LRAs to induce different cellular reservoirs remains poorly understood. The latent HIV reservoir is formed early in acute infection^{8, 9}, is inherently stable^{10, 11} and, at present, no cellular markers have been identified that are capable of distinguishing the very rare CD4 T cells containing latent replication-competent proviruses (in the order of 1 per million resting CD4 T cells^{12–15}) from uninfected, bystander cells. These factors have made both studying and targeting the latent viral reservoir for elimination highly challenging. The CD4 lineage presents tremendous heterogeneity *in vivo*, as do other cell types that might contribute to viral reservoirs (such as some myeloid cells¹⁶). Regardless of the strategy pursued to achieve a HIV cure, a deeper understanding of the cells that support HIV replication *in vivo* and serve as long-lived latent viral reservoirs in ART-treated subjects is required both to eliminate residual virus and to inform the development of a vaccine capable of eliminating HIV-infected cells.

Development of the protocol and comparisons with other methods

Research into HIV reservoirs has been limited by the sensitivity, specificity and caveats of available strategies used to detect and phenotype such cells. Standard techniques include, but

are not limited to: the use of *in vitro* cell lines and lab-adapted viruses to model infection; measurements of viral DNA by PCR for total or integrated viral genomes^{13, 17, 18}; and the Quantitative Viral Outgrowth Assay (QVOA)^{12, 19}. Work performed using *in vitro* infections has provided a wealth of information, but is limited by the requirement of many models for cellular activation to enable efficient infection and/or the propagation of cells *in vitro*, both of which alter cell characteristics; and the significant differences between lab-adapted viruses and the transmitted/founder or chronic viruses circulating in the population. Not all integrated HIV proviruses are replication-competent, i.e. able to produce fully infectious virus; indeed over 90% of integrated HIV proviruses may contain deletions or mutations that preclude replication competence^{20, 21}. Due to the prevalence and rapid accumulation of these defective/dysfunctional HIV genomes, PCR-based estimates represent the maximal viral reservoir size, even in individuals who initiated ART during acute infection^{21, 22}. At the opposite end of the spectrum, the QVOA represents a minimal estimate, as not all replication-competent viruses are reactivated following a single round of stimulation²⁰. Recent work has resulted in additional mRNA-based techniques, such as the Tat/Rev Induced Limiting Dilution Assay (TILDA)²³, which have begun to close the gap between these two sets of measures. However, the above techniques rely either on population-level, rather than single-cell, analysis to detect viral reservoirs, resulting in a loss of crucial information; or on limiting dilution strategy assays in which cell phenotypes cannot be determined retrospectively.

We thus sought to develop a flow-cytometry based protocol for the single-cell identification and characterisation of HIV viral reservoirs in primary samples from HIV-infected individuals¹⁵. Antibodies against HIV Gag proteins have been used previously to study *in vitro* infection, but are limited by high non-specific binding, which prevents sensitive identification of HIV-infected cells at frequencies lower than 1,000 events per million¹⁵. By combining classical HIV Gag protein detection with RNA fluorescent *in situ* hybridization for HIV Gag and Pol mRNAs (mRNA Flow-FISH) we developed the HIV^{RNA/Gag} assay, with which we are able to identify HIV^{RNA+/Gag+} CD4 T cells in the range of 0.5–1 events per million. This gain in specificity dramatically changes the scope of questions that can be addressed; it allows identification of HIV-infected CD4 T cells directly *ex vivo* in primary clinical samples from HIV-infected individuals, which was simply not possible with previous techniques.

To be identified by the HIV^{RNA/Gag} assay, cells must contain virus able to transcribe viral mRNAs and translate viral protein. Therefore, we define this population as the translation-competent reservoir in HIV-infected subjects with ongoing viral replication. In ART-treated, virally-suppressed individuals, the assay identifies the translation-competent latent reservoir in cells following latency reversal. This assay effectively narrows down the gap between the maximal and minimal estimates of the reservoir size mentioned above, although the characteristics of the reservoirs measured are distinct.

A key advantage and novel feature of the HIV^{RNA/Gag} assay is that it enables concurrent phenotyping of both the HIV-infected CD4 T cells that are maintaining infection in viremic individuals, and of the CD4 T cells which reactivate virus in response to latency reversing agents, at a single cell level. This type of information has previously been inferred only at a

population level, for example by sorting subsets of CD4 T cells and determining the relative reservoir size.

Limitations

Identification of latent HIV reservoirs in primary samples is limited by the rarity of these cells in ART-treated individuals (in the order of 1 per million resting CD4 T cells). We have observed that the application of the Poisson distribution to the detection of these rare events can be a key source of variability and dictates the starting number of cells required. In the hypothetical examples in Figure 1, the true frequency of HIV^{RNA+/Gag+} events is 2 per million CD4 T cells (the median size of the latent translation-competent reservoir detected following PMA/ionomycin stimulation in our cohort¹⁵). However, if 1×10^6 events are acquired on a flow cytometer, the probability of observing a frequency that differs by more than 2-fold from the true value (e.g. <1 or >4 HIV^{RNA+/Gag+} events per million CD4 T cells) is 18.8 %. (Figure 1A) When 3×10^6 events are collected, the probability of detecting a frequency of HIV^{RNA+/Gag+} events outside of this bracket falls to 7.1 % (Figure 1B), and at 10×10^6 events collected it is 0.5 % (Figure 1C). Thus, the more events acquired, the higher the accuracy of the assay. While it is in principle possible to acquire 10×10^6 or more events by setting up several tubes per condition and merging the FACS data files, this is frequently impractical in terms of cell numbers, operating time and costs. Therefore, we routinely acquire between $2\text{--}4 \times 10^6$ CD4 T cells per subject, per condition.

To enable the collection of $2\text{--}4 \times 10^6$ CD4 T cells at the final step of acquisition on the flow cytometer, we begin with $100\text{--}200 \times 10^6$ PBMCs, depending on the individual's CD4 T cell count and the number of conditions to be tested. We aim to put into culture, for each donor and condition, at least 15×10^6 CD4 T cells to take into account the cell loss associated with an overnight culture and the effects of the agents required for latency reversal. Following this culture, we then start each HIV^{RNA/Gag} assay with 10×10^6 CD4 T cells to account for an observed ~70 % cell loss throughout the protocol. This cell number requirement may be a significant limitation for studies where leukaphereses or large blood draws are not obtained. It should be noted that this requirement for high cell numbers is not specific to this assay, but a reflection of the mathematical Poisson law. Thus, any other assay aiming at detection very rare events, such as the QVOA, has similar requirements for high starting numbers of cells.

Where cell numbers are not limiting, we recommend that each user determines the number of events that need to be analysed based on the expected frequency of their population of interest and taking into account Poisson distribution, as well as the expected losses indicated above.

The probe sets indicated here against HIV Gag and Pol (see “Reagents”) and used by our group¹⁵ were designed against the Clade B isolate JR-CSF. Each probe set contains 20 individual probes, so 40 total, which recognise regions along the whole length of the target mRNA. This provides redundancy and tolerance for sequence variation, as not all probes need to bind to generate a signal. However, the more probes that bind, the brighter the signal (higher mean fluorescence intensity, MFI). Therefore, when adapting the assay to detect alternative, shorter or more variable mRNAs, the number of probes may have a significant impact on the sensitivity of the assay. In our North American cohorts, where the majority of

individuals are infected with Clade B isolates, the redundancy in the HIV^{RNA/Gag} assay has been sufficient to account for inter-donor variability. However, for studies on samples outside of North America and Europe we would recommend designing a probe set against the consensus sequence for the circulating clade.

As discussed previously, the HIV^{RNA/Gag} assay narrows down the gap between the maximal and minimal estimates of the reservoir size inferred by alternative methods. However, some caveats remain. As mentioned, the QVOA is limited to detection of latently infected cells that reactivate virus upon a single round of stimulation, and therefore may underestimate the size of the latent reservoir²⁰. As a similar stimulation technique is applied in the HIV^{RNA/Gag} assay, the same applies here. Furthermore, it should be noted that the detection of a provirus by the HIV^{RNA/Gag} assay does not guarantee that this virus is replication competent. Detection is dependent on production of Gag protein and GagPol mRNA, defining a “translation-competent reservoir”, however a provirus with a deletion outside of GagPol would be detected with this assay but could be unable to initiate a productive infection. This translation-competent reservoir is likely to contain a lower proportion of defective proviruses (and thus a higher frequency of replication-competent proviruses) than alternative PCR-based measurements such as integrated HIV DNA and TILDA. However, it likely still represents an over-estimate of the true replication-competent reservoir size.

Applications of the protocol

The version of the HIV^{RNA/Gag} assay described¹⁵ could be applied to questions involving the HIV replication cycle, restriction factor, viral pathogenesis or effectiveness of intervention/cure therapies. Regarding the HIV cure/latency field, the detection of the translation-competent viral reservoir has significant implications for: i) quantification of latent reservoir size; ii) the efficacy assessment of cure strategies that rely on a reduction in the size of the latent reservoir, rather than complete eradication of latent proviruses; iii) phenotyping of the viral reservoir; and iv) the definition of HIV-infected CD4 cells as expressing HIV proteins, thus having the potential of being efficiently recognized by the adaptive immune system.

For the HIV^{RNA/Gag} assay described here we chose to use probes against both HIV Gag and Pol since: 1) these regions have been shown to be relatively well conserved between clinical isolates; 2) the length of these mRNAs allows for a relatively high number of mRNA-specific probes (40 total) which increases the redundancy in the system for inter-subject sequence variability; and 3) using a high number of probes amplifies the potential low copy number of HIV mRNAs per cell and increases the signal-to-noise ratio, so increasing the detection limit in terms of RNA copy number. However, a key advantage of the HIV^{RNA/Gag} technique is its versatility: it can easily be adapted to detect other HIV genes and proteins, including multiplexing different genes; flow panels can be adapted to quantify vast numbers of surface and intracellular markers - albeit with careful optimization; and – importantly - easy translation to other tissues/cell types (e.g. lymphoid tissues) and/or other species/viruses (e.g. SIV detection in rhesus macaques). This type of mRNA Flow-FISH assay can also be used to detect cytokines²⁴, host factors and/or other pathogen related RNAs. Lastly, the assay could be transferred to alternative cell types; the protocol here focuses on the CD4

T cell reservoir. However, cells of the myeloid lineage (including macrophages) have been proposed as important cellular reservoirs²⁵ and these subsets could in principle be investigated by the same technique, provided the user undertakes careful validation of the protocol. Therefore it represents a powerful tool that can be applied to address a broad range of experimental questions, both within the HIV field and in immunological studies in general.

The version of the HIV^{RNA/Gag} assay described here focuses on the use of a commercially available RNA Flow-FISH assay (Human PrimeFlow, Affymetrix/eBioscience, see “Reagents”) to detect HIV reservoirs *ex vivo*. However, alternative Flow-FISH techniques have previously been used to identify HIV-infected alveolar macrophages²⁶, study HIV-infected cell lines^{27, 28} and detect HIV reservoirs in primary samples²⁹. These methods utilize single mRNA staining only and are thus limited specificity and accuracy. Therefore, the lessons learned from the development of this assay could easily be translated to an alternative product or an in-house system.

Biohazardous materials

Samples from human peripheral blood represent a Class 2 material; therefore appropriate precautions should be taken according to institutional and governmental guidelines when handling these materials. HIV-1 is a Class 2 human pathogen, so samples from HIV-1-infected individuals should be handled in at least a Biosafety Level (BSL) 2 facility. In the culture conditions used for the standard experiment, a spreading infection is restricted by the presence of anti-retrovirals. However, we use BSL-3 guidelines when working with such samples. Appropriate Personal Protective Equipment (PPE) including double gloves and eye protection are worn at all times. All solid waste is inactivated by autoclave incineration; all liquid waste is inactivated with VirkonTM or similar and autoclaved.

Experimental design

The protocol to detect HIV viral translation-competent reservoirs can be broken down into two major parts. Sample preparation, Part I, is highly adaptable and can be extensively modified according to the experimental question. Part II comprises the specific steps of the HIV^{RNA/Gag} assay; Part IIA describes protein antigen detection and Part IIB gene-specific mRNA detection. While the core steps in Part II are constant, the assay can be adapted for detection of different protein antigens or mRNAs.

The procedure described here is for the identification of latent translation-competent reservoirs from HIV-infected, ART-treated individuals. In the example described in the “Procedure” section and associated tables, there are 12 samples total: samples from 3 different donors (one HIV-uninfected, two HIV-infected), each of which are tested unstimulated or stimulated with a latency reversing agent at two different doses; plus three control tubes where *in vitro* infected CD4 T cells may be used. **As controls, we recommend:** A) a HIV-Gag protein FMO; B) an mRNA negative control using an irrelevant or scrambled mRNA probe; and C) a positive control mRNA. Controls A and B can be used to confirm the gating strategy defined using the HIV-uninfected biological control; Control C is useful to determine if the assay worked, in the absence of GagPol mRNA staining.

This protocol uses a 1.5-ml tube-based version of the assay. The assay can also be performed in a 96-well plate format, however our group has not validated this version of the assay to the same extent as the tube-based protocol. We therefore recommend using the 1.5-ml tube version for initial implementation and careful side-by-side comparison before the investigator considers switching to a 96-well plate-based assay. See Box 1 for further discussion on the adaptation of the protocol to 96-well format.

Part I (4–6 hours plus overnight incubation, for standard experiment); Sample preparation (Steps 1–24, see Figure 2)—Sample preparation is dependent on the experimental question to be addressed, however in all cases the cells of interest must be in suspension to move into Part II. It is important to ensure the cell preparation is of good quality, with high viability if possible, to ensure reasonable cell recovery throughout the protocol. For detection of translation-competent reservoirs in HIV-infected individuals *ex vivo*, or following reactivation, we isolate CD4 T cells from freeze-thawed PBMC samples isolated previously. CD4 T cells are either rested or stimulated overnight with a latency-reversing agent of interest. Samples from uninfected donors, treated in parallel, are used as biological controls and to determine the flow cytometry gating strategy. For identification of the translation-competent latent reservoir following treatment with LRAs *in vitro*, it is important to include a donor matched, untreated condition. Additional relevant biological controls may be required depending on the experiment set up. See Box 2 for an *ex vivo* autologous infection protocol that can be used to prepare HIV-positive control samples.

Part IIA (0.5 days); Protein antigen detection (Steps 25–67, see Figure 3)—Following stimulation/reactivation CD4 T cells are collected, washed and aliquoted for staining. Samples are stained according to the experimental question with a mixture of surface antibody markers. As a minimum for primary cells, we recommend the inclusion of antibodies against basic phenotypic markers (CD3, CD4) and an exclusion “dump” channel (CD8, CD14, CD19), as well as the addition of a viability dye to exclude dead cells and decrease background (see Table 1 for reagent set up for this panel and Table 2 for alternative panel options). Following fixation and permeabilization, samples are stained intracellularly for HIV-1 Gag protein as a minimum, but may also be stained for additional intracellular antigens concurrently. When staining for multiple markers, it is appropriate to include the same controls as for any multi-parametric flow cytometry analysis, such as fluorescent-minus-one (FMO) controls. In FMO controls, the sample is stained for all markers (both mRNA and protein) except one and this is repeated for all markers. These controls can then be used to define the threshold of positivity when designing a gating strategy. Of note, for GagPol mRNA and Gag protein only, we recommend that gates be drawn using a biological control (e.g. an uninfected donor treated identically to the HIV-infected donors of interest) rather than FMOs. Following a second fixation, the protocol may be paused overnight. However, in general, for operator ease, we proceed to Part IIB and pause there at the second pause point.

Part IIB (1.5 days); mRNA detection, signal amplification, sample acquisition and analysis. (Steps 68–110, see Figure 4)—Samples are labelled for gene-specific mRNAs of interest, and then stored overnight; this is the second pause point. The following

day, the signal is amplified using a branched dsDNA system and the amplified signal is labelled with a fluorescent dye. For detection of translation-competent reservoirs, we use probes designed against the GagPol region of the HIV-1 strain JR-CSF. Additional controls that may be included to monitor mRNA staining include a probe against a house-keeping gene (RPL13A is recommended). Negative control probes (either scrambled, or against an irrelevant mRNA such as bacterial DapB; see Reagents) can be used; however we have found that for the HIV^{RNA/Gag} assay, HIV-uninfected samples treated identically to HIV-positive samples enable the most accurate gating. Samples are acquired on a flow cytometer according to standard operating procedures^{30, 31}. Correct compensation controls are essential and are discussed in Box 3. A 1 laser machine can be used to detect the HIV-1 Gag protein and GagPol mRNA without additional antibody stains (see Table 2 for suggested panels/fluorochrome combinations), but this is not recommended for primary cells.

MATERIALS

REAGENTS

- Peripheral CD4 T cells isolated from HIV-infected and uninfected donors. ! **Caution** - Any study protocols involving human subjects must confirm to institutional and governmental ethical guidelines. ! **Caution** - HIV-1 is a Class 2 human pathogen and HIV-infected samples should be handled in a Biosafety Level 2 (BSL-2) facility, in accordance with safe working practices. Gloves and protective eyewear should be worn.
- Human PrimeFlow 3-plex kit (Affymetrix eBioscience, Cat#88-18009, 40 or 100 tests). Kit includes: Fixation Buffers 1A+B, 10× Permeabilization Buffer, RNasin I, RNasin II, Fixation Buffer II, Wash Buffer, Target Probe Diluent, Preamplification and Amplification buffers, Label Probe Diluent, Label Probe Mix, and Positive control (RPL13A) mRNA probes. ! **Caution** – Fixation buffers contain paraformaldehyde (PFA). PFA is an irritant; avoid exposure to skin or eyes.
- RPMI 1640 medium (Gibco by Life Technologies, Cat#11875-093, 500 ml)
- HEPES (Life Technologies, 1 M, Cat#15630-080, 100 ml)
- Penicillin-streptomycin (Gibco by Life Technologies, 10,000 U/ml, Cat#15140-122, 100 ml)
- DPBS pH 7.4, no Ca²⁺/Mg²⁺ (Gibco by Life Technologies, 1×, Cat#14190-144, 500 ml)
- FBS (Seradigm by VWR, Cat#1500-500, 500ml)
- EasySep Human CD4+ T cell isolation kit (Stem Cell, Cat#19052, for 1 × 10⁹ cells)
- The “Big Easy” EasySep Magnet (StemCell, Cat#18001, for isolating 4 × 10⁸ cells)
- EDTA UltraPure (Invitrogen, 0.5M, Cat#15575-038, 100ml)

- Trypan blue (Gibco by Life Technologies, 0.4 %, Cat#15250-061, 100ml)
- S7 nuclease (Roche/Sigma Aldrich, 15,000U, Cat#10107921001)
- Sterile RNase-free H₂O (Wisent Bioproducts, Cat#809-115-CL, 500ml)
- Zidovudine (AZT, NIH AIDS reagent database, Cat#8435, 20 mg)
- T20 (Trimeris/Roche via NIH AIDS reagent database, Cat#9845, 5 mg)
- PMA (Sigma-Aldrich, Cat#P1585, use at 50 ng/ml)
- Ionomycin (Sigma-Aldrich, Cat#I9657, use at 0.5 µg/ml)
- Bryostatins 1 (Enzo, Cat#BML-ST103-0010, 10 µg, use at 10 nM)
- FcR Block (Miltenyi Biotec, Cat#130-059-901, 2 ml)
- BD CompBeads Plus (BD Biosciences, Cat#560497, 6 ml)
- OneComp eBeads (eBioscience, Cat#01-1111-42)

Suggested antibodies, dyes and probes (See Table 2 for suggested Panel information) for all panels

- Anti-HIV-1 Gag p24 (Beckman Coulter, Clone KC57/FH190-1-1, RD-1 (PE), Cat# 6604665).
- IgG Isotype control (BioLegend, Clone MOPC-21, For Type 1 control use AF467, Cat# 400130; For Type 4 control use AF488, Cat# 400129)
- HIV-1 Gag mRNA (Affymetrix eBioscience, JR-CSF target sequence, Type 1 Cat#VF1-13962, Type 4 Cat #VF4-18312, 20 pairs of branched DNA probes, probe length median[range] = 23[17–30]nts)
- HIV-1 Pol mRNA (Affymetrix eBioscience, JR-CSF target sequence, Type 1 Cat# VF1-13961, Type 4 Cat # VF4-18314, 20 pairs of branched DNA probes, probe length median[range] = 25[18–30]nts)
- RPL13A Positive control mRNA (provided with 3-plex version of Human PrimeFlow kit; can also be purchased from Affymetrix eBioscience as Type 1 Cat#VA1-13100, Type 4 Cat#VA4-13187).
- Scrambled negative control mRNA (Affymetrix eBioscience, Type 1 Cat#VF1-16506, Type 4 Cat#VF4-19835)

Suggested antibodies, dyes and probes (See Table 2 for suggested Panel information) for Panel B

- Anti-CD3 (BioLegend, Clone OKT3, BV605, Cat# 317322)
- Anti-CD4 (BD Biosciences, Clone RPA-T4, Pe-Cy7, Cat# 560649)
- Anti-CD8 (BioLegend, Clone SK1, BV510, Cat# 344732)
- Anti-CD14 (BioLegend, Clone M5E2, BV510, Cat# 301842)
- Anti-CD19 (BioLegend, Clone H1B19, BV510, Cat# 302242)

- Fixable Viability Dye (eBioscience, eFluor780, Cat# 65-0865)

Suggested antibodies, dyes and probes (See Table 2 for suggested Panel information) for Panel C

- Anti-CD3 (BD Biosciences, Clone UCHT1, BUV395, Cat# 563548)
- Anti-CD4 (BD Biosciences, Clone SK3, BUV496, Cat# 564651)
- Anti-CD8 (BioLegend, Clone SK1, BV510, Cat# 344732)
- Anti-CD14 (BioLegend, Clone M5E2, BV510, Cat# 301842)
- Anti-CD19 (BioLegend, Clone H1B19, BV510, Cat# 302242)
- Anti-CD27 (BD Biosciences, Clone L128, BV605, Cat# 562655)
- Anti-CD45RA (BioLegend, Clone HI100, BV711, Cat#304138)
- Anti-CD278 (ICOS), (eBioscience, Clone ISA-3, PE-Cy7, Cat#25-9948)
- Anti-CD279 (PD-1), (BioLegend, Clone EH12.2H7, BV421, Cat#329920)
- Anti-CXCR5 (BD Biosciences, Clone RF8B2, BB515, Cat#564624, stain during culture)
- Fixable Viability Dye (eBioscience, eFluor506, Cat# 65-0866)

Plastic wear

- 15-ml falcon tubes, sterile (Corning Falcon, Cat#353096)
- Plate, 24 wells, sterile, tissue culture treated (Corning Falcon, Cat#353047)
- 14-ml FACS tubes, sterile (Corning Falcon, Cat#352057)
- Sterile, RNase free, low binding tips (all Ranin), p1000 (Cat#17007954); p200 (Cat#17007961); p20 (Cat#17007957)
- Sterile serological pipettes (all Starstedt), 5-ml (Cat#86.1253.001); 10-ml (Cat#86.1254.001); 25-ml (Cat#86.1685.001)
- Kova Glastic SL 10W GRID (Counting chambers, VWR, Cat#CA36200-020)
- RNase-free, low binding 1.5-ml tubes (provided with Human PrimeFlow kit above) **Critical** – Low-binding RNase free tubes greatly increase cell recovery and should be used throughout the protocol.
- 0.5-ml tubes sterile (Starstedt, Cat#72.730.005)
- 1.5-ml tubes, RNase-free sterile (For reagent preparation, Starstedt, Cat#72.692.405)

EQUIPMENT

- Tissue culture facilities in at least a BSL-2 are required.

- Centrifuge equipped with swinging bucket rotor, able to cool to 4 °C (e.g. VWR Thermo Heraeus Multifuge Benchtop Centrifuge 1XR, Cat #97039-270). **Critical** – The use of a swinging-bucket centrifuge is key. Use of a fixed angle rotor will result in poor cell recovery.
- Centrifuge adaptors for 15-ml falcon tubes and 1.5-ml eppendorfs.
- Tissue culture incubator (37 °C, 5% CO₂; e.g. Sanyo Professional CO₂ incubator MCO19AICUVH)
- Aspirator (recommended, Mandel Vacusafe Comfort with Vacuboy, Cat # TM-158310).
- Benchtop PCR hood (recommended, AirClean Systems PCR/RNA Workstation AC648DBC)
- Light microscope (suitable for cell counting and assessment of cell viability/activation, e.g. Fisher Scientific Wilovert 30)
- Hybridization oven, able to maintain 40 °C (e.g. Shel Lab 1330FM Forced Air Oven). **Critical** – Temperature stability is important. The oven used must be able to maintain a stable temperature with limited fluctuations.
- Flow cytometer with the capacity to detect at least 2 colors; see Table 2 for example panels for set-ups of different laser number (e.g. BD Biosciences, LSR II)
- Flow cytometry analysis software (e.g. Tree Star FlowJo)

REAGENT SETUP

- **Heat inactivated FBS.** Complement-inactivate FBS by heating to 56 °C for 30 min in a water bath. Aliquot FBS into single use aliquots and store at –20 °C for up to 1 year.
- **Complete culture medium (R10).** Prepare R10 by making up 10 % (vol/vol) heat-inactivated FBS, 50U/ml penicillin/streptomycin, 10 mM HEPES in RPMI 1640 Medium with Phenol Red. Prepare the medium in advance and store at 4 °C for up to 2 weeks.
- **R10 + ARVs.** Prepare R10 as above. Add AZT to a final concentration of 5 µM and T20 at a final concentration of 7.5 µg/ml. Use fresh.
- **EasySep CD4 Isolation Buffer.** Prepare by making up 2 % (vol/vol) heat-inactivated FBS, 1 mM EDTA in sterile PBS pH7.4. Prepare in advance and store at 4 °C for up to 2 weeks.
- **S7 nuclease.** Add 3ml of sterile PBS pH7.4 to dried pellet. Store at 4 °C for up to 1 month.
- **2% FBS/PBS.** Prepare by making up 2 % (vol/vol) heat-inactivated FBS. Prepare in advance and store at 4 °C for up to 2 weeks.

- **AZT.** Reconstitute with DMSO to give a concentration of 50 mM. Aliquot and store at -20°C for 1 year.
- **T20.** Reconstitute with sterile PBS pH7.4 to give a concentration of 2 mg/ml. Aliquot and store at -20°C for 1 year.
- **PMA.** Reconstitute with DMSO to give a concentration of 1 mg/ml. Aliquot and store at -80°C for 1 year.
- **Ionomycin.** Reconstitute with DMSO to give a concentration of 0.5 mg/ml. Aliquot and store at -80°C for 1 year.
- **Bryostatins 1.** Reconstitute with DMSO to give a concentration of 25 μM . Aliquot and store at -80°C for 1 year.

EQUIPMENT SETUP

- **Hybridization oven.** The hybridisation oven should be calibrated to maintain a stable temperature of 40°C . We recommend the oven be turned on at least 24 hr before use.
- **Flow cytometer.** The flow cytometer used should be routinely calibrated and cleaned. CS&T should be run frequently. Quality assurance for multicolor flow cytometry is discussed elsewhere^{30, 31}.

PROCEDURE

Part I: Cell thawing, negative CD4 T cell isolation and stimulation. Timing 4–6 h + overnight incubation

- 1 *Cell thawing.* Prepare cold, labelled, 15-ml conical tubes and pre-cool the centrifuge to 4°C . At least 1 conical tube is required for each donor sample to be thawed, with a maximum of 200×10^6 PBMCs per tube to avoid cell clumping. Work with a maximum of 2 donors at any time. Multiple cryovials of frozen PBMCs from one **donor** may be needed to meet cell number requirements (i.e. 2 vials containing 50×10^6 PBMCs each to have 100×10^6 starting PBMCs). Work with a maximum 4 cryovials at any point.
- 2 Thaw required **donor** sample cryovials by warming in a 37°C water bath, until floating ice is visible. Transfer contents of each vial to a cold 15-ml conical tube prepared in Step 1. Wash cryovial with 1ml of cold FBS and add to the 15-ml conical tube. Add Nuclease S7 to each conical tube (20 μl per ml of PBMC suspension, minimum 40 μl), mix by tapping the tube and incubate for 20 sec. Add cold FBS to a final volume of 10 ml.
- 3 Centrifuge the conical tube at $420g$ for 10 min at 4°C .
- 4 Discard the supernatant and gently resuspend the pellet in 1 ml of cold R10 and top up to 10 ml with R10. If there are multiple conical tubes for a single donor, resuspend each of the pellets in 1 ml of cold R10 and combine into a single conical tube. Top this tube up to 10 ml with R10, then split the sample back out

into the starting number of conical tubes. Top all conical tubes up to 10 ml with R10. This will ensure that the PBMCs are split evenly across all conical tubes, so only one conical tube will need to be counted per donor, while keeping the number of cells per conical under 200×10^6 . Centrifuge the conical tubes at $420g$ for 10 min at 4°C .

- 5 Discard the supernatant and gently resuspend the pellet in 1 ml of cold EasySep CD4 Isolation Buffer and top up to 10 ml with EasySep CD4 Isolation Buffer.

Critical step – If you do not wish to isolate CD4 T cells, for example for detection of HIV reservoirs in total PBMCs, perform Step 5 in cold R10, count cells as in Steps 6+7, then proceed to Step 19. The presence of CD8 T cells in a reactivated culture may have an adverse effect on the detection of the reactivated latent viral reservoirs, therefore we recommend performing a CD8 depletion (for example using Dynabeads™ CD8 Positive Isolation Kit, ThermoFisher, Cat#11333D) as a minimum.

- 6 *Cell counting.* Prepare a 0.5-ml tube with 90 μl of Trypan blue. Remove a 10 μl aliquot of cell suspension and add to the 0.5-ml tube containing Trypan Blue. Mix by vortexing and transfer 10 μl to a Kova Glasik counting chamber. Count both the live cells (Trypan Blue negative) and any dead cells (Trypan Blue positive). Calculate total cell number and record the viability (given as %live

cells) using the formula: $100 - \left(\frac{\text{dead cells}}{\text{live cells} + \text{dead cells}} \times 100 \right)$

? Troubleshooting

- 7 Centrifuge the conical tube at $420g$ for 10 min at 4°C .
- 8 *CD4 T cell isolation.* Discard the supernatant and gently resuspend pellet in EasySep CD4 Isolation Buffer at 20 μl per 10^6 cells (i.e. at $50 \times 10^6/\text{ml}$). Remove any clumps of dead cells with a pipette.
- 9 Transfer cell suspension to a 14-ml FACS tube. PBMCs from multiple conical tubes from the same donor can be recombined into one 14-ml FACS tube. If total PBMC number is $>400 \times 10^6$, aliquot between multiple tubes.
- 10 Add 1 μl per 10^6 PBMCs of Stem Cell Biotinylated Antibody Cocktail from EasySep CD4 Isolation kit. Mix well by pipetting. Incubate for 10 min at room temperature (RT; 20°C).
- 11 Add 2 μl per 10^6 PBMCs of StemCell Bead Cocktail. Mix well by pipetting. Incubate for 5 min at RT.
- 12 Add EasySep CD4 Isolation Buffer to a final volume of 10 ml per 14-ml FACS tube.
- 13 Transfer 14-ml FACS tube to a “Big Easy” StemCell isolation magnet. Remove the cap. Incubate for 5 min at RT.

- 14 Prepare a fresh 15-ml conical tube with 2.5 ml of R10. Carefully pick up the magnet with the 14-ml FACS tube in place and slowly pour the unbound cell fraction into the fresh 15-ml conical containing R10.
- 15 Centrifuge the conical tube at 540g for 5 min at 4 °C.
- 16 Discard the supernatant and gently resuspend the pellet in 1 ml of cold R10 and top up to 10ml with R10.
- 17 Count the isolated CD4 T cells as in Step 6.

? Troubleshooting

- 18 Centrifuge the conical tube at 540g for 5 min at 4 °C.
- 19 *CD4 T cell stimulation/reactivation of latent reservoirs.* Discard the supernatant and gently resuspend the pellet at 2×10^6 CD4 T cells/ml in R10 + ARVs, (see Reagent Set Up).
- 20 Plate onto a sterile, 24 well, tissue culture treated plate at 1 ml per well.
Critical step – Use only the middle 8 wells and fill the outer wells with 1ml of sterile PBS at RT to limit loss by evaporation
Critical step – The number of CD4 T cells per donor per condition is crucial for the accurate detection of latent translation-competent reservoirs. For detection of very rare events (~ 1 per 10^6) we recommend starting with at least 16×10^6 cells, i.e. 8 wells at 2×10^6 /ml.
- 21 Rest the cells at 37 °C with 5 % CO₂ in a tissue culture incubator for 3 hr.
- 22 Prepare the latency reversing agents as desired. For example, for PMA/ionomycin stimulation use at 50 ng/ml and 0.5 µg/ml respectively. For Bryostatin-1 use at 10 nM.
Critical step – We recommend titrating these reagents to maximize cell activation and minimize toxicity in the time frame studied.
- 23 Add latency reversing agents to each well for the appropriate final concentration. Mix by pipetting gently, or swirling the plate.
- 24 Stimulate cells overnight for 12 – 18 hr at 37 °C with 5 % CO₂.
Critical step – Optimal length of incubation is dependent on LRA used and should be determined by the user.

Part IIA: Cell collection and preparation, viability stain, surface antibody stain and intracellular stain. Timing 4–8 h

- 25 *Cell collection and preparation.* As soon as incubation time ends, place all plates at 4 °C. Cool centrifuge to 4 °C. Prepare cold 15-ml conical tubes for each donor/condition. We recommend checking the cultures under a microscope and taking note of any loss in viability and activation induced by any stimuli added.

- 26** Collect cells by pipetting gently up and down to mix with a 1 ml pipette. Transfer to 15-ml conical. Identical wells can be combined.
- 27** Wash wells with cold R10. Add 1 ml to a well, pipette gently on the base of the well to lift any remaining cells and combine into 15-ml conical. Repeat for all donors and conditions.
- Critical step** – As soon as one 15-ml conical is full, place at 4 °C to limit RNA degradation.
- 28** Centrifuge the conical tubes at 540g for 5 min at 4 °C.
- 29** Discard the supernatant and gently resuspend the pellet in 1 ml of cold R10 and top up to 10 ml with R10. Centrifuge the conical tubes at 540g for 5 min at 4 °C.
- 30** Discard the supernatant and gently resuspend the pellet in 1 ml of cold 2% FBS/PBS.
- 31** Count the isolated CD4 T cells as in Step 6. In particular, take note of the cell viability. Samples with a low viability <50–60 % may produce high background and show poor cell recovery throughout the protocol.
- ? Troubleshooting**
- 32** Top up with 10 ml of cold 2% FBS/PBS. Centrifuge the conical tubes at 540g for 5 min at 4 °C.
- 33** Discard supernatant and resuspend the pellets at 10×10^6 /ml in cold sterile PBS. Aliquot 1ml into PrimeFlow 1.5-ml low binding RNase-free tubes for viability staining as described in Steps 36–40.
- Critical step** – If no viability stain is required, resuspend in cold 2% FBS/PBS, rather than PBS, follow Steps 34 and 35, then proceed to Step 40 for surface staining. We recommend a viability stain be used for all primary cell samples.
- 34** Centrifuge tubes (600g, 5 min, 4 °C).
- 35** Remove 900 µl with a pipette, or using an aspirator. The line on the tube at 100 µl can be used as a guide. Resuspend the cells in the residual volume either by pipetting gently or vortexing on a low speed.
- Critical step** – We have observed that vortexing, particularly for samples from HIV-infected, untreated individuals, has a negative impact on cell viability. We would recommend that for fragile samples, resuspension is performed carefully and by gentle pipetting.
- 36** *Viability stain.* Prepare a stock of Fixable Viability stain. 100 µl of this stock is required per test. See Table 1 for example calculations.
- 37** Add 100 µl of the Fixable Viability stain to the residual 100 µl left in each tube from Step 35, giving a volume of 200 µl per tube. Mix well by pipetting.
- 38** Incubate at 4 °C for 20 min in the dark.
- 39** Add 800 µl of cold 2% FBS/PBS and invert tubes 3 times to wash.

- 40** Centrifuge, discard supernatant and resuspend as in Steps 34 + 35.
- 41** *FcR block.* Add 1.4 µl of FcR block into the residual volume in each tube. If FcR block is not required, go to Step 43.
- Critical step** – We recommend the use of FcR block particularly when PBMCs are used as the starting cell population, to limit non-specific antibody binding. If no surface stain is required, proceed to Step 48 for fixation.
- 42** Incubate at 4 °C for 10 min in the dark.
- 43** *Surface antibody stain.* During the incubation, prepare a mix of titrated, surface stain antibodies.
- Critical step** – Antibody selection will affect the quality of the cell staining. See Box 4 for a detailed discussion.
- Critical step** – Using the optimal antibody concentration to maximize the background/noise ratio is key. We advise titration of all antibodies used. As a minimum, we recommend antibody staining for basic phenotypic markers (e.g. CD3, CD4, exclusion) for cell identification.
- Critical step** – If two or more Brilliant Violet (BV), Brilliant Ultra-violet (BUV) and Brilliant Blue (BB) fluorochromes are included in the panel, non-specific interactions may be observed between these colors. Brilliant Stain Buffer (BD Bioscience, Cat # 563794) can be used in preparation of the antibody mixes. We have not observed any issues when using this buffer with PrimeFlow™, however we highly recommend that each investigator validate their specific antibody combination with and without this buffer.
- 44** Add surface antibody mix directly to the residual 100 µl. Mix well by pipetting.
- 45** Incubate at 4 °C for 30 min in the dark.
- 46** Add 1 ml of cold 2% FBS/PBS and invert tubes 3 times to wash.
- 47** Centrifuge, discard supernatant and resuspend as in Steps 34 + 35, except here, remove at least 1 ml to get back to the 100 µl residual volume. This volume will now be used for all further steps.
- 48** *Fixation I and permeabilization.* Make up Fixation Buffer I by mixing equal parts of Fixation Buffer 1A and 1B. 1 ml is required per tube. See Table 1 for example preparation calculations. Do not vortex – mix by gently inverting the tube. Prepare and use fresh. Store at 4 °C until use.
- 49** Add 1 ml of Fixation Buffer I to the 100 µl residual volume. Invert to mix.
- 50** Incubate for 30 min at 4 °C in the dark.
- 51** Centrifuge at 800g for 5 min at 4 °C
- Critical step** – Note the increased spin speed after this fixation step. Failure to increase the spin speed will result in cell loss. Use 800g for all following steps.
- 52** Discard supernatant and resuspend as in Step 35.

- 53** Prepare a stock of 1 × Permeabilization Buffer plus RNAsin. This buffer should be prepared fresh and stored at 4 °C before use. Do not vortex – mix by inverting. 3 ml is required per sample. See Table 1 for example preparation calculations.
- 54** Add 1 ml of cold Permeabilization Buffer to the 100 µl residual volume in each tube. Invert to mix.
- 55** Spin, discard supernatant and resuspend as in Steps 51 + 52.
- 56** Repeat Steps 54+55, such that each tube is washed twice in Permeabilization Buffer.
- 57** *Intracellular antibody stain.* Prepare intracellular antibody stains. If using additional intracellular stains as well as the minimum anti-HIV-1 Gag antibody KC57 RD-1, prepare one mix for the additional stain excluding anti-HIV-1 Gag.
- Critical step** – If not performing an intracellular stain, proceed to Step 65. This is not recommended for detection of translation-competent reservoirs.
- 58** Add 2 µl of anti-HIV-1 Gag antibody KC57 RD-1 directly to the residual 100 µl in each tube. Mix by pipetting gently.
- 59** Incubate at RT for 30 min in the dark.
- 60** If using additional intracellular antibody stains, add this mix directly to the residual 100µl volume. If not, proceed to Step 61.
- 61** Incubate for a further 30 min at 4 °C in the dark, so that the samples are stained with anti-Gag KC57 for 1 hr total.
- Critical step** – Anti-Gag KC57-RD-1 requires a longer staining time than most ICS antibodies. We have found that staining with anti-Gag KC57-RD-1 for 30 minutes at RT, prior to staining with additional intracellular antibodies is optimal to maximize anti-Gag signal, while minimising background for additional intracellular antibodies.
- 62** During this incubation warm a 50 ml aliquot of Wash Buffer to RT.
- 63** Add 1ml of Permeabilization buffer to the residual volume and invert 3 times to mix.
- 64** Spin, discard supernatant and resuspend as in Steps 51 + 52.
- 65** *Fixation II.* Prepare Fixation Buffer II by diluting 1:8 in Wash Buffer at RT. 1 ml is required per sample. See Table 1 for example calculations. Do not vortex-invert to mix.
- 66** Add 1 ml of RT Fixation Buffer II to the 100 µl residual volume and invert 3 times to mix.
- 67** Incubate for 1 hr at RT in the dark.
- Pause Point** – As an alternative to this incubation, samples can be stored overnight in Fixation Buffer 2 at 4 °C. However, we have observed that longer-term storage in Fixation Buffers increases background and negatively impacts

fluorochrome stability. Therefore we would recommend continuing to a later Pause Point.

Critical step – From this point forward all buffers should be at RT or warmer; reagents and steps should be carried out at RT or above.

Part IIB: Labelling mRNA, amplification, labelling amplified signal and data acquisition.

Timing 1.5 days

- 68** *Labelling mRNA.* During this incubation, thaw mRNA target probes at RT and prepare mRNA Target Probe Mixes in Target Diluent. See Table 1 for example calculations. Mixes should be warmed to 40 °C before use.

Critical step – mRNA Target Probe mix preparation is critical. The target probes should be added directly into the appropriate volume of Target Diluent—do not pipette down the sides. Mixes should be well-mixed by pipetting. The liquids are viscous and difficult to work with. We recommend using low-binding RNase-free tips where possible and prepare additional reagent to take into account pipetting error. The number of additional tests we routinely prepare for is shown in Table 3.

Critical step – PrimeFlow probes are available in three colors (Types) – Type 1 (AF647), 4 (AF488) and 6 (AF750). Types 1 and 4 are recommended for low-copy number RNAs or RNAs where the expression level is not known. Antibodies and mRNA probes cannot be used on fluorochromes with overlapping spectra (i.e. a FITC-tagged antibody cannot be used with a Type 4 AF488 probe set).

- 69** Centrifuge cells in Fixation Buffer 2 at 800g for 5 min at RT.
- 70** Discard supernatant and resuspend as in Step 35.
- 71** Add 1 ml of RT Wash Buffer to the residual 100 µl volume. Invert 3 times to mix.
- 72** Centrifuge, discard supernatant and resuspend as in Steps 69 + 70.
- 73** Repeat Steps 71+72 so that samples are washed twice in RT Wash Buffer.

Pause Point – To pause the protocol, prepare Storage Wash Buffer as in Reagent Set Up. See Table 1 for example calculations. Use in place of standard Wash Buffer for Step 73. Place samples at 4 °C for overnight storage. If this Pause Point is used, warm up samples to RT and resuspend pellets in the residual 100 µl volume before proceeding. Also warm Wash Buffer to RT.

- 74** Add 100 µl of the warm Target Probe Mix directly into the 100 µl residual volume, do not add down the sides of tubes. Mix gently by pipetting until the two liquids no longer appear separate. Vortex briefly to mix, with 2 pulses on a low speed. Where possible, work in the dark.

Critical step – Ensure that the cell pellets are well resuspended in the residual 100 µl before addition of the Target Probe Mix.

- 75** Place the samples into a metal heat block within an oven pre-heated to 40 °C.
- 76** Incubate for 1 hr at 40 °C.
- Critical step** – The correct temperature is very important. We recommend monitoring the temperature at all times. A tolerance of 40 °C +/- 1 °C is acceptable. The oven used should be well calibrated and the temperature stable.
- 77** Remove the metal heat block containing the tubes from the oven. Invert the whole heat block, taking care to hold the tubes in place, to mix.
- 78** Incubate for a further 1 hr at 40 °C.
- 79** Add 1 ml of RT Wash Buffer to the residual 200 µl volume. Invert 3 times to mix.
- 80** Centrifuge, discard supernatant and resuspend as in Steps 69 + 70.
- 81** Repeat Steps 79 + 80, so that the samples are washed twice.
- Pause Point** – Prepare Storage Wash Buffer as in Reagent Set Up. See Table 1 for example calculations. Use in place of standard Wash Buffer for Step 81. Place samples at 4 °C for overnight storage. If this Pause Point is used, warm up samples to RT and resuspend pellets in the residual 100 µl volume before proceeding. Also warm Wash Buffer to RT.
- 82** *Amplification.* Aliquot the required volume of Pre-Amplification Mix into a 1.5-ml RNase-free tube. Place in the heat block and warm to 40 °C.
- 83** Add 100 µl of the warm Pre-Amplification Mix directly into the 100 µl residual volume, do not add down the sides of tubes. Mix gently by pipetting until the two liquids no longer appear separate. Vortex briefly to mix, with 2 pulses on a low speed. Where possible, work in the dark.
- Critical step** – Ensure that the cell pellets are well resuspended in the residual 100 µl and at RT before addition of the Pre-Amplification Mix.
- 84** Place the samples into a metal heat block within an oven pre-heated to 40 °C.
- 85** Incubate for 1.5 hr at 40 °C.
- 86** Aliquot the required volume of Amplification Mix into a 1.5-ml RNase-free tube. Place in the heat block and warm to 40 °C.
- 87** Add 1 ml of RT Wash Buffer to the 200 µl residual volume. Invert three times to mix.
- 88** Centrifuge, discard supernatant and resuspend as in Steps 69 + 70.
- 89** Repeat Steps 87 + 88 twice, so that the samples are washed three times.
- 90** Add 100 µl of the warm Amplification Mix directly into the 100 µl residual volume, do not add down the sides of tubes. Mix gently by pipetting until the two liquids no longer appear separate. Vortex briefly to mix, with 2 pulses on a low speed. Where possible, work in the dark.

- 91** Incubate for 1.5 hr at 40 °C.
- 92** During this incubation, aliquot the required volume of Label Probe Diluent into a 1.5-ml RNase-free tube. See Table 1 for example calculations. Place in the heat block and warm to 40 °C. Thaw the Label Probes mix in the dark on ice.
- 93** Add 1 ml of RT Wash Buffer to the 200 µl residual volume. Invert three times to mix.
- 94** Centrifuge, discard supernatant and resuspend as in Steps 69 + 70.
- 95** Repeat Steps 93 + 94 twice, so that the samples are washed two times.
- 96** *Labelling amplified signal.* Prepare the Label Probe Mix as described in Reagent Set Up. See Table 1 for example calculations. Ensure the Label Probe diluent is at 40 °C before use and that the Label Probes have completely thawed. Mix by pipetting gently – do not vortex. We recommend limiting the number of freeze/thaws for the label probe. If >5 freeze/thaws is expected, aliquots should be prepared.
- 97** Add 100 µl of the warm Label Probe mix directly into the 100 µl residual volume, do not add down the sides of tubes. Mix gently by pipetting. Vortex briefly, with 2 pulses on a low speed.
- 98** Incubate for 1 hr at 40 °C.
- 99** Add 1 ml of RT Wash Buffer to the 200 µl residual volume. Invert three times to mix.
- 100** Centrifuge, discard supernatant and resuspend as in Steps 69 + 70.
- 101** Repeat wash in Steps 99 and 100 with wash buffer.
- 102** *Acquisition.* For acquisition on a flow cytometer, complete a final wash with either Storage Buffer, or 2% FBS/PBS.
- Pause Point** – Samples can be stored overnight in 2% FBS/PBS, or for 1 week in storage buffer, depending on the fluorochromes used. We recommend acquiring the samples as soon as possible.
- 103** Acquire the samples on a flow cytometer equipped with the appropriate lasers and filter sets for the panel used. In the examples shown in Figures 5–8 and Supplementary Figure 1, samples were acquired on a modified 5-laser BD LSRII.
- Critical step** – Ensure that the flow cytometer is clean before use – false positive events will affect the limit of detection of the assay.
- Critical step** – We recommend running samples slowly to decrease the electronic abort rate. In our hands, diluting samples to ~2,000 events/second and running at the lowest speed setting on a BD LSRII allows for maximal cell and event recovery.

Critical step – Collecting a large number of events (2–4 million) takes up considerable memory and may cause issues with acquisition software; therefore we recommend collecting data only within the ‘Lymphocyte’ or P1 gate to minimise data usage.

? Troubleshooting

Data analysis: Timing 1–4h

- 104** Analyze data using FlowJo Versions 9 and 10 for Mac.
- 105** Gate on lymphocytes based on SSC-A versus FSC-A using a restrictive gate as shown in Supplementary Figure 1A.

Critical step – A restrictive gate here helps eliminate autofluorescent cells.

- 106** Exclude doublets using SSC-H/W and FSC-H/W as in Supplementary Figure 1B.
- 107** Exclude dead/dying cells using a viability stain as shown in Supplementary Figure 1C.
- 108** For the identification of CD4 T cells, gate on exclusion channel negative (e.g. CD8, CD14, CD19) events as in Supplementary Figure 1D. If a stimulation that may induce CD3 and/or CD4 downregulation (such as PMA/ionomycin) is used proceed directly to Step 110.

Critical step – We recommend the use of FMOs to guide gating of most phenotypic protein markers (except for HIV Gag protein – see Step 110). Autofluorescence is increased with the protocol, so FMOs must undergo the complete protocol.

- 109** Where CD3 is not down-regulated by the conditions used, gate CD3⁺ events as shown in Supplementary Figure 1E.
- 110** Gate GagPol mRNA⁺ Gag Protein⁺ double positive cells (the HIV^{RNA+/Gag+} population) using a HIV-uninfected control donor (UC) sample as a guide. See Figures 5–7 for example gating using UC donor samples.

Critical step – This sample should be treated identically and stained in parallel to the samples of interest. Cell number is also important – the same number of cells should be used for the UC as the samples of interest.

TIMING

Steps 1–7 – *Thawing of PBMC samples*, 1–3 hours depending on sample number

Steps 8–18 – *CD4 T cell isolation*, 1–1.5 hours depending on sample number

Steps 19–24 – *Reactivation of reservoirs*, 15–21 hours depending on LRA used.

Steps 25–35 – *Sample collection and preparation*, 0.5–3 hours depending on sample number

Steps 36–42 – *Viability stain and FcR block*, 1.5 hours

Steps 43–47 – *Surface antibody stain*, 1 hour

Steps 48–56 – *Fixation I and Permeabilization*, 1.5 hours

Steps 57–64 – *Intracellular stain*, 2 hours

Steps 65–67 – *Fixation II*, 2 hours

Steps 68–81 – *mRNA labelling*, 2 hours

Steps 82–95 – *Amplification of mRNA signal*, 4 hours

Steps 96–101 – *Labelling amplified signal*, 2 hours

Steps 102–103 – *Sample acquisition*, 2–6 hours

Step 104–110 – *Data analysis*, 1–4 hours

TROUBLESHOOTING

Troubleshooting advice can be found in Table 4.

ANTICIPATED RESULTS

Positive control samples can be used to optimize the HIV^{RNA/Gag} assay in individual labs and are recommended for users unfamiliar with RNA Flow FISH or any of the steps within. Example staining for RPL13A housekeeping gene mRNA on expanded CD4 T cells, stained with a basic panel such as Panel A from Table 2 is shown in Figure 5A. RPL13A is stained with a Type 1 (AF647) probe here. As shown in the histogram, a majority of the cell population is clearly identified as positive for this mRNA. Example GagPol mRNA staining is shown in Figure 5BC. Here, CD4 T cells from a HIV-uninfected negative control donor (UC, B) or a HIV-infected donor (HIV⁺, C) were activated *in vitro*, then cultured to allow an autologous spreading infection to be established as detailed in Box 2. Samples were stained with Panel B from Table 2. We expect very low background staining to be observed for the UC. For the HIV⁺ sample, when gating on total CD3⁺ CD4 T cells, a 0.5 – 18 % of cells can be expected to be HIV^{RNA+} depending on the donor. When gating on CD3⁺ CD4 dim cells, the frequency is increased – in the example shown around 50% of the cells are HIV^{RNA+}. The mRNA⁺ population is clear, at least one log above background, giving a distinct peak.

Example GagPol mRNA and Gag protein staining of CD4 T cells for an UC, a HIV-infected, untreated, chronic progressing individuals (CP) and two HIV-infected, ART-treated participants (ART-T) is shown in Figure 6. Samples were gated as in Supplementary Figure 1, except that no gating was performed based on CD3 as this marker was downregulated with stimulation. The false positive rate observed in UC is exceptionally low. We observed a total of 3 false positive HIV^{RNA+/Gag+} cells from a total of 30.5×10^6 UC donor CD4 T cells tested; an average of 0.1 false positive HIV^{RNA+/Gag+} events per 10^6 cells. The frequencies detected in HIV-infected primary samples vary considerably, depending on participant

factors including viral load and ART, as well as the stimulation used¹⁵. In samples from CP, we readily detected HIV^{RNA+/Gag+} CD4 T cells in the absence of stimulation (median[range] = 123[1.5–230]/10⁶ CD4 T cells). The size of this population was increased upon stimulation with PMA/ionomycin (median[range] = 311[3.6–768]/10⁶). In contrast, in samples from ART-T individuals in the absence of stimulation, HIV^{RNA+/Gag+} CD4 T cells were rare or absent (median[range] = 0.55[0–2.6]/10⁶). However, following stimulation, the latent translation-competent reservoir could be detected in CD4 T cells from all but one of 14 ART-T donors (median[range] = 3.56[1.52–660]/10⁶)¹⁵.

The dramatic gain in specificity with the HIV^{RNA/Gag} assay as compared to standard flow cytometry-based methods is due to the simultaneous detection of HIV mRNAs and proteins. As illustrated in Figure 7, high background staining is observed in the representative HIV-uninfected control (UC) when analysis is performed using HIV Gag protein or GagPol mRNA staining alone (Figure 7A, B). This background is sufficient to effectively mask the signal from two HIV-infected, untreated CPs, in particular for CP2, who has a low viral load (<10,000 copies/ml). However, dual staining for both Gag protein and GagPol mRNA results in a decrease in background for the UC (0 events detected in 2×10^6 CD4 for this UC) and enables identification of rare HIV^{RNA+/Gag+} CD4 T cells in both CPs at very low frequencies (13 per million for CP2, Figure 7C, D).

A major advantage of the HIV^{RNA/Gag} assay is that it can be used to characterize and phenotype HIV reservoirs on a single cell level. In Figure 8, example flow cytometry plots are shown for samples from an ART-T individual, where isolated CD4 T cells were stimulated with the LRA Bryostatin-1 (10 nM, 18 hr) and stained as in Panel C from Table 2. CD4 T cells were gated first as in Supplementary Figure 1, except that no gating was performed based on CD3 as this marker was downregulated with stimulation. HIV^{RNA+/Gag+} events were then gated as in Figure 8A. These HIV^{RNA+/Gag+} events, which represent the bryostatin-reactivated translation-competent reservoir, can then be analysed for expression of additional phenotypic markers such as CD45RA and CD27, shown in Figure 8B as compared to the total CD4 phenotype.

Samples that have been processed with the HIV^{RNA/Gag} assay can be used in downstream applications, including FACS and microscopy. This technique can be used to identify and sort very rare populations of cells, enabling microscopy analysis of a pure population of interest. Example images from HIV-infected CD4 T cells processed as in Box 5, sorted and analysed by microscopy are shown in Figure 9.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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BOX 1**Adaptation to a 96 well plate format**

The version of the protocol described here and validated extensively in our laboratory uses a 1.5-ml tube version of the assay. However, the approach can be modified to a 96-well plate format. We note that this version of the assay has not been validated to the same extent as the tube-based protocol by our group. The key steps are the same between assays. However, the plate-based assay is adapted to work with lower volumes and with a dry pellet (rather than a 100 µl residual volume). For example, in Step 33, samples should be resuspended at $10\text{--}50 \times 10^6/\text{ml}$, such that plating 200 µl onto each well of a v-bottom plate provides $2\text{--}10 \times 10^6$ cells per well. All wash steps should be performed with 200 µl per well; an additional wash is required to take into account any incomplete first washes (i.e. 100 µl wash onto 100 µl residual volume) and pellets are resuspended in a fresh 100 µl of the appropriate buffer. When using an antibody stain, the dry pellets can either be resuspended in 100 µl of buffer and proceed as though this is the residual volume, or in an antibody mix pre-prepared in 100 µl of the appropriate buffer. Spins are performed at 500g (1000g after fixation) for 4 minutes. Following spins, samples can be decanted by pipetting of the residual liquid or carefully flicking the plate.

When deciding between the tube or plate versions of the assay, there are multiple factors to consider. We have observed that cell loss may be greater, and more variable, when samples are processed in a plate as compared to tubes. Therefore for precious samples, working with tubes may be preferable. There may also be sample transfer between adjacent wells, therefore separation of samples on a plate is important to limit background. We have also observed that autofluorescent/non-specific background in mRNA channels may be greater in plates compared to tubes, which can negatively impact the signal:noise ratio. The major advantages of the plate-based assay are operator ease of use and by extension an increase in the maximum number of samples that can be processed per day. The protocol is significantly shortened by flicking of plates to wash, rather than aspiration of individual tubes. Given the potential advantages and disadvantages, we therefore strongly recommend that the investigator compares the tube and plate protocols in parallel, in their laboratory and for their specific purpose, to determine the version best suited for their study.

BOX 2***Ex vivo* viral propagation for positive control CD4 T cells**

CD4 T cells infected with a lab-adapted virus (including VSVG pseudotyped viruses) using standard methods, e.g. magnetofection³², can be used as a positive control for HIV^{mRNA/Protein} assay. Alternatively CD4 T cells infected with autologous virus following an *ex vivo* expansion, as described below, can be used:

- 1** Isolate CD4 T cells by negative selection as described in Steps 8–19 in the main protocol and resuspend at $2 \times 10^6/\text{ml}$ in R20 (RPMI 1640 with 20% vol/vol FBS).
- 2** Plate CD4 T cells into 24 well plates, 1 ml per well. Use only central wells; fill edge wells with 1 ml RT PBS.
- 3** Rest for 2 hours at 37 °C.
- 4** Stimulate CD4 T cells with PHA-L (10 µg/ml) and IL-2 (100 U/ml) for 36–40 hours.
- 5** Collect activated CD4 T cells by gently pipetting the 1 ml volume up and down in the well to break up activation clumps. Transfer the 1 ml to a 15 conical tube. Repeat for all replicate wells. Wash out each well with 1 ml of warm R10 and combine with the CD4 T cell suspension. Spin down.
- 7** Wash CD4 T cells twice in warm R10 to remove PHA.
- 6** Count cells using the method described in Step 6 of the main protocol.
- 7** Replate at $2 \times 10^6/\text{ml}$ in R20.

Critical step – As cell clumping due to activation may limit counting accuracy, we assume no cell loss over the incubation in Step 4 and replate CD4 T cells in the starting volume from Step 1.

- 8** Maintain cells for 6–7 days at $\sim 2 \times 10^6/\text{ml}$, by splitting 1:2 as required (with a maximum of every other day). Maintain IL-2 at 100 U/ml throughout the culture.

BOX 3**Effective compensation**

As with general flow cytometry experiments, the correct compensation is key to accurate interpretation of the results. For surface and intracellular antibodies, we recommend the use of eBioscience OneComp Beads. All beads are stained at the same time as the intracellular antibody stain to mimic as closely as possible any change in fluorochrome signal or stability over time. As fixation is known to alter fluorochromes, the antibody-stained beads are fixed for 1 hour at RT with a solution of 2% paraformaldehyde, washed with 2% FBS/PBS, then stored at 4 °C until use. There are two options for the compensation of the mRNA stain. The first is to use cells from the experiment stained only with a positive control mRNA which is highly expressed by the cells of interest, such as RPL13A. This has the advantage that the autofluorescence and background is most similar to the cells of interest. However, we have found that the signal from the GagPol mRNA probe set is consistently brighter than that with the positive control, leading to compensation issues. Therefore, we stain BD CompBeads Plus with an isotype control antibody conjugated to the same fluorochrome as the mRNA (AF647 for Type 1, AF488 for Type 4 and AF750 for Type 6). It is key that the same fluorochrome is used for compensation as is used for the mRNA probe type i.e. APC is not an appropriate substitute for AF647. These beads have a larger size than eBioscience OneComp beads and we have found that this provides more accurate compensation.

BOX 4**Antibody selection**

As with general flow cytometry experiments, antibody (both in terms of the clone and fluorochrome) selection is crucial for obtaining optimal results. First, with regard to monoclonal antibody selection, we have observed that some clones do not withstand the PrimeFlow procedure as well as others, resulting in a loss of signal. For example the anti-CD4 clone RPA-T4 is more stable than SK3, but the latter can still be used. We recommend that each user test different clones against antigen of interest before selection. Some fluorochromes are incompatible with the assay, including any PerCP or -Cy5 conjugates and Qdots. Signal from dim fluorochromes (such as V500, or FITC) may be masked by the increase in background associated with the assay. We have found that the BV dyes (BD, BioLegend) generally work well. However, we have observed degradation over time with some (BV650 in particular). Fluorochrome ‘spreading’ into other channels is also increased compared to standard flow cytometry, for example BV605 spreading into PE. These fluorochromes can be used, but with careful antigen selection. We highly recommend the use of FMO (fluorescent minus one) controls to identify any potential issues. We have observed some limited interactions between specific BV dyes when multiple dyes are used in the same mix, as reported by BD Bioscience. This can be overcome by the addition of BD Horizon Brilliant Buffer (Cat # 563794) to the antibody mix. We recommend that each user determine the requirement of this buffer in their antibody panel of interest.

BOX 5**Sorting HIV^{RNA/Gag} assay samples for microscopy**

HIV mRNA and protein can be visualized in CD4 T cells from HIV-infected subjects by microscopy using the HIV^{RNA/Gag} assay. In our case, isolated CD4 T cells infected with autologous virus after an *ex vivo* expansion for 7 days were used as in Box 2. This Box continues from Step 102 of the main procedure. Process samples as described in the complete, detailed protocol. Samples should be stained with a minimal panel such as Panel B in Table 2, except that staining for CD3 and CD4 is not required.

1. Following Steps 1–102 of the main Procedure, store cells overnight in storage buffer if needed.
2. Coat μ -Slide VI 0.4 slides with Poly-L-lysine (0.01 %, sigma), 30 min, RT. Aspirate Poly-L-lysine solution and wash slides with PBS. Dry at RT overnight. Slides can be stored at 4 °C until use.
3. Sort live, CD8[−]CD14[−]CD19[−] cells based on HIV^{mRNA/Protein} expression pattern as desired (e.g. low/high intensity mRNA staining¹⁵). Centrifuge (5 min, 800 g, RT) and resuspend cells in storage buffer. Cells can be stored at 4 °C for short term or frozen at −20 °C for long term storage. In the example shown in Figure 9, cells were sorted using a BD FACS Aria and BD DIVA.
4. Quench with glycine (100 nM, 10 min, RT) to decrease autofluoresence.
5. Wash cells with PBS, centrifuge (800g, 5 min, RT), discard supernatant and resuspend.
6. Stain nucleus with DAPI (1–100 ng/ml, 2 min, RT). The optimal DAPI concentration may vary depending on cell type and microscope set up, therefore we recommend each user titrate their own stock.
7. Wash cells with PBS, centrifuge (5 min, 800g, RT), discard supernatant and resuspend.
8. Plate cells onto Poly-L-lysine coated slides prepared in Step 5.
9. Image cells by confocal microscopy using appropriate filter sets (AF647 for HIV GagPol mRNA Type 1, PE for HIV Gag protein, DAPI for nuclear staining). We recommend using mRNA or Gag protein single stained cells as controls.

Figure 1.

Impact of Poisson distribution on the detection of rare HIV^{RNA+}/Gag⁺ events. Models are based on an actual frequency of 2 HIV^{RNA+}/Gag⁺ events (dashed line) per 10^6 CD4 T cells and demonstrate the Poisson distribution of possible readings when either (a) 1×10^6 , (b) 3×10^6 , or (c) 10×10^6 CD4 T cell events are acquired.

Figure 2.

Part I. Schematic of CD4 T cell isolation and stimulation. PBMCs are thawed and CD4 T cells are negatively isolated by magnetic isolation. Cells are counted, resuspended in RPMI + 10% FCS + 50 U/ml Penicillin/Streptomycin (R10) with the antiretrovirals (ARVs) T20 and AZT, plated onto 24 well plates and rested for 3 hr at 37 °C. Latency reversing agents (LRA) are added to appropriate wells, with some wells left unstimulated (UN) as negative controls, and cells are incubated for a further 12–18 hr at 37 °C.

Figure 3.

Part IIA. CD4 T cells are collected, washed, viability checked and resuspended for the HIV^{RNA}/Gag assay. Cells are stained with a viability stain and surface antibody stain, then fixed and permeabilized. CD4 T cells are then stained intracellularly for HIV Gag and fixed a final time.

Figure 4.

Part IIB. GagPol mRNA is labelled, amplified using a two-step dsDNA amplification system and the amplified probe is labelled with a fluorescent marker. Samples can be stored short-term, or acquired by flow cytometry immediately, and then analyzed.

Figure 5.

Representative staining of mRNA positive controls processed as described in the complete protocol, demonstrating the expected median fluorescent intensities (MFI) and frequencies of mRNA⁺ cells. Frequencies of mRNA⁺ CD4 T cells are indicated above the black histogram gate and shown as a percentage from parent population. Samples were acquired on a modified 5-laser BD LSRII and analyzed using FlowJo Versions 9 and 10 for Mac. **(a)** CD4 T cells labelled for the housekeeping mRNA RPL13A. The majority of cells are positive for the housekeeping gene. **(bc)** CD4 T cells from an uninfected control (UC, b) or a HIV-infected individual (HIV⁺, c) were cultured to establish a spreading infection *in vitro* as described in Box 2 and labelled for GagPol mRNA. Low background staining is shown in (b) for the UC. In (c) staining is shown for total CD3⁺ CD4 T cells, or CD3⁺ CD4^{dim} T cells, demonstrating the increased frequency of HIV mRNA⁺ CD4 T cells in the CD4^{dim} population.

Figure 6.

Example flow cytometry plots from Step 104–110 showing primary CD4 T cell samples processed with the HIV^{RNA}/Gag Assay, demonstrating expected staining patterns and frequencies of HIV^{RNA}+Gag+ cells. Samples were acquired on a modified 5-laser BD LSRII and analyzed using FlowJo Versions 9 and 10 for Mac. Plots are shown for either unstimulated (blue) samples, or samples following stimulation with PMA/ionomycin (PMA/iono, red). A HIV-uninfected negative control donor (UC, 2×10^6 CD4 analyzed per condition) is used for gating as described in Step 110 and illustrates expected low background. Detection of HIV^{RNA}+Gag+ events in HIV-infected, untreated chronic progressor samples (CP, $4\text{--}8 \times 10^5$ CD4 analyzed per condition) define the CD4 T cells infected with translation-competent virus and maintaining an active *in vivo* infection. In HIV-infected, virally-suppressed ART-treated donors (ART-T, $1\text{--}2 \times 10^6$ CD4 analyzed per condition), HIV^{RNA}+Gag+ CD4 T cells detected following stimulation with PMA/iono represent the translation competent latent reservoir. Numbers shown are events per 10^6 CD4 T cells.

Figure 7.

Comparison of single mRNA compared to dual mRNA and protein staining on subject samples. Samples from one uninfected control (UC) and two chronic progressors (CP) were processed as in the complete protocol. Events within gates are shown in color (red/blue/purple) and overlaid onto total events in grey. As shown in (ab) single stains for either Gag protein (a) or GagPol mRNA (b) result in a high background in the UC, which prevent accurate detection of low frequency HIV^{Gag+} or HIV^{RNA+} events in CP samples. In comparison, dual staining for Gag protein and GagPol mRNA staining as shown in (c) enables identification of low frequencies of HIV^{RNA+/Gag+} cells. This is summarized in (d)

where populations and gates from (a–c) are overlaid. Viral loads (VL) are indicated as vRNA copies/ml. Numbers indicate positive events per million CD4 T cells. All gates were drawn based on the UC GagPol mRNA⁺ Gag Protein⁺ gate in (c). In all cases, 2×10^6 CD4 T cells were analyzed by flow cytometry. Samples were acquired on a modified 5-laser BD LSRII and analyzed using FlowJo Versions 9 and 10 for Mac.

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Figure 8.

Example HIV^{RNA/Gag} Assay staining and concurrent phenotyping of the translation-competent latent viral reservoir following LRA-induced reactivation for a virally suppressed, ART-treated individual. Such staining can be used to identify the phenotype of the CD4 T cells able to respond to LRA stimulation by production of HIV mRNAs and protein.

Samples were processed as in the complete protocol, using Panel C from Table 2. **(a)**

Example HIV^{RNA/Gag} Assay staining demonstrating detection of the translation-competent latent reservoir. **(b)** Example flow cytometry plot demonstrating the use of markers CD45RA and CD27 to phenotype the translation-competent latent viral reservoir.

CD45RA⁻CD27⁻ cells are classified as effector memory; CD45RA⁻CD27⁺ as central/transitional memory; CD45RA⁺CD27⁺ as naïve and CD45RA⁺CD27⁻ as terminally differentiated. HIV^{RNA+/Gag+} events in purple are overlaid onto total CD4 events in grey. 6×10^5 CD4 T cells were analyzed by flow cytometry. Samples were acquired on a modified 5-laser BD LSRII and analyzed using FlowJo Versions 9 and 10 for Mac.

Figure 9.

The HIV^{mRNA}/Gag assay enables the microscopy analysis of rare populations of HIV-infected CD4 T cells, by sorting the rare populations of interest prior to microscopy. In the two examples shown here, CD4 T cells were processed with the full HIV^{mRNA}/Gag assay protocol from Steps 1–102, then the rare HIV^{mRNA}/Gag⁺ CD4 T cells were sorted and imaged as described in Box 5. Two populations were sorted; CD4 T cells positive for HIV GagPol mRNA only (HIV^{mRNA}/Gag⁻, top panel) and CD4 T cells positive for both HIV GagPol mRNA and Gag protein (HIV^{mRNA}/Gag⁺, lower panel). Images are shown first as single channels (DAPI in blue, GagPol mRNA in green, Gag Protein in red). Overlay analysis can be used to determine the cellular localization and co-localization of GagPol mRNAs and Gag protein. For example, in the HIV^{mRNA}/Gag⁻ population, GagPol mRNA expression is restricted to the nucleus. In comparison, in the HIV^{mRNA}/Gag⁺ population, GagPol mRNA is found in both the nucleus and the cytoplasm, with Gag protein found only in the cytoplasm. This suggests that the HIV^{mRNA}/Gag⁻ population may represent an early stage in viral life cycle, or infection with defective viruses, while the HIV^{mRNA}/Gag⁺ population represents productively infected CD4 T cells. Scale bar represents 10 μ m.

TABLE 1

Example reagent preparation.

Reagent	Preparation	For 1 tube	For 12 tubes*
Viability stain	1:500 in cold PBS (2 × stock)	100µl (99.8µl PBS + 0.2µl Viability stain)	1300µl (1297.4µl PBS + 2.6µl Viability stain)
Surface stain Use immediately	Anti-CD3 BV605, anti-CD4 PE-Cy7, anti-CD8, CD14 and CD19 BV510.	19µl (Anti-CD3 BV605 (5µl), anti-CD4 PE-Cy7 (5µl) anti-CD8, CD14 and CD19 BV510 (all 3µl))	247µl (Anti-CD3 BV605 (65µl), anti-CD4 PE-Cy7 (65µl) anti-CD8, CD14 and CD19 BV510 (all 39µl))
Fixation I Use immediately	1 part Fixation Buffer 1A for 1 part Fixation buffer 1B	1 ml (500 µl Fixation Buffer 1A + 500 µl 1B)	13 ml (6.5 ml Fixation Buffer 1A + 6.5 ml Fixation Buffer 1B)
Permeabilization Buffer Use immediately	1:10 Permeabilization Buffer, 1:1000 RNasin I, 1:100 RNasin II in H ₂ O	3 ml (300 µl Permeabilization buffer, 3 µl RNasin I, 30 µl RNasin II + 2.67 ml H ₂ O)	39 ml (3.9 ml Permeabilization buffer, 39 µl RNasin I, 390 µl RNasin II + 34.71 ml H ₂ O)
Fixation II Use immediately	1:8 Fixation Buffer 2 in Wash Buffer	1 ml (125 µl Fixation Buffer 2 + 875 µl Wash Buffer)	13 ml (1.625 ml Fixation Buffer 2 + 11.375 ml Wash Buffer)
GagPol probes Warm to 40 °C	1:20 Target mRNA probes in Target Probe Diluent	100 µl (5 µl Gag probe + 5µl Pol probe + 90 µl Target Probe Diluent.	1.3 ml (65 µl Gag probe + 65µl Pol probe + 1.170 ml Target Probe Diluent.
Overnight storage buffer	1:1000 RNasin I in Wash Buffer	1 ml (1 µl RNasin I + 999 µl wash buffer)	13 ml (13 µl RNasin I + 12.987 ml wash buffer)
Label probes Warm to 40 °C	1:100 Label Probes in Label Probe Diluent	100 µl (1 µl + 99 µl)	1.3 ml (13 µl + 1.287 ml)

* Sufficient reagent for one spare test is prepared to take into account pipetting error.

TABLE 2

Panel options for 1, 3/4, or 5 laser flow cytometers. Please see Reagents for recommended clone and purchasing information.

Fluorochrome	Panel A (1 laser)	Panel B (3/4 lasers)	Panel C (5 lasers)
	<i>Basic detection of HIV mRNA and protein – recommended for cell lines.</i>	<i>Minimum panel for CD4 T cell HIV translation-competent reservoir detection</i>	<i>Identification of memory phenotype of HIV^{RNA+/Gag+} CD4</i>
BUV395			CD3
BUV496			CD4
AF488	GagPol mRNA (Type 4)		CXCR5 *
PE	Gag Protein	Gag Protein	Gag Protein
PE-Cy7		CD4	ICOS
BV421			PD-1
BV510/eF506		Exclusion	Exclusion + Viability stain
BV605		CD3	CD27
BV711			CD45RA
AF647		GagPol mRNA (Type 1)	GagPol mRNA (Type 1)
eF780		Viability stain	

* Stained during stimulation.

TABLE 3

Guidelines for preparation of Target Probes, Amplification buffers and Label Probes.

Number of Samples/Tubes	Number of extra Tests prepared
1	+ 0.2
2 – 3	+ 0.5
4–10	+ 1
10–20	+ 2

Table 4

Troubleshooting

PROBLEM	STEP(S)	POSSIBLE REASON	SOLUTION
Poor cell recovery or high debris	6, 17, 31, 103 + 104–110	Low viability of starting cells	Improve cell culture and preparation.
		Incorrect spin speeds	Check spin speeds (note increased speed after fixation) in all centrifugations, particularly Step 51 onwards.
		Low starting cell number	Increase cell starting number.
		Harsh cell treatment	Limit vortexing of samples and handle carefully throughout the protocol, see Step 35 for correct procedure.
Poor mRNA staining	103 + 104–110	Oven temperature incorrect in Steps 76, 78, 85, 91 & 98.	Recalibrate oven.
		Oven temperature unstable in Steps 76, 78, 85, 91 & 98.	Carefully monitor oven temperature throughout protocol
		Incorrect amount of mRNA Target Probes used in Step 68.	Use Target Probes at 1:20
		Incorrect amount of Label Probes used in Step 96.	Use Label Probes at 1:100
		High residual volume in tubes, following washes in Steps 73, 81, 89 & 95.	Maintain residual volume at 100 µl.
		mRNA degradation in Steps 1–64. See Step 27 for example.	Ensure cells are in the growth phase before use. Work at 4 °C before fixation.
		Poor cell permeabilization and/or fixation in Steps 48, 53 & 65.	Check Reagent Setup (see Table 1). Use buffers prepared on the same day. Check incubation times and temperatures.
		Poor washing in Steps 71–73, 79–81, 87–89, 93–95, 99–101	Ensure all wash steps are followed, using the appropriate buffer at the required temperature.
High background in mRNA channel/high MFI of mRNA negative population	103 + 104–110	Low viability/number of starting cells in Steps 1–24 and 36–40. Check viability in Step 31.	Improve cell culture and preparation. Use Viability stain to exclude dead cells.
		Incorrect amount of mRNA Target Probes used in Step 68.	Use Target Probes at 1:20
		Incorrect amount of Target Probes used in Step 96.	Use at 1:100
		Unclean flow machine/samples run too fast.	Clean the flow cytometer well before use. Run samples at ~2,000 events/second.
		Low residual volume following washes in Steps 73, 81, 89 & 95.	Residual volume should be 100 µl.
		Incorrect fixation time in Steps 50 + 76.	Check fixation time – longer fixes can increase background.
		Poor washing in Steps 71–73, 79–81, 87–89, 93–95, 99–101.	Ensure all wash steps are followed, using the appropriate buffer at the required temperature.
No HIV^{RNA}/Gag⁺ cells detected	103 + 104–110	Low starting cell number in Step 23.	See “Limitations” for discussion of starting cell number.
		Poor mRNA staining.	Run a positive control sample to rule out assay issues.

PROBLEM	STEP(S)	POSSIBLE REASON	SOLUTION
Poor antibody staining	103 + 104–110	Clone unstable during protocol.	Test new antibody clones. See Box 4 for further information.
		Fluorochrome unstable during protocol.	Test additional fluorochromes. See Box 4 for further information.
		Incorrect incubation times/temperatures in Steps 43 & 61.	For anti-Gag KC57, stain for 1 hr (30 min at RT, 30 min at 4 °C)
		Non-optimal antibody concentration in Step 43.	Titrate all antibodies before use.
		Incorrect compensation.	See Box 4 for details
		Interactions between BV, BUV and BB polymer dyes.	Prepare antibody mixes with BD Horizon Brilliant Stain Buffer (Cat #563794) and compare to standard mix to determine if this buffer is required.