Intact Proviral DNA Assay (IPDA)

Introduction

The HIV/SIV field is moving closer to achieving “functional cures” and continues to devise new approaches for targeting the latent reservoir of virus. A sensitive and reliable method for quantifying the latent reservoir is critical for evaluating the effectiveness of these strategies.

Many approaches have been used for this purpose, ranging from PCR-based assays that likely overestimate the reservoir size, to cellular based assays, such as the “gold-standard” quantitative viral outgrowth assay (QVOA) which quantifies replication-competent proviruses in a sample (Finzi et al. 1997; Siliciano and Siliciano 2005; Laird et al. 2013; Eriksson et al. 2013) but may underestimate reservoir size by failing to induce all potentially inducible virus in a given assay (Ho et al. 2013; Hosmane et al. 2017). In 2019, Bruner et al. (Bruner et al. 2019), described the intact proviral DNA assay (IPDA), a sensitive and robust method for quantifying cells harboring full-length HIV proviruses. This assay does not require reactivation of virus for quantification; instead the IPDA specifically targets complete genomes without APOBEC hypermutation. Bender et al. (Bender et al. 2019) developed an analogous assay to evaluate SIV genomes present in cells from infected macaques. The IPDA not only offers many benefits of PCR-based methods (sensitive, cost-effective, requires minimal number of input cells) but also provides additional information (beyond the presence of viral sequence) about the potential for the virus to reactivate. While no reservoir assay is perfect, the IPDA provides a reasonable approximation of HIV/SIV reservoir size, requiring a minimal number of cells, and at a reasonable cost.

The Virology Services (VS) Unit of the Wisconsin National Primate Research Center (WNPRC) adapted the IPDA to offer to clients on a fee-for-service basis. While the assay offered by VS is based on that developed by Bender et al. (Bender et al. 2019), it has been modified for optimal implementation in our laboratory. A key difference is that the assay was originally developed on the BioRad QX-200 droplet digital (ddPCR) platform, but we have adapted the assay for use on ThermoFisher’s Absolute Q digital PCR (dPCR) system. dPCR offers several advantages over older ddPCR approaches. For example, while both approaches separate samples into thousands of partitions, allowing for interrogation of a single molecule of DNA per partition, dPCR is much more reliable and reproducible than ddPCR. ddPCR generates droplets in a water-in-oil emulsion to sequester DNA molecules and reagents, whereas dPCR physically separates the sample and reagents into nanowells on a microtiter plate. The droplets generated in ddPCR are fragile and susceptible to shearing, often leading to suboptimal numbers of droplets. The physical separation in dPCR on the other hand has proven extremely robust and reproducible, reliably filling over 20,000 (usually over 20,400) partitions. Additionally, because the Absolute Q instrument can detect up to 4 targets with

different color fluorophores in a single reaction, we have been able to further multiplex the reactions in the assay for more efficient use of sample.

Principle

The first step of the IPDA is to isolate cellular DNA from the samples of interest. We then test extracted DNA in a series of PCR reactions targeting both a host gene as well as specific regions in the SIV genome. Two targets in the SIV genome, one in Pol and one in Env, are used (Figure 1) and were carefully selected to discriminate between intact provirus and virus that is likely to contain deletions (Bender et al., 2019). The presence of both the Pol and Env targets on a single molecule of DNA is strong indication that the genome remains intact, without deletions. When only the Pol or Env target is detected, this indicates that a viral genome is present but contains one or more deletions and is, therefore, unlikely to reactivate. Bender et al., confirmed by full genome sequencing that 90% of the DNA molecules found to be positive for these two amplicons were indeed intact.

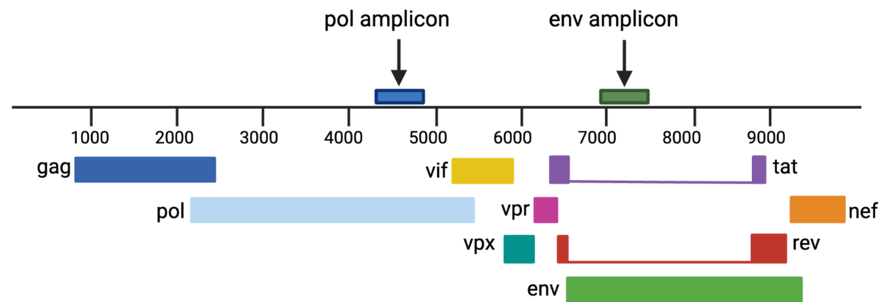

Figure 1: The IPDA targets two amplicons in the SIV genome; one in Pol and one in Env. The presence of both targets on a given provirus is a strong indication that the provirus lacks large internal deletions.

While internal deletions in the viral genome are the most common mutation rendering SIV genomes defective, they are not the only type of mutation that makes proviruses replication incompetent (Mudd 2024). APOBEC3G hypermutation is another significant source of defective SIV genomes (Bender et al. 2019). In fact, SIV infections have a higher degree of APOBEC3G-mediated hypermutation than HIV infections, with certain positions displaying >80% G to A mutations (Bender et al. 2019). The IPDA, therefore, seeks to account for APOBEC3G A-to-G hypermutation when enumerating intact SIV genomes. This is accomplished using an unlabeled competitor probe that binds the hypermutated sequence within the Pol and Env amplicons (Figure 2). This hypermutated competitor probe will outcompete the labeled probe that targets the wild-type sequence in the presence of hypermutated virus. Because the hypermutated probe is unlabeled, it will not contribute to the signal detected in dPCR, effectively "sequestering" hypermutated genomes that are unlikely to encode replication-competent proviruses thus preventing them from being erroneously counted

in the assay. It is important to note that while APOBEC3G hypermutation is accounted for when enumerating intact provirus, it is not possible to count the number of proviruses containing hypermutated sequence.

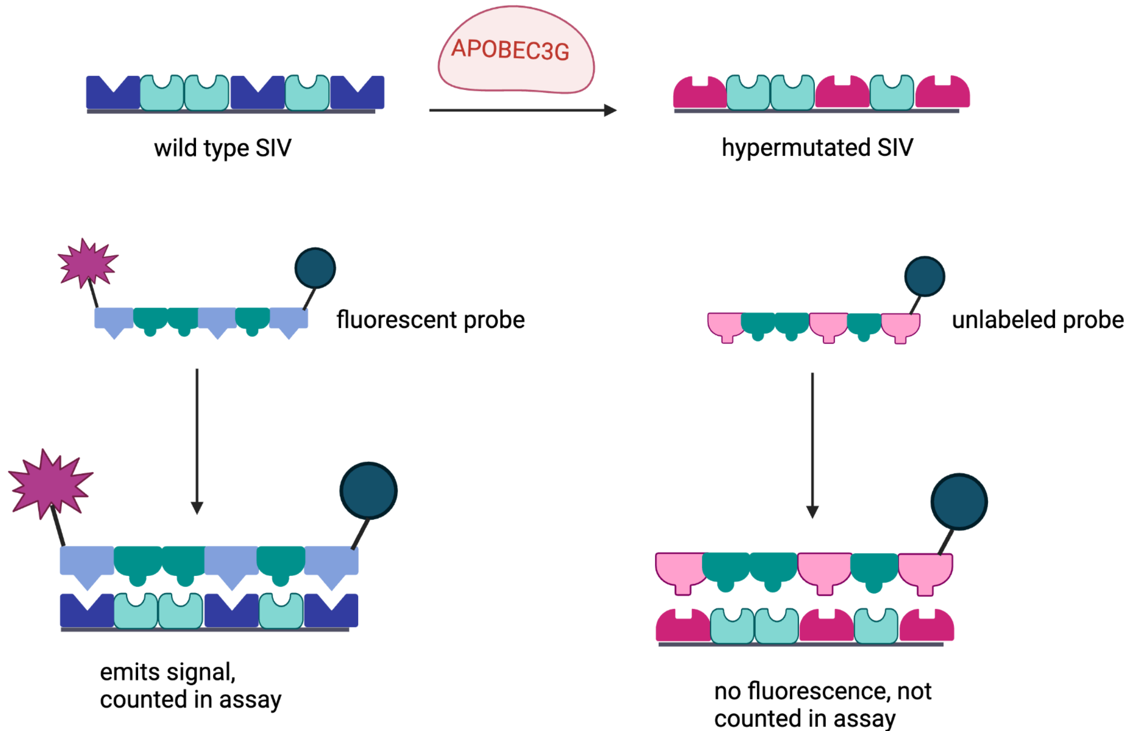

Figure 2: The IPDA accounts for APOBEC mediated hypermutation by including an unlabeled competitor probe in the reaction. The fluorescent probe compliments wild type SIV sequence while the unlabeled probe matches the hypermutated SIV sequence. When provirus contains hypermutations it will preferentially bind the unlabeled probe (thus preventing it from binding the labeled probe) and not emit a signal, and therefore not be counted in the assay.

We normalize SIV genomes to cellular genomes so that results can be reported as SIV genomes per million cells. Cellular genomes are enumerated using 2 assays targeting the RPP30 gene in the macaque genome. Not only are these targets used to measure cell equivalents, but they are also used to determine the DNA shearing index (DSI). The DSI is used to account for random shearing of DNA during the isolation process and PCR that could separate the two SIV targets in an otherwise intact provirus (Figure 3). The two targets in the RPP30 gene are the same distance apart as the SIV Pol and Env targets (both ~2 kb), therefore shearing between those amplicons serves as a proxy for shearing between the SIV targets.

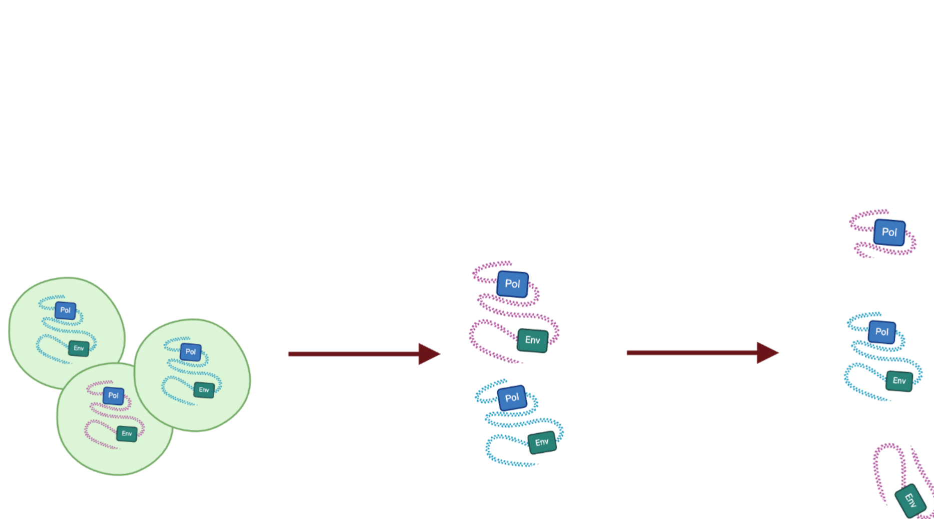

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Figure 3: DNA is isolated from cells for the IPDA. During the process of both isolation and PCR amplification DNA can be sheared, potentially separating the Pol and Env amplicons that may have otherwise been present on a single SIV provirus. This is accounted for in the IPDA by calculating the DNA shearing index (DSI).

Sample requirements

- Minimum 2-5 million cells (PBMC, specific cell population etc.)

OR

- Minimum 6 ml EDTA blood (*note that there is an additional charge for isolating PBMC from whole blood)

Results

The Absolute Q instrument generates scatter plots showing fluorescence of positive partitions for each target, as shown in Figure 4 (single channel dPCR results). Typically these reactions show a clear distinction between positive and negative partitions for all reactions used in the IPDA assay.

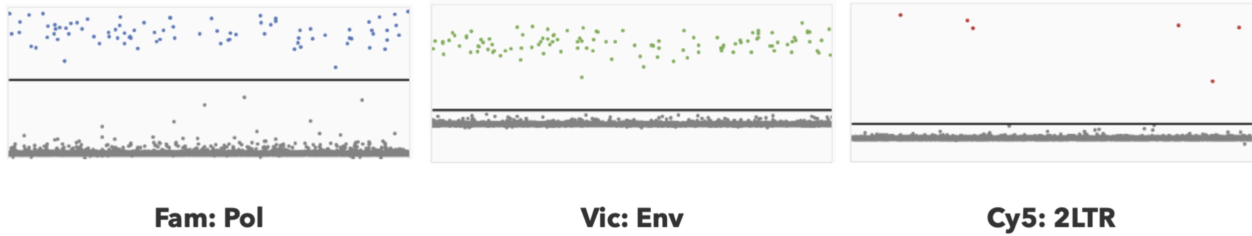

Figure 4: Raw data from the Absolute Q dPCR instrument is visualized as single channel dot plots. Thresholds for each channel have been set based on the negative control sample in that experiment.

After setting thresholds for calling positive partitions (based on negative control samples), we enumerate partitions that are positive for 1 or both SIV targets. This is also visualized using 2D dot plots comparing fluorescence in 2 channels for each partition (Figure 5).

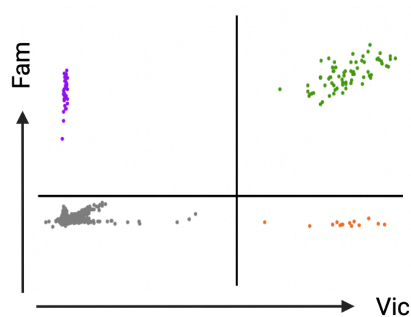

Figure 5: Individual partitions are assessed for fluorescence in each channel. This is illustrated in 2D plots comparing multiple channels. Partitions containing molecules of DNA with both the Pol (Fam) and Env (Vic) targets are found in the upper right quadrant.

IPDA Report

We will return a report detailing relevant sample information, QC data and calculated results (including the intact provirus per million cells, DSI, % intact provirus, number of 2 LTR circles etc.) This is illustrated in the sample report below:

IPDA assay: Sample info

Sample ID	Sample date	Sample notes	Sample type	DNA concentration (ng/ul)	A_{260}/A_{280}
Animal 1	2024-07-31	SIV VL = undetectable	PBMC	87.5	1.97
Negative CTL	2021-03-26	SIV naive control	PBMC	55.7	1.93
Positive CTL	2024-04-05	Viremic animal SIV VL = 2E+07 copies/ml	PBMC	105.45	1.90

IPDA assay: Results

Sample ID	Cell eq/ml	Partitions interrogated* (replicates tested)	DSI	Intact provirus/ million cells**	2 LTR circles/ million cells	Total SIV DNA/ million cells	Pol+,Env- / million cells	Pol-,Env+ / million cells	SIV DNA composition
Animal 1	2.60E+06	101666 (5)	0.292291774	49.55	0	61.36	1.53	10.29	Intact SIV: 80.7% Env+, Pol deletion: 2.5% Env deletion, Pol+: 16.8%
Negative CTL	1.43E+06	20423 (1)	0.2125	0	0	0			
Positive CTL	3.68E+06	20428 (1)	0.172236504	1.28E+04 (Avg = 1.16E+04)	2.07E+03	1.69E+04	613.65	1416.10	Intact SIV: 88% Env+, Pol deletion: 8.4% Env deletion, Pol+: 3.6%

*Env/Pol/2LTR reaction

**2 LTR circles subtracted

References:

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