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Enzymatic Assay for Rapid Measurement of Antiretroviral Drug Levels

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Cite This: ACS Sens. 2020, 5, 952−959 Read Online

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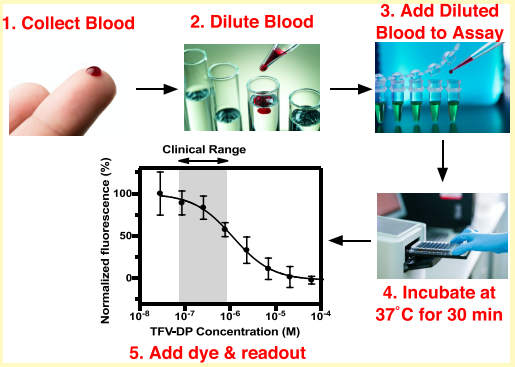
ABSTRACT: Poor adherence to pre-exposure prophylaxis (PrEP) and antiretroviral therapy (ART) can lead to human immunodeficiency virus (HIV) acquisition and emergence of drug-resistant infections, respectively. Measurement of antiviral drug levels provides objective adherence information that may help prevent adverse health outcomes. Gold-standard drug-level measurement by liquid chromatography/mass spectrometry is centralized, heavily instrumented, and expensive and is thus unsuitable and unavailable for routine use in clinical settings. We developed the REverSe TRanscrIptase Chain Termination (RESTRICT) assay as a rapid and accessible measurement of drug levels indicative of long-term adherence to PrEP and ART. The assay uses designer single-stranded DNA templates and intercalating fluorescent dyes to measure complementary DNA (cDNA) formation by reverse transcriptase in the presence of nucleotide reverse transcriptase inhibitor drugs. We optimized the RESTRICT assay using aqueous solutions of tenofovir diphosphate (TFV-DP), a metabolite that indicates long-term adherence to ART and PrEP, at concentrations over 2 orders of magnitude above and below the clinically relevant range. We used dilution in water as a simple sample preparation strategy to detect TFV-DP spiked into whole blood and accurately distinguished TFV-DP drug levels corresponding to low and high PrEP adherences. The RESTRICT assay is a fast and accessible test that could be useful for patients and clinicians to measure and improve ART and PrEP adherence.



KEYWORDS: *human immunodeficiency virus (HIV), pre-exposure prophylaxis (PrEP), adherence, enzymatic assay, drug-level measurement, antiretroviral*

For nearly 40 million people living with HIV (PLHIV) and millions more at risk of acquiring HIV,[1] antiretroviral therapy (ART) and pre-exposure prophylaxis (PrEP) can extend the length and quality of life and prevent HIV infection.[2] As access to ART and PrEP improves globally, medication adherence increasingly becomes a challenge in HIV treatment and prevention.[3] Poor ART adherence leads to viral rebound, emergence of drug resistance, and treatment failure.[4] Poor PrEP adherence reduces individual- and community-level HIV prevention benefits. Roughly 30% of PLHIV receiving ART do not maintain sufficient adherence,[2,5−7] and non-adherence rates were higher in several PrEP trials.[3,8] Poor adherence occurs for several reasons including barriers to care or medication, medication side effects, psychological problems, and poor provider−patient relationships.[9] Clinicians, patients, and patient advocates need tools to accurately measure antiretroviral drug levels and assess interventions to improve health outcomes.[3,10]

There are several approaches to measuring ART and PrEP adherence. Subjective measures of adherence, such as self-reports and surveys,[3,11] pill counts and tracking of pharmacy refills,[3,12,13] and wireless pill containers,[14,15] do not provide proof of pill ingestion, limiting their accuracy.[3] Digital pills with radio-frequency transmitters embedded in gel caps provide proof of pill ingestion and information about short- and long-term adherence patterns.[16] Digital pills require an individual to wear an RFID receiver that transmits the signal to a cloud-based server and require modification of the medication, which may trigger additional regulatory review and may be cost-prohibitive in global health settings.[3]

Quantifying concentrations of antiretroviral drugs and their metabolites is an objective approach to measure ART and PrEP adherence.[3] Tenofovir disoproxil fumarate (TDF) is used in all PrEP regimens currently recommended by health organizations (e.g., WHO and U.S. Centers for Disease Control), and tenofovir-based treatment regimens are used in over 90% of all ART regimens.[17] TDF is hydrolyzed into

Received: November 7, 2019
Accepted: April 6, 2020
Published: April 6, 2020

https://dx.doi.org/10.1021/acssensors.9b02198
ACS Sens. 2020, 5, 952−959

tenofovir (TFV) and phosphorylated intracellularly by nucleotide kinases into tenofovir diphosphate (TFV-DP).[18] TFV-DP is a nucleotide reverse transcriptase inhibitor (NRTI) that terminates the DNA chain when HIV reverse transcriptase (HIV RT) synthesizes complementary DNA (cDNA). TFV has a short half-life (15 h) in plasma and is detectable for up to 7 days.[18,19] TFV measurement is susceptible to the "white coat" effect, where one is unable to correctly identify patients who take their medications just before a doctor's office visit.[20] Conversely, TFV-DP has a longer half-life (17 days) and accumulates 25-fold in red blood cells (RBCs) and thus provides adherence information over 1−2 months.[19] TFV-DP concentrations are associated with health outcomes such as viral suppression[21] and PrEP efficacy.[22]

Immunoassays were recently developed to measure TFV.[23−26] Competitive immunoassays accurately classified recent dosage (≤24 h)[27] and identified nonadherence that was sustained for more than 7 days.[26] However, all of the HIV adherence monitoring immunoassays developed so far have targeted TFV and as such are susceptible to the white coat effect.[25,28]

TFV-DP drug levels can be measured accurately by liquid chromatography/mass spectrometry (LC/MS).[19,29] Median TFV-DP concentrations ranged from 15 to 170 fmol/10^6 RBCs depending on adherence.[19] Pharmacokinetic studies with LC/MS demonstrated that PrEP clients taking ≥ 4 doses/week are considered to maintain long-term adherence and are protected from HIV infection.[29] Nevertheless, LC/MS requires significant capital investment, extensive sample preparation, trained personnel, and cold reagent storage and is unsuitable for routine clinical use.[28]

In this paper, we develop an enzymatic assay, termed REverSe TRanscrIptase Chain Termination (RESTRICT), for ART and PrEP long-term adherence monitoring. The assay is inspired by the mechanism of action of TFV-DP on HIV RT and infers drug levels from DNA polymerization. Enzyme inhibition assays targeting RT were originally developed in the context of HIV detection,[30] enzyme characterization,[31,32] drug screening,[33−35] and drug resistance monitoring.[33,36,37] There are a few reports describing the use of enzyme inhibition assays to measure metabolites of antiretroviral drugs for therapeutic drug monitoring.[38−40] These early assays all measured the incorporation of radio-labeled nucleotides into RNA templates in the presence of antiretroviral drugs extracted from peripheral blood mononuclear cells (PBMCs), which requires labor-intensive and heavily instrumented sample preparation and assay readout that are difficult to implement in routine clinical use. Early enzymatic assays for therapeutic drug monitoring also only targeted metabolites with short half-lives (hours) that were not indicative of long-term adherence.

Building on recent reports of the accumulation of TFV-DP in red blood cells (RBCs) and pharmacokinetic data about drug levels corresponding to ART and PrEP adherence,[19,41,42] we designed the RESTRICT assay to measure antiretroviral drug levels in RBCs and used whole blood dilution as a simple sample preparation strategy. We developed and optimized the RESTRICT assay using designer single-stranded DNA templates, primers, and intercalating fluorescence dyes to measure TFV-DP spiked in buffer and blood at clinically relevant concentrations. The RESTRICT assay accurately distinguished TFV-DP concentrations in blood corresponding to low and high long-term PrEP adherence in less than 1 h.

EXPERIMENTAL SECTION

RT Activity Characterization. We determined optimal assay conditions for RT activity to minimize assay time and reagent concentration, using RT enzyme obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 p66/p51 reverse transcriptase recombinant protein from Dr. Stuart Le Grice and Dr. Jennifer T. Miller.[43] Reactions were carried out in a buffer containing 60 mM Tris (77-86-1, Sigma-Aldrich), 30 mM KCl (7447-40-7, Sigma-Aldrich), 8 mM $MgCl_2$ (7786-30-3, Sigma-Aldrich), and 10 mM dithiothreitol (20−265, Sigma-Aldrich) buffered to pH 8.0 using HCl (7647-01-0, Acros Organics).

We used a DNA template for the RESTRICT assay because DNA is more stable and more inexpensive than RNA and because HIV RT has DNA-dependent DNA polymerase activity. The DNA template had a 20 nt primer binding site complementary to the 16S rRNA Forward primer AGA GTT TGA TCC TGG CTC AG (51-01-19-06, Integrated DNA Technologies, Coralville, IA) followed by 45 TTCA repeats for a total template length of 200 nt. The DNA template was designed using NUPACK software[44] to preferentially include T bases because TFV-DP is a deoxyadenosine triphosphate (dATP) analogue and thus will bind to T's in the DNA template. The template was also designed to be free from secondary structures that could lead to unwanted pausing of the RT enzyme.[45]

To characterize RT activity, master mixes consisting of final concentrations of 5 nM DNA template, 5 nM primer, 50 μM deoxynucleotides (dNTPs) (D7295, Sigma-Aldrich), and RT enzyme concentrations of 25, 50, 100, and 200 nM were prepared in black, flat-bottom polystyrene 384-well plates with nonbinding surfaces (3575, Corning). RT enzyme was added as the last step in assay setup after which microwell plates were immediately incubated at 37°C in a microplate reader (SpectraMax iD3, Molecular Devices). Assays were stopped by the manual addition of 40 μL of PicoGreen intercalating dye (P7581, ThermoFisher Scientific) diluted 1:400 in 1× TE (10128-588, VWR). Reactions were quenched at 16 min intervals up to a total time of 128 min. PicoGreen was incubated for 1 min before reading out the assay signal with the microplate reader. Assays were run in triplicate unless otherwise specified.

Data was analyzed using GraphPad Prism 8.1 software (GraphPad Software Inc.). The fluorescence intensity from the RT activity assay as a function of time was fit to an exponential curve. Fluorescence intensity as a function of RT concentration was fit to a four-parameter logistic regression curve that follows the familiar symmetrical sigmoidal shape of enzymatic assays. The four-parameter logistic curve fits take the form

$$y = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} \tag{1}$$

For RT activity assays, y represented the fluorescence intensity, while x represented the enzyme concentration.

RESTRICT Assay in Buffer. We conducted RESTRICT assays with TFV-DP (166403-66-3, BOC Sciences Inc.) using 5 μL of DNA template, 5 μL of primer, 20 μL of dNTP solution, 5 μL of TFV-DP, and 5 μL of HIV-1 RT. We varied reagent concentrations to optimize experimental conditions (see Table S1 in the Supporting Information). Serial dilutions of TFV-DP in buffer spanning a concentration range of 1−10 000 nM were prepared to span 2 orders of magnitude above and below the clinically relevant range for adherence measurement, as described in pharmacokinetic studies.[19,41] RESTRICT assay optimization experiments were completed at 100, 300, 1560, and 6250 nM dNTP concentrations.

Fluorescence from the RESTRICT assay was normalized to compare data points gathered at different dNTP concentrations as follows

$$\tilde{F}(j) = \frac{F(j) - F_{\min}}{F_{\max} - F_{\min}} \tag{2}$$

where the subscripts max and min denote the maximum and minimum measured fluorescence values, respectively. Maximum

https://dx.doi.org/10.1021/acssensors.9b02198
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fluorescence was obtained in "No TFV-DP" controls where TFV-DP was replaced with buffer, while minimum fluorescence was obtained in "No RT" controls where RT was replaced with buffer.

RESTRICT assay data were fit to four-parameter logistic regression curves. The 50% inhibition concentration (IC_{50})—the concentration of the drug required to achieve 50% inhibition of its target enzyme in vitro was obtained using eq 1, where the parameter x was the TFV-DP concentration and the parameter C represented the IC_{50}.

RESTRICT Assay in Blood. HIV-negative, human whole blood (BioIVT, Westbury, NY) was diluted in nuclease-free water (3098, Sigma-Aldrich) to lyse RBCs and reduce unwanted inhibition of RT activity by blood components such as hemoglobin and immunoglobulins. Blood was mixed with water by vortexing and incubating for 5 min to lyse RBCs.

Determining Optimal Blood Dilution for RESTRICT. Serial dilutions of whole blood in water were prepared at concentrations ranging from 2 to 10.0%. Five microliters of diluted whole blood at each concentration was added to 35 μL of master mix (at 500 nM dNTP) to measure RT activity in the presence of diluted blood. Assays were stopped by adding PicoGreen and reading out with the plate reader as described previously. Baseline correction was carried out by subtracting the average fluorescence from No RT controls from the fluorescence obtained from each RT activity assays.

RESTRICT Assays in 0.25% Blood. We added 5 μL of TFV-DP spiked in 2% blood to 35 μL of master mix so that the final concentration of blood in the RESTRICT assay was 0.25%. We prepared serial dilutions of TFV-DP in diluted blood to correspond with a concentration range of 5.7−11 000 fmol/10^6 RBCs in whole blood and thus cover the clinical range for TFV-DP adherence measurement (see Table S2 in the Supporting Information). Master mixes for the RESTRICT assay in blood contained 2 nM DNA template, 20 nM primer, 100 nM dNTP, and 100 nM of HIV-1 RT. Data corresponding to high and low TFV-DP concentrations within the clinical range for adherence measurement were compared using an unpaired t-test in GraphPad Prism.

RESULTS AND DISCUSSION

The RESTRICT assay measures the extent of cDNA synthesized by HIV RT as a function of the concentration of nucleotide reverse transcriptase inhibitor (NRTI) drugs (Figure 1). RT forms a double-stranded DNA (dsDNA) by polymerizing free nucleotides complementary to a DNA template starting from a region of the template that is hybridized to a primer. At low NRTI concentrations relative to dNTP concentration, RT is unlikely to incorporate NRTIs into the cDNA chain and polymerizes the single-stranded template into full-length dsDNA strands that bind to many intercalating dye molecules and provide a high assay signal. Conversely, at high NRTI concentrations, RT is very likely to incorporate NRTIs into the cDNA chain early, resulting in chain termination and formation of short DNA fragments that bind to few intercalating dye molecules and provide a low assay signal. At moderate NRTI concentrations, the length of the dsDNA product varies and follows a sigmoidal relationship characteristic of enzyme inhibition assays, as shown in Figure 1. In this way, the fluorescence readout from the RESTRICT assay is used to distinguish low, medium, and high NRTI concentrations.

RT Activity Characterization. To characterize RT activity, we determined the effect of RT concentration and assay time on cDNA production as measured by the output fluorescence of an RT activity assay (Figure 2A). At RT concentrations of 50, 100, and 200 nM, the fluorescence intensity increases with time until ~60 min, when it plateaus. The fluorescence intensity remains flat over time at 25 nM RT. When optimizing enzyme inhibition assays, it is desirable to

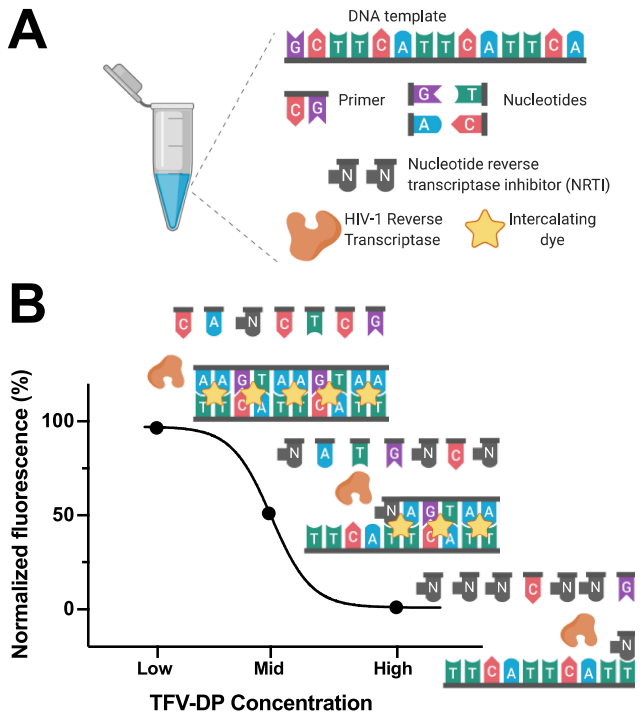

Figure 1. Overview of RESTRICT assay. (A) RESTRICT assay requires nucleic acid templates, primers, nucleotides, nucleotide reverse transcriptase inhibitors (NRTIs), HIV-1 reverse transcriptase enzyme (HIV RT), and intercalating dye. (B) Assay measures complementary DNA (cDNA) synthesis by HIV RT. At low NRTI concentrations, RT forms full-length double-stranded DNA (dsDNA) products that provide high fluorescence with intercalating dye. At intermediate NRTI concentrations, RT forms dsDNA fragments that provide intermediate fluorescence, while at high NRTI concentrations, very little (if any) dsDNA is formed resulting in low fluorescence.

choose an assay time where the RT activity provides measurable fluorescence over baseline levels. The 30 min incubation time provided a strong signal over background levels.

We measured the fluorescence intensity at 8 RT enzyme concentrations to characterize the effect of RT concentration on assay output fluorescence (Figure 2B). The fluorescence intensity remains at the same level as the negative control (no RT) until ~25 nM RT when it begins to increase significantly and then plateaus above ~200 nM RT. A concentration of 100 nM RT provides an optimal signal over background levels without using excess RT.

The fluorescence output as a function of DNA template concentration was used to determine the lowest template and dNTP concentrations required to measure fluorescence (Figure 2C). There is a linear relationship between template concentration and fluorescence intensity. The lowest detectable concentration, above background signal, was 0.25 nM of the DNA template.

RESTRICT Assays in Buffer. We performed RESTRICT assays with TFV-DP in buffer at concentrations spanning 2 orders of magnitude above and below the clinical range for PrEP adherence. The RESTRICT assay generates sigmoidal-shaped curves representative of enzyme inhibition assays as a function of TFV-DP concentration (Figure 3A). As dNTP concentration increases, the fluorescence intensity from the RESTRICT assay increases. This shift in the vertical direction

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ACS Sens. 2020, 5, 952−959

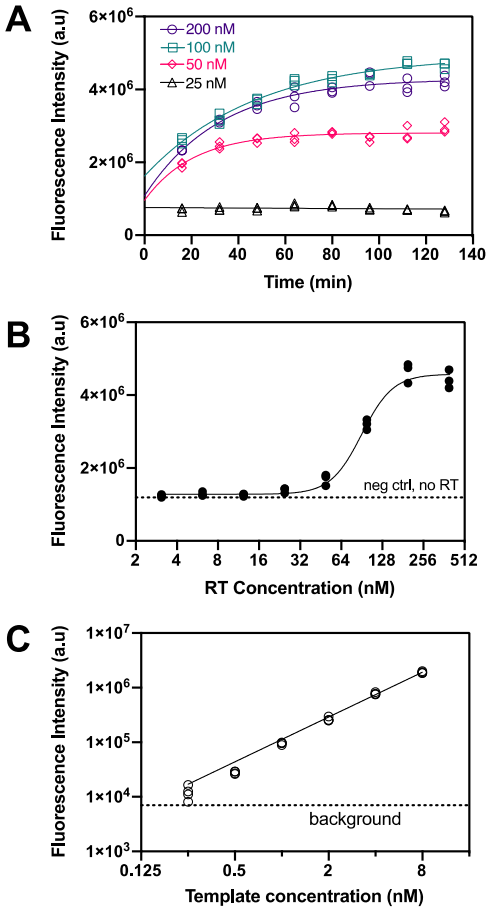

Figure 2. RT activity characterization. (A) Fluorescence intensity over time at different RT concentrations. Lines are exponential fits. N = 3. (B) Effect of RT concentration on fluorescence intensity after 30 min incubation. Fluorescence intensity plateaus above 100 nM RT. The line is a four-parameter logistic regression fit. N = 3. (C) Effect of template concentration on fluorescence intensity with 100 nM RT and 30 min incubation time. The line is a linear fit of the data. N = 4.

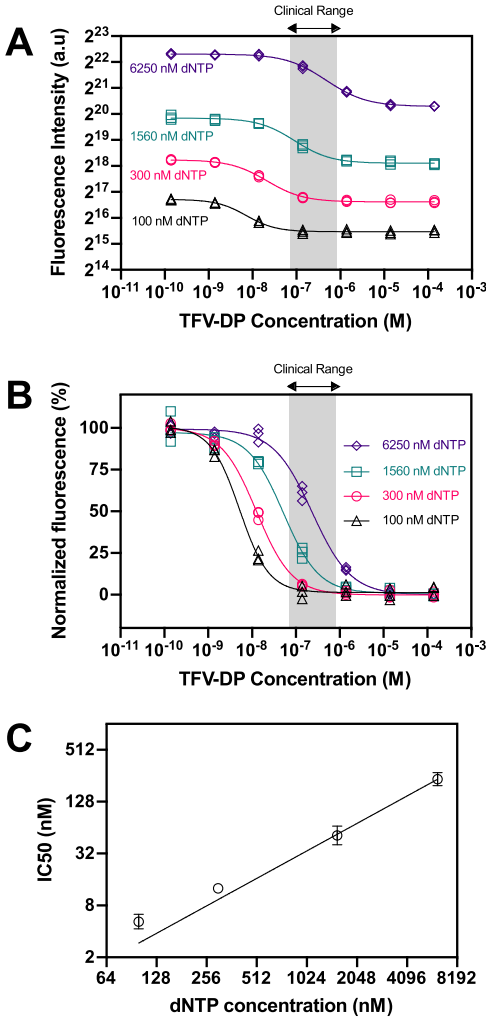

Figure 3. RESTRICT assay in buffer. (A) RESTRICT assays with TFV-DP at different dNTP concentrations. Fluorescence intensity increases, and the curve shifts toward larger TFV-DP concentration as dNTP concentration increases. (B) Normalized data showing that inhibition curves shift right, toward higher TFV-DP as dNTP concentration increases. Gray shaded region indicates the clinical range for PrEP adherence. (C) Graph of dNTP concentration versus IC_{50} values. N = 3; error bars indicate 95% confidence intervals.

is because we kept a fixed 50 to 1 ratio of dNTP to DNA template in all our experiments conducted at 100, 300, 1560, and 6250 nM dNTPs. As shown in Figure 2C, there is a linear relationship between fluorescence intensity and DNA template concentration since more intercalating dye molecules can be incorporated when there are higher DNA template concentrations.

We also see a shift in the horizontal direction as dNTP concentration increases. We normalized the RESTRICT assay data to more easily compare inhibition curves at different dNTP concentrations (Figure 3B). As dNTP concentration increases, the inhibition curves shift right, toward higher IC_{50} values. The IC50 shifts to higher IC_{50} because dNTP and TFV-DP compete for incorporation into the growing DNA strand and inhibition by lower TFV-DP concentrations can only be detected when there are lower dNTP concentrations.

Figure 3C shows the measured IC_{50} values as a function of dNTP concentration and indicates that IC_{50} values increase linearly as a function of dNTP concentration. This linear relationship allows us to design and optimize the RESTRICT assay to have a target IC_{50} value within the clinically relevant concentration range. We designed and optimized the RESTRICT assay to operate at concentration ranges up to 2 orders of magnitude lower than the clinical range (Figure 3B), allowing us to dilute complex samples (like blood) while still retaining the ability to detect clinically relevant concentrations.

RESTRICT Assay in Blood. We chose dilution in water as a simple and user-friendly strategy for sample preparation[46] because it both lyses RBCs and also reduces the concentration of confounding blood matrix components that may suppress reverse transcriptase activity.[46,47]

Determining Optimal Blood Dilution for RESTRICT. The net fluorescence intensity, i.e., the difference between fluorescence from each RT activity assay and the background signal from no enzyme controls, decreases as blood fraction increases and is indistinguishable from the background at a 1.88% final concentration of blood (Figure 4A). This is because diluting blood reduces the nonspecific inhibition of the RT enzyme by blood matrix components. However, the trade-off is that greater dilution also decreases the concentration of the analyte (TFV-DP) in the sample.

To detect TFV-DP in diluted blood, RESTRICT assays need to be performed at lower IC_{50} values compared to buffer.

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ACS Sens. 2020, 5, 952−959

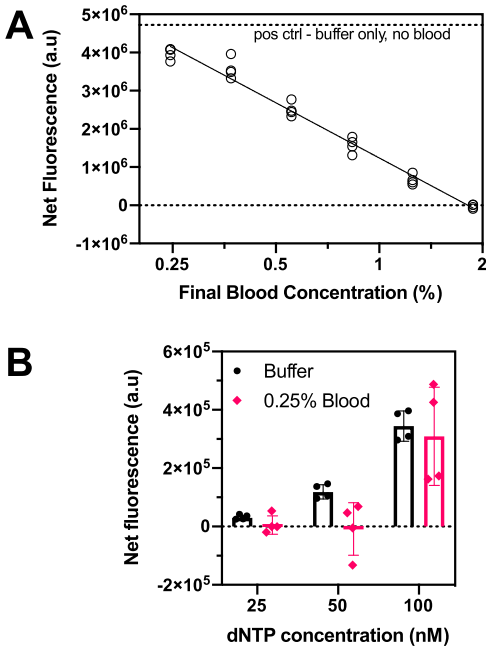

Figure 4. Determining optimal blood dilution for RESTRICT assay. (A) RT activity assay with 500 nM dNTP and diluted whole blood spiked into the assay at various final concentrations to determine how much dilution was required to minimize nonspecific RT inhibition by blood matrix components. (B) RT activity assay with a 0.25% final blood concentration at low dNTP concentrations to determine the lowest dNTP concentration at which RT activity was detectable in blood.

The inhibition curve is shifted to lower TFV-DP concentrations by decreasing dNTP concentration (Figure 3B,C). Figure 2C shows that the lowest dNTP concentration at which we could detect a measurable fluorescence from RT activity in buffer was 25 nM. Anticipating that RT activity in blood would be more variable than in buffer, we ran RT activity assays in diluted blood to determine the lowest dNTP concentration at which we could perform RT activity assays. We chose a final concentration of 0.25% blood (dilution factor 400×) to minimize nonspecific inhibition (Figure 4A), where there was only a 20% decrease in fluorescence intensity in blood compared with that of buffer.

The net fluorescence intensity was measured in aqueous buffer and in 0.25% blood at dNTP concentrations of 25, 50, and 100 nM (Figure 4B). Here, the net fluorescence is the difference between the fluorescence measured from each data point minus the signal from a "no RT enzyme" control at the same conditions to account for variations in background signal. Figure 4B shows that there was a measurable fluorescence signal at 25 nM dNTP in buffer that increased gradually as the dNTP concentration was increased to 50 and 100 nM, consistent with Figure 2C. Conversely, the variation in RT activity when 0.25% blood was introduced resulted in a net zero fluorescence at both 25 and 50 nM dNTP concentration, with RT activity in 0.25% blood only measurable at 100 nM dNTP. Thus, we determined that the lowest dNTP concentration that we could work with in 0.25% whole blood was 100 nM.

RESTRICT Assays in 0.25% Blood. We evaluated the RESTRICT assay for semiquantitative measurement of clinically relevant TFV-DP concentrations spiked in diluted whole blood. Figure 5A shows the steps required to complete

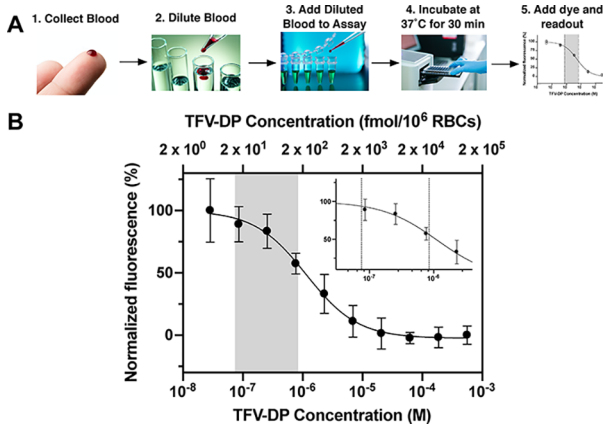

Figure 5. RESTRICT assay in diluted whole blood. (A) Flowchart for RESTRICT assay in blood. (B) Inhibition curve with TFV-DP spiked in diluted whole blood (0.25% final concentration) and 100 nM dNTP. Gray shaded region and the inset show clinical range for TFV-DP adherence. $N = 4$; error bars indicate one standard deviation.

the RESTRICT assay in blood. The entire assay, from sample collection to assay readout, was completed in less than 1 h and required <5 μL of blood. Figure 5B shows data from a RESTRICT assay with 0.25% blood, 100 nM dNTP, at various TFV-DP concentrations around the clinical range for PrEP adherence. The RESTRICT assay data in diluted blood (Figure 5B) followed the expected sigmoidal shape of enzyme inhibition assays seen in buffer (Figure 3B); however, there was greater variation in fluorescence intensities in blood compared with that of buffer. The coefficient of variation of normalized fluorescence from the RESTRICT assay was 11% in blood and only 4% in buffer. This is expected given that blood is a complex sample that contains inhibitors that can suppress reverse transcriptase activity and autofluorescent components that can confound the intercalating dye signal.

The RESTRICT assay in Figure 5B overlaps with the clinical range for TFV-DP adherence, although the IC_{50} of the curve is not located exactly at the center of the clinical range, which would maximize the ability to distinguish low and high TFV-DP concentrations within the clinical range. Improved sample preparation to remove unwanted RT inhibition by blood components could allow the use of greater amounts of blood in the assay and enable further optimization of the RESTRICT assay to shift the inhibition curve to the center of the clinical range and reduce the variation when the assay is carried out with blood samples.

Nevertheless, the RESTRICT assay in blood could distinguish drug levels within the clinical range for PrEP adherence measurement. Median TFV-DP concentrations in RBCs range from 15 to 170 fmol/10^6 RBCs depending on adherence.[19] As shown in Table 1, the p-value is 0.013 for the unpaired *t*-test, comparing fluorescence at 16.9 fmol/10^6 RBCs TFV-DP, corresponding to low adherence (1 dose per week), with the fluorescence at 152.3 fmol/10^6 RBCs TFV-DP, corresponding to high adherence (7 doses per week). These data demonstrate that the RESTRICT assay accurately distinguishes TFV-DP drug levels in blood corresponding to low and high PrEP adherence with high statistical confidence.

Potential Use Cases for the RESTRICT Assay. The RESTRICT assay could provide information on antiretroviral drug levels prior to treatment failure and thus could be a useful and objective tool for monitoring long-term adherence to ART

https://dx.doi.org/10.1021/acssensors.9b02198
ACS Sens. 2020, 5, 952−959

Table 1. Comparison between RESTRICT Assay Results at Low and High Concentrations within the Clinical Range for TFV-DP Adherence Measurement[a]

TFV-DP concentration (nM)	85.4	768
TFV-DP concentration (fmol/10^6 RBCs)	16.9	152.3
corresponding dosage per week	1	7
corresponding adherence level	low	high
normalized fluorescence (%)	89.0	57.5
95% confidence interval	66.4−111.5	44.1−70.6
P-value for unpaired *t*-test	0.013	

[a]$N = 4$.

and PrEP in clinical practice and implementation studies. For example, the RESTRICT assay could be used to identify patients with low antiretroviral drug levels (<2 doses per week)[29,41] who are at risk of treatment failure. Objective measures of adherence could be used to compare the effectiveness of behavioral interventions designed to improve medication adherence and HIV treatment and prevention outcomes.[3]

The RESTRICT assay could also be used in conjunction with HIV viral load tests to identify patients at risk of viral rebound or development of drug resistance. Recent work shows that patients with high viral loads and moderately high TFV-DP drug levels are likely to have drug-resistant infections.[48] In low- and middle-income countries, where drug resistance tests are inaccessible, HIV-positive ART patients who exhibit high viral load levels are often switched to more expensive second- or third-line drug regimens.[49] The RESTRICT assay could be a useful tool to determine if poor adherence is a contributor to high viral load and prevent unnecessary use of the second- and third-line drugs. Furthermore, in settings where viral load measurements are expensive and inaccessible, the RESTRICT assay could be a rapid and inexpensive test that could be performed regularly to determine the risk of treatment failure.

Limitations of the RESTRICT Assay. The assay has some limitations that might preclude its use in particular contexts. For example, the assay does not yet distinguish between inhibition by NRTIs like TFV-DP and non-nucleoside reverse transcriptase inhibitors (NNRTIs) like efavirenz that are sometimes included in ART regimens. For example, patients may falsely appear adherent if they took a recent dose of NNRTIs even though they have not been regularly taking their NRTIs. This limitation is not a typical concern in PrEP clients since NNRTIs are not used in PrEP. Furthermore, as first-line ART regimens in most low- and middle-income countries switch from NNRTIs to dolutegravir (an integrase inhibitor),[50] this limitation might not be a long-term concern. Also, false-positive results might be encountered as a result of impaired drug clearance, such as in renal/hepatic impairment. ART drug doses are usually set based on a patient's creatinine clearance (CrCl), which is an indicator of renal function in patients.[51] Clinicians would be able to identify false-positive results that arise due to impaired drug clearance by checking patient's CrCl levels.

SUMMARY

We showed that the RESTRICT enzymatic assay can be used to measure antiretroviral drug levels indicative of long-term PrEP and ART adherence. The assay measures cDNA formation by RT in the presence of TFV-DP. At higher TFV-DP concentrations, cDNA chain termination occurs resulting in lower fluorescence signals from intercalating dye. We developed and optimized the RESTRICT assay at TFV-DP concentrations 2 orders of magnitude above and below the clinical range for PrEP adherence. We showed that there is a linear relationship between dNTP concentration and the IC_{50} of the RESTRICT assay and that decreasing dNTP concentration shifts the RESTRICT assay to lower TFV-DP concentrations. We spiked TFV-DP into diluted whole blood at concentrations within the clinical range for adherence measurement and demonstrated that the assay could distinguish concentrations corresponding to low and high PrEP adherence in less than 1 h. The RESTRICT assay could be a useful test for rapid and accessible measurement of long-term antiretroviral drug levels to identify patients at risk of treatment failure. This work is innovative because it develops a new category of adherence measurement test that could allow patients and clinicians to monitor and improve long-term ART and PrEP adherence and health-care outcomes. Ongoing work is aimed at testing clinical samples and benchmarking the RESTRICT assay against drug-level measurement by LC/MS.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.9b02198.

Tables providing additional details on the experimental conditions (volumes and concentrations of reagents used to prepare master mix for RESTRICT assays and TFV-DP serial dilutions in 2% whole blood) (PDF)

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https://dx.doi.org/10.1021/acssensors.9b02198
ACS Sens. 2020, 5, 952−959

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful for funding from the NIH (R01AI136648, R21AI127200, R01EB022630). Dr. Olanrewaju thanks the Mistletoe Research Foundation for funding and support. Part of this work was conducted using equipment in the Biochemical Diagnostics Foundry for Translational Research supported by the M.J Murdock Charitable Trust. The authors are also grateful for helpful conversations with Jay Rutherford, Bob Atkinson, and Enos Kline at the University of Washington, as well as Rebecca Sandlin and Mehmet Toner at Harvard Medical School. The research reported in this publication was supported by the University of Washington/Fred Hutch Center for AIDS Research, an NIH-funded program under award number AI027757, which is supported by the following NIH Institutes and Centers: NIAID, NCI, NIMH, NIDA, NICHD, NHLBI, NIA, NIGMS, and NIDDK. Figure 1 was created with BioRender.com.

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https://dx.doi.org/10.1021/acssensors.9b02198
ACS Sens. 2020, 5, 952−959