Quantitative SIV Viral Outgrowth Assay

Introduction

The Virology Services Unit of the Wisconsin National Primate Research Center (WNPRC) is dedicated to supporting virological research in non-human primates (NHP). HIV/AIDS is a main focus of NHP virology research at WNPRC. We offer a range of services to support NHP AIDS research, including molecular diagnostics for detecting and quantifying SIV/SHIV DNA and RNA and production of custom SIV stocks. Our standard SIV plasma viral load assay can detect as few as 100 copies vRNA/ml plasma. However, with the promise and increasing emphasis on cure research, there is new interest in methods for quantifying the SIV latent reservoir in infected animals to assess the outcomes of various interventions.

Multiple "reservoir assays" exist, but none is perfect. In fact, different approaches can give very different estimates of "reservoir size" when testing the same sample [1-3]. Reservoir assays can be broken down into 2 main types: PCR-based assays and cell-culture-based assays. Each assay has distinct advantages and disadvantages so it is important to consider the aims of the study when choosing which assay(s) to use for quantifying the reservoir (**Figure 1**).

PCR-based assays are relatively quick to perform and very sensitive, but provide no information about the ability of the virus they measure to replicate and infect new cells. A substantial proportion of integrated proviruses have internal deletions or other mutations that render them incapable of producing infectious progeny upon reactivation [4]. Therefore, PCR-based assays that only detect viral nucleic acids tend to overestimate the size of the reservoir.

Cell-culture-based assays are more cumbersome to perform and take longer to return results than PCR-based assays. Since cell culture assays require infection of new cells (and typically, several rounds of infection) to amplify virus, they only detect replication-competent virus. This may give a more realistic measure of the "functional" reservoir, i.e., the component of the latent provirus population that is actually capable producing infectious progeny. However, it is now known that cell culture assays tend to underestimate the size of the reservoir because not all replication-competent proviruses may be activated in a given assay [4].

The distinction between replication-competent and incompetent viruses is a biologically important

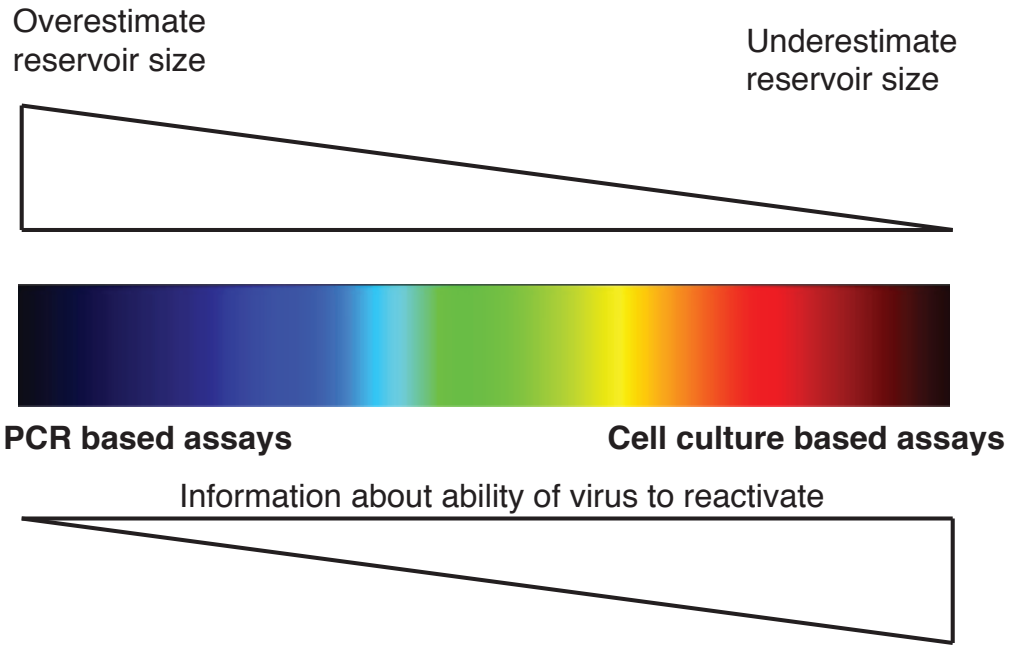

Figure 1: HIV/SIV reservoir assays exist in a "spectrum". On one end you have the PCR based assays which are quick to perform and sensitive, but tell you nothing about the ability of the virus to replicate. On the other end of the spectrum are culture based assays. Since these assays require infection of new cells to amplify virus, they only detect replication competent virus. However not all potentially inducible virus will be activated in a given assay. As a result the culture based assays underestimate the viral reservoir. PCR based assays on the other hand dramatically overestimate the reservoir size. This is because they measure all virus present, not just that which is capable of replicating and infecting new cells. A large proportion of provirus contains mutations, deletions, or for some other reason is not capable of completing its life cycle.

one: because not all proviruses are replication-competent, it may not be necessary to eliminate all detectable HIV DNA from an individual in order to achieve a "functional cure." Instead, a more relevant and achievable goal may be to reduce or eliminate replication-competent viruses from the latent reservoir. For this reason, assays that specifically detect functional proviruses may provide the best assessment of the impact of a given intervention on the reservoir.

Assays that detect functional proviruses typically involve stimulating PBMC or purified cell subsets with a mitogen to reverse viral latency and then culturing these cells with susceptible target cells. Activated proviruses release infectious progeny, which, after several replication cycles, become detectable by confirmatory QRT-PCR or antigen capture assays. Virology Services provides a quantitative viral outgrowth assay (QVOA), which uses techniques initially developed by Robert Siliciano and colleagues [5-7]. We consulted with Dr. Siliciano and Dr. Joel Blankson in establishing our technique.

Samples

The QVOA can be performed on a variety of cell types. Most commonly the assay is set up using CD4 T cells isolated from PBMC. Lymph node cells have also been used successfully in QVOA assays. Regardless of sample type, CD4 T cells are selected by depletion of non-CD4+ T cells, leaving the population of interest untouched.

A minimum of 16 ml EDTA blood or 30 million PBMC from either rhesus or cynomolgus macaques are required to perform the QVOA.

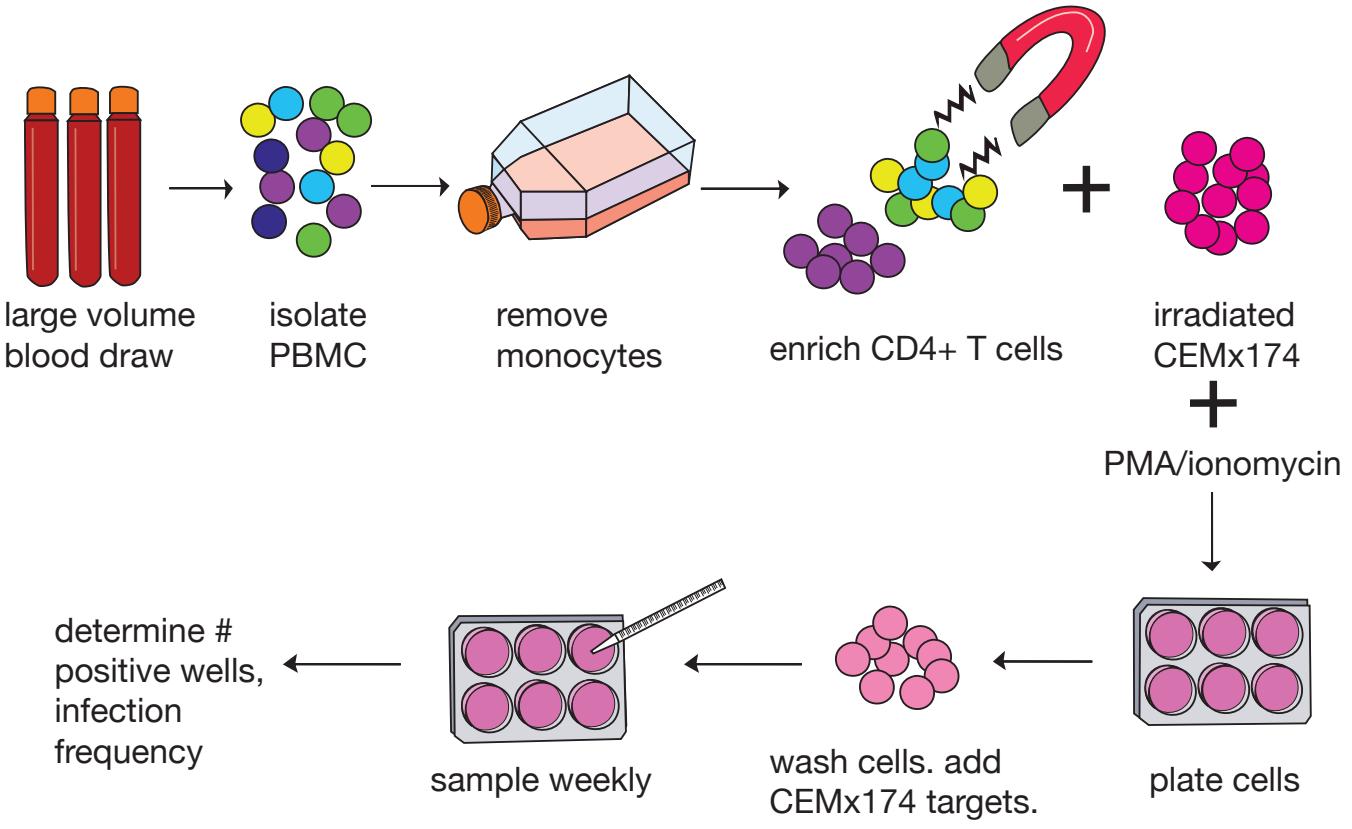

Figure 2: This schematic illustrates the protocol for performing the QVOA. Briefly, PBMC are isolated from whole blood, then enriched for CD4+ T cells. These CD4+ T cells are stimulated with irradiated CEMx174 cells and PMA/Ionomycin overnight. The next day cells are washed to remove the mitogen and then plated in the presence of fresh CEMx174 cells which serve as target cells for stimulated virus to infect. After 1-2 weeks cultures are sampled and tested by QRT-PCR for the presence of virus.

Assay description

To increase the probability of finding cells harboring latent provirus, samples are enriched for CD4 T cells. This is done by indirectly labeling non-CD4+ T cells using a biotin conjugated antibody cocktail and anti-biotin microbeads. Labeled cells are then removed by magnetic separation, leaving a highly pure population of untouched CD4+ cells. CD4 T cells are stimulated overnight by culturing with both irradiated CEMx174 cells and PMA/ionomycin. Following stimulation cells are plated and cultured for 1-2 weeks, at which point the supernatant is collected and tested for SIV RNA using our sensitive QRT-PCR assay. The process of performing a QVOA assay is illustrated in **Figure 2**.

There are 2 varieties of QVOA assays to fit different sample types. For samples that are likely to contain substantial proportions of latently infected cells (1 in 10,000 cells or more; e.g. from viremic animals) we will plate and test limiting dilutions of cells (in duplicate). Samples that are suspected to have very few latently infected (e.g., from elite controllers/elite suppressors, or animals on suppressive ARV therapy), cells will be plated in as many replicate wells as possible containing 1-2 million cells per well, which increases the likelihood of detecting rare replicating viruses.

QVOA results

Data from QVOA assays are reported in infectious units per million cells (IUPM). This concentration is determined using a maximum likelihood approach, as described [8]. We use an online calculator developed by Dr. Siliciano's group for this purpose (http://silicianolab.johnshopkins.edu/). The calculator provides a maximum likelihood estimate based on the number of cells plated, number of wells, and a dilution factor if applicable.

A sample QVOA report provided by Virology Services is shown in **Table 1**. In this example an elite controller (EC) was tested along with an animal on combination antiretroviral therapy (cART). Both animals had plasma viral loads below the limit of detection of our plasma viral load assay. As has been observed previously [9], the elite controller maintains a reservoir that is about 1 log smaller than that of the animal on antiretroviral therapy.

animal	assay type	cells per well	number of replicates	positive wells	IUPM
EC	replicate wells	2E+06	8	2/8	0.143841
cART	replicate wells	1E+06	3	2/3	1.098612

Table 1. Representative QVOA assay data report.

The QVOA report will detail the type of assay performed (dilution series or replicate wells), the number of cells plated per well, number of wells tested and the number of wells that produced virus. From this we calculate and report the IUPM.

QVOA cost

Typical costs for the QVOA assay are between $800-$1000 per sample. This price includes all assay-associated costs, including materials and labor, for testing approximately 30 million PBMC (pre-CD4 enrichment) plated in 8-12 wells. Prices depend on the type of assay and number of wells plated; economies of scale can be realized if multiple samples can be tested at once. Please contact us for more specific pricing for your samples.

Contact us

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