Flow cytometry assay for simultaneous detection of HIV RNA and Gag protein

Single-cell characterization of viral translation-competent reservoirs in HIV-infected individuals.

Baxter AE, Niessl J, Fromentin R et al. (2016) *Cell Host Microbe* 20:368–380.

Dramatic advances in antiretroviral therapy (ART) have enabled the currently 71 million HIV-infected individuals worldwide to lead relatively normal lives, transforming HIV infection into a chronic but manageable disease. ART, however, mandates lifelong treatment, with interruption leading to immediate viral rebound and disease progression. Recent years have seen a push for HIV curative initiatives to relieve infected individuals of the multifaceted burdens of prolonged therapy. These strategies have been hampered by the fact that HIV establishes latent reservoirs in predominantly transcriptionally silent T cells that are impervious to currently available ART.

Studies suggest that a feasible approach to achieving a functional cure for HIV might entail a "shock and kill" tactic: using latency-reversing agents (LRA) to reactivate HIV reservoirs while simultaneously improving the host immune system to kill cells with reactivated virus [1]. A major hurdle in this approach has been establishing robust, high-throughput assays to identify HIV reservoirs or cells harboring viral RNA following LRA administration. The current gold standard for identifying HIV reservoirs capable of producing infectious virions is the quantitative viral outgrowth assay (QVOA) [2]. Unfortunately, QVOA is a time-consuming, labor-intensive, and costly method, impeding its widespread adaptation. Furthermore, the QVOA underestimates HIV reservoirs and, most importantly, offers no pertinent immunophenotypic information about the cells with reactivated HIV. Other methods include PCR-based assays such as droplet digital PCR (ddPCR) [3] and the Tat/rev-induced limiting dilution assay (TILDA) [4].

Multiple studies have identified HIV RNA as a potential biomarker of HIV reservoirs and an indicator of latency reversal during the "shock and kill" assays. A step toward identifying and optimizing HIV curative measures would be provided by a single-cell assay permitting robust HIV RNA detection and multiparametric characterization of infected cells expressing reactivated HIV RNA.

Baxter and colleagues have recently reported their use of the Invitrogen™ PrimeFlow™ RNA Assay (Figure 1) to detect viral translation–competent reservoirs in HIV-infected individuals [5]. The PrimeFlow RNA assay is a flow cytometry–based *in situ* hybridization assay that combines sensitive branched DNA (bDNA) amplification with single-cell resolution. Moreover, the PrimeFlow RNA assay is compatible with simultaneous immunophenotyping for cell-surface and intracellular proteins.

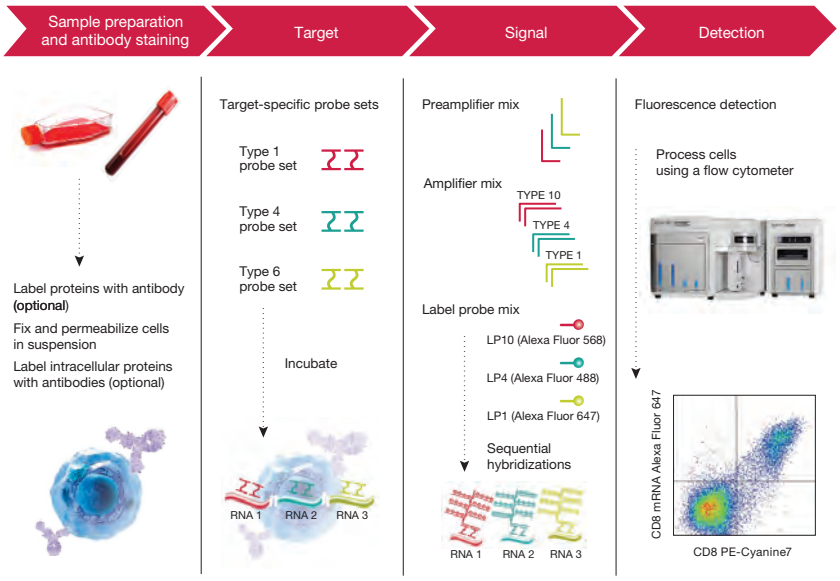

Figure 1. The PrimeFlow RNA Assay workflow. The workflow for the Invitrogen™ PrimeFlow™ RNA Assay Kit (Cat. No. 88-18005-204) starts with optional antibody labeling of cell-surface proteins followed by fixation, permeabilization, and optional antibody labeling of intracellular proteins. Next, the fixed and permeabilized cells are hybridized with RNA-specific target probes; up to 4 different RNA targets can be detected in a single experiment. This hybridization is then detected after branched DNA (bDNA) signal amplification using preamplifiers, amplifiers, and label probes, which comprise oligonucleotides conjugated to highly fluorescent Alexa Fluor™ 488, Alexa Fluor™ 568, Alexa Fluor™ 647, or Alexa Fluor™ 750 dyes. Labeled cells are analyzed on a standard flow cytometer.

Simultaneous HIV RNA and protein detection in T cells

In their recent publication, Baxter et al. describe the detection of CD4 T cells expressing HIV RNA (using probe sets against both the *GAG* and *POL* genes) and Gag protein (using anti-Gag antibody). They report that, after culturing CD4 T cells from untreated HIV-infected individuals (UNT) *in vitro* for 7–10 days, they could readily detect CD4 T cells positive for both HIV RNA and protein ($HIV^{RNA+/Gag+}$); after addition of antiretrovirals to this T cell culture (UNT + ARVs), $HIV^{RNA+/Gag+}$ T cells were undetectable.

They also examined CD4 T cells from untreated HIV-infected individuals (UNT) and ART-treated HIV-infected individuals (Tx) after stimulation with LRAs *in vitro*. After reservoir reactivation using PMA/ionomycin or a protein kinase C (PKC) agonist such as bryostatin and ingenol, translation-competent virus could be detected using the PrimeFlow RNA assay. In addition, the estimated reservoir size in the $HIV^{RNA+/Gag+}$ T cells correlated well with the size of reservoirs measured by orthogonal assays.

The PrimeFlow RNA assay is compatible with immunophenotyping, allowing these researchers to further characterize the T cells expressing reactivated virus. The central memory T cell (T_{CM}) subset had previously been identified as the predominant harbor of HIV reservoirs in infected individuals on ART. Using the PrimeFlow RNA assay, Baxter et al. confirmed that the T_{CM} subset in untreated infected individuals also harbored high levels of HIV, and that $HIV^{RNA+/Gag+}$ cells expressed the co-inhibitory markers PD-1, CTLA4, and TIGIT, contributing to T cell exhaustion, a hallmark of HIV infections.

Conclusions and potential for future HIV research

Baxter et al. showed that the PrimeFlow RNA assay could be used to assess the size of HIV reservoirs, to determine the efficacy of LRAs, and to establish the phenotype of cells expressing reactivated virus. Their conclusions complement those of other studies using the PrimeFlow RNA assay to study the kinetics of HIV transcription and translation [6] and to characterize subpopulations of CD4 T cells that transcribe HIV RNA [7]. ■

References

1. Deeks SG (2012) *Nature* 487:439–440.
2. Laird GM, Eisele EE, Rabi SA et al. (2013) *PLoS Pathog* 9:e1003398.
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6. Martrus G, Niehrs A, Cornelis R et al. (2016) *J Virol* 90:9018–9028.
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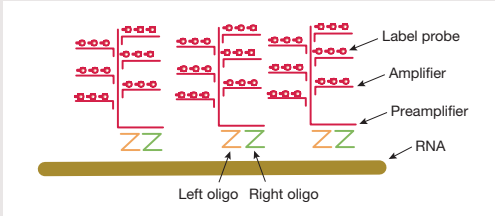
More about the PrimeFlow RNA assay

With the Invitrogen™ PrimeFlow™ RNA Assay Kit, researchers can reveal the dynamics of RNA transcription together with protein expression patterns at the single-cell level by multicolor flow cytometry. The PrimeFlow RNA assay employs fluorescence *in situ* hybridization (FISH) with branched DNA (bDNA) signal amplification for the simultaneous detection of up to 4 RNA targets, and it can be used in combination with immunolabeling of both cell-surface and intracellular proteins using fluorophore-conjugated antibodies.

In the PrimeFlow RNA assay workflow, cells are first labeled with cell-surface antibodies, fixed and permeabilized, and then labeled with intracellular antibodies. Next, these cells are hybridized with oligonucleotide probes specific for the RNA targets. Hybridized targets are detected after bDNA amplification, which is achieved through sequential hybridization steps with preamplifiers, amplifiers, and fluorophore-conjugated label probes. A fully assembled amplification "tree" has 400 label probe–binding sites, and can produce >8,000-fold signal amplification.

With target-specific probe sets, the PrimeFlow RNA assay can be used to detect miRNA, lncRNA, and mRNA, as well as vRNA and telomere DNA. The PrimeFlow RNA Assay Kit provides reagents for detecting up to 4 RNA transcripts in mammalian cells optionally labeled with antibodies that recognize cell-surface or intracellular proteins. For more information, including catalog probe sets and ordering guidelines, visit **thermofisher.com/primeflowbp77**.



Branched DNA (bDNA) amplification scheme used in the PrimeFlow RNA assay.

Product	Quantity	Cat. No.
PrimeFlow™ RNA Assay Kit	40 tests	88-18005-204
	100 tests	88-18005-210