Title

REverSe TRanscrIptase Chain Termination (RESTRICT) for Selective Measurement of Nucleotide Analogs Used in HIV Care and Prevention

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Abstract (150 words)

Sufficient drug concentrations are required for efficacy of antiretroviral drugs used in human immunodeficiency virus (HIV) care and prevention. Measurement of nucleotide analogs, included in most HIV medication regimens, enables monitoring of short- and long-term adherence and the risk of treatment failure. The REverSe TRanscrIptase Chain Termination (RESTRICT) assay rapidly infers the concentration of intracellular nucleotide analogs based on the inhibition of DNA synthesis by HIV reverse transcriptase (RT) enzyme. Here, we introduce a probabilistic predictive model for RESTRICT and demonstrate selective measurement of multiple nucleotide analogs using DNA templates designed according to the chemical structure of each drug. We measure clinically relevant concentrations of tenofovir diphosphate (TFV-DP), emtricitabine triphosphate (FTC-TP), and azidothymidine triphosphate (AZT-TP) with agreement between experiment and theory. RESTRICT represents a new class of activity-based assays for therapeutic drug monitoring and precision dosing in HIV care and could be extended to other diseases treated with nucleotide analogs.

The development and availability of antiretroviral drugs has decreased the number of deaths related to Human Immunodeficiency Virus (HIV) by 40% over the past decade.[1] However, sufficient drug concentrations are required to obtain the individual and population-level benefits of antiretroviral therapy (ART) and pre-exposure prophylaxis (PrEP) regimens for HIV treatment and prevention, respectively.[2–5] In several PrEP trials and implementation studies, concentrations of antiretroviral medications in blood were correlated with PrEP efficacy.[6] Similarly, viral suppression was associated with antiretroviral concentration among people living with HIV receiving ART.[5] Regular monitoring of antiretroviral drug levels and provision of appropriate counselling/feedback could promote ART and PrEP adherence, increase antiretroviral drug concentrations, and improve HIV care.[4]

Nucleotide analogs are a suitable target for antiretroviral drug monitoring because of their inclusion in most ART and PrEP regimens[7] and their favorable pharmacokinetics.[8] Nucleotide analogs terminate DNA synthesis by HIV reverse transcriptase (RT) and prevent HIV replication. Tenofovir diphosphate (TFV-DP), a deoxyadenosine analog, is used in over 80% of ART regimens and in all approved oral PrEP regimens.[7,9] TFV-DP has a 17-day half-life in red blood cells (RBCs) and accumulates 25-fold at steady state compared to the start of drug ingestion, which enables monitoring of long-term medication adherence over a 1-3 month period.[10–12] Meanwhile, emtricitabine triphosphate (FTC-TP), a deoxycytidine analog, is also included in several ART regimens and in all approved oral PrEP regimens. FTC-TP has a 35-hour half-life in RBCs and provides information about recent (1-week) adherence.[13] Measurement of TFV-DP and FTC-TP concentrations can provide information about short-term and long-term adherence and enable investigation of their implications in clinical practice and behavioral science studies.[5,10,11,13–16]

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is the gold standard for measuring nucleotide analogs,[10,11,17,18] and was used in directly observed therapy trials to determine TFV-DP concentrations corresponding to low (2 doses/week), intermediate (4 doses/week), and high (7 doses/week) PrEP adherence.[10,11] LC-MS/MS was used to establish FTC-TP thresholds corresponding to recent medication ingestion.[10,11,13] Although LC-MS/MS provides accurate and quantitative information about TFV-DP and FTC-TP concentrations, it requires complex sample preparation, highly trained operators, and resource intensive equipment that limit its use to centralized and highly capable laboratories. This hinders routine measurement of antiretroviral concentrations and could delay counselling and interventions in behavioral science studies.[4,19] There are ongoing efforts to miniaturize LC-MS/MS instruments and to integrate the required sample preparation steps into miniaturized cartridges.[20–22] However, the per-unit cost and complexity of current miniaturized LC-MS/MS systems still exceeds requirements in low and middle-income settings.

A rapid and inexpensive test that measures nucleotide analogs indicative of long- and short-term adherence in point-of-care (POC) settings like a doctor's office, patient's home, or event setting could be beneficial in monitoring and improving ART and PrEP outcomes.[4,23] Immunoassays and lateral flow assays for measuring antiretroviral drugs were recently developed.[24–27] Most immunoassays for HIV adherence measurement target tenofovir (TFV), a metabolite of TFV-DP in plasma and urine.[12] TFV has a 15-hour half-life in plasma and indicates recent ingestion of medication within the prior 48 hours. The short half-life of TFV makes its measurement susceptible to conflating recent medication ingestion (≤1 week) with long-term (1-3 month) adherence. We recently developed an immunoassay for measuring clinically relevant concentrations of both TFV and TFV-DP;[28] however, additional sample preparation is required to separate RBCs from plasma because the antibodies used could not distinguish between TFV and TFV-DP. There is still a strong need for assays that can selectively measure biomarkers of short- and long-term in POC settings.[4,29]

We recently developed the REverSe TranscrIptase Chain Termination (RESTRICT) assay for detection of nucleotide analogs based on their enzyme inhibition activity.[30] Unlike immunoassays, RESTRICT directly measures the pharmacologic activity of the analyte. RESTRICT uses DNA templates, nucleotides, recombinant HIV-1 reverse transcriptase, primers, and intercalating dyes to infer the concentration of nucleotide analogs based on the inhibition of DNA synthesis by recombinant HIV RT (**Fig.1**). RESTRICT is completed in less than one hour and uses readily available nucleic acid analysis reagents including DNA templates, primers, nucleotides, and intercalating dyes. DNA chain termination occurs at high nucleotide analog concentration resulting in low DNA synthesis and low fluorescence reported by the intercalating dye. Meanwhile at low nucleotide analog concentration, there is extensive DNA synthesis and high fluorescence from the intercalating dye.

We use dilution in water as a simple and user-friendly sample preparation mechanism to release intracellular TFV-DP from RBCs and decrease non-specific inhibition by blood matrix components.[31] In a pilot clinical evaluation, RESTRICT measured clinically relevant TFV-DP concentrations in whole blood and identified participants with TFV-DP concentrations corresponding to adequate adherence (≥700 fmol per 3 mm dried blood spot punch) among PrEP clients. RESTRICT represents a new class of activity-based diagnostic assays with potential for rapid, POC measurement of antiretroviral drugs used in HIV treatment and prevention.

In this paper, we demonstrate that RESTRICT can selectively measure multiple nucleotide analogs based on their chemical structure. Guided by a probabilistic model of RT inhibition, we investigate the impact of assay parameters including nucleotide analog affinity, nucleotide concentration, template length, and template sequence on RESTRICT. We demonstrate selective detection of biomarkers of either long-term or short-term adherence without cross-reactivity using DNA templates that are rich in the endogenous nucleotide that the drug mimics.

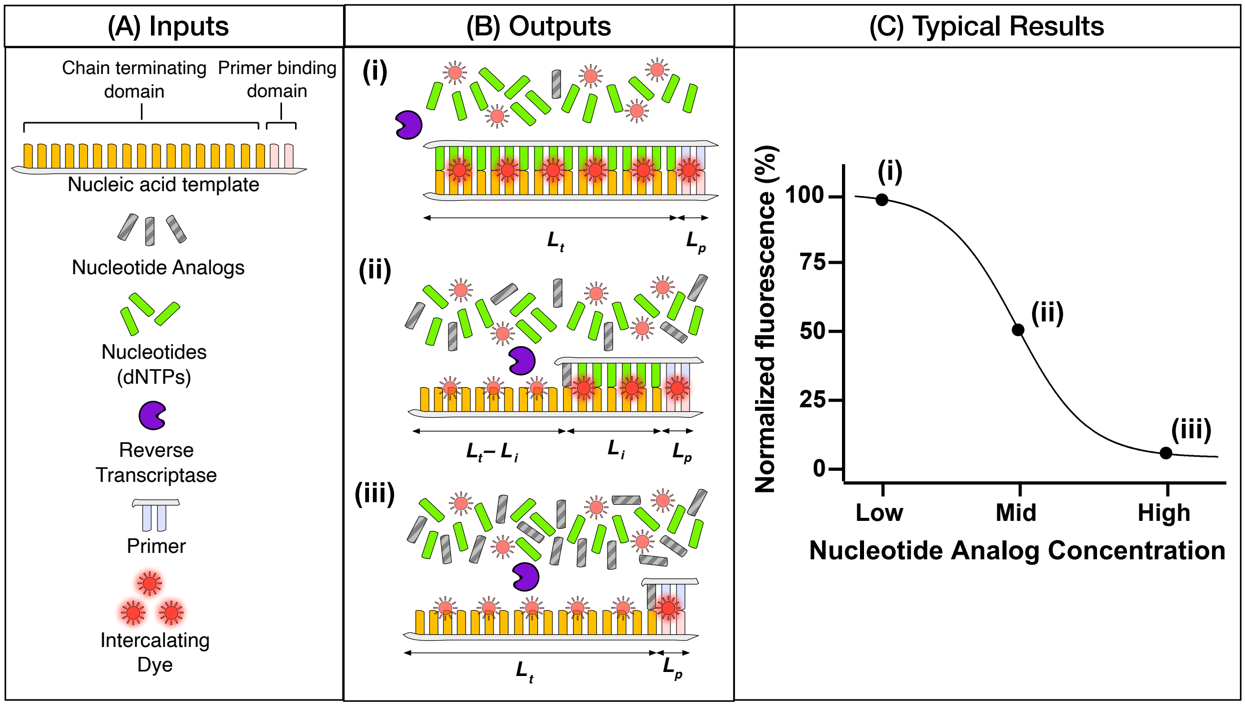

Fig. 1. Summary of inputs and outputs of RESTRICT assay. (A) RESTRICT uses nucleic acid templates, nucleotides, recombinant HIV-1 reverse transcriptase, primers, and intercalating dyes to measure the concentration of nucleotide analogs. The nucleic acid templates have primer binding domains to initiate DNA synthesis and chain terminating domains designed to bind specific nucleotide analogs**. (B)** The key contributions to fluorescence output of the RESTRICT assay come from (i) full-length dsDNA products, (ii) fragment dsDNA products, and (iii) unpolymerized ssDNA template. **(C)** Typical sigmoidal fluorescence versus concentration profile obtained from RESTRICT assays illustrating the high, intermediate, and low fluorescence obtained from the different assay end products

Theory

Overview and key assumptions: We present a probabilistic reaction equilibrium model of RESTRICT. We assume that incorporation of dNTP or nucleotide analog occurs as a random probabilistic event that depends primarily on their relative concentrations. For simplicity, we ignore time-dependent changes in reagent concentration and assume that dNTP and nucleotide analog are available in excess for DNA chain elongation or termination. This model recapitulates experimental results and allows investigation of the role of assay parameters such as nucleotide analog affinity, dNTP concentration, template length, and template sequence on RESTRICT assay performance.

Nucleic acid templates used in the RESTRICT assay consist of a primer binding domain and a chain terminating domain (**Fig.1A**). The primer binding domain is located on the 3' end of the template and guides primer initiation of DNA synthesis. The chain terminating domain is located directly downstream of the primer binding domain and is designed to enable preferential insertion of specific nucleotide analogs. For example, an assay designed to detect TFV-DP, a deoxyadenosine analog, will use a DNA template rich with thymidine (T) bases to provide ample opportunity for TFV-DP insertion. Although the ideal chain terminating domain for TFV-DP detection is a homopolymeric template consisting of a string of T's, in practice, HIV RT exhibits template preferences and does not efficiently polymerize Poly(dT) templates,[32,33] so the template sequence must be optimized.

There are several contributions to the output fluorescence signal. The largest signal fraction originates from the interaction between intercalating fluorescent dyes and double stranded DNA (dsDNA) products. There are smaller magnitude contributions from unpolymerized single stranded DNA (ssDNA) templates and primers. Intercalating dyes provide significantly greater fluorescence when bound to dsDNA; however, they also produce a measurable fluorescence signal when bound to ssDNA (**Fig.1B**). For example, PicoGreen® used in our experiments, provides 11 times greater fluorescence when bound to dsDNA compared to ssDNA.[34,35] The theoretical model also accounts for fluorescence from unpolymerized ssDNA. In the sections below, we provide calculations of the fluorescence contributions of different assay end products.

Full-length dsDNA products: For a nucleic-acid template with chain terminating domain length, $\boldsymbol{L_t}$, and $\boldsymbol{n}$ bases complementary to the target nucleotide analog ($\boldsymbol{n \le L_t}$), the formation of full-length dsDNA requires $\boldsymbol{n}$ consecutive dNTP insertion events. Assuming these are all independent dNTP insertion events in the presence of excess dNTP and nucleotide analog concentration, the probability of full-length dsDNA formation can be expressed as,

$$P_{dNTP,n} = \left(\frac{[dNTP]}{[dNTP] + K_{aff} \cdot [NA]}\right)^{n} \quad (1)$$

where $[\boldsymbol{NA}]$ is nucleotide analog concentration, $[\boldsymbol{dNTP}]$ is nucleotide concentration, and $\boldsymbol{K_{aff}}$ is the relative affinity of RT enzyme for the nucleotide analog compared to dNTP.

The fluorescence from full-length dsDNA products, $\boldsymbol{F_{full}}$, depends on the length of the DNA template and the fluorescence properties of the intercalating dye and can be expressed as,

$$F_{full} = C_t \cdot K_{dye} \cdot L_t \cdot P_{dNTP,n} \quad (2)$$

where $\boldsymbol{K_{dye}}$ is the fluorescence per double-stranded base-pair per unit concentration of the intercalating dye, and $\boldsymbol{C_t}$ is the concentration of DNA template.

Fragment dsDNA products: There is a distribution of dsDNA fragments sizes during RESTRICT assays as previously observed in experimental studies of RT activity.[32] Partial-length dsDNA fragments contribute to the total fluorescence in the RESTRICT assay (**Fig.1B**). Like full-length DNA, fluorescence from dsDNA fragments depends on the probability of formation of a dsDNA fragment and the length of the DNA fragment. For a given pair of nucleotide and nucleotide analog concentrations, we can calculate the probability of chain termination at any of the $\boldsymbol{n}$ possible insertion sites for nucleotide analogs in the DNA template. The probability of formation of a dsDNA fragment is,

$$P_{frag,i} = P_{dNTP,i-1} \cdot P_{NA} \quad (3)$$

where the index $\boldsymbol{i \geq 1}$ counts the bases where it is possible to insert nucleotide analog and the maximum possible value of $\boldsymbol{i}$ is the total number of bases in the template, or simply $\boldsymbol{n}$. $\boldsymbol{P_{dNTP,i-1}}$

is the probability that dNTP molecules were incorporated in the nucleic acid template at all bases preceding the base $\boldsymbol{i}$ where nucleotide analog was inserted and is calculated using equation (1). $\boldsymbol{P_{NA}}$ is the probability of a single nucleotide analog insertion event and can be expressed as,

$$P_{NA} = 1 - P_{dNTP,n=1} = \left(\frac{K_{aff} \cdot [NA]}{[dNTP] + K_{aff} \cdot [NA]}\right) \tag{4}$$

To determine the total fluorescence contribution from dsDNA fragments, we calculate the sum of fluorescence from all the dsDNA fragments. Adapting equation (3) and summing the fluorescence from individual dsDNA fragments, the fluorescence from dsDNA fragments can be expressed as,

$$F_{dsDNA-frag} = C_t \cdot K_{dye} \cdot \sum_{i=1}^{n-1} \left(P_{dNTP,i-1} \cdot P_{NA} \cdot L_i\right) \tag{5}$$

where each termination site $\boldsymbol{i}$ and the resulting dsDNA fragment of length, $\boldsymbol{L_i}$, is known since we know the exact sequence of DNA templates used in the assay.

Unpolymerized ssDNA: We also account for the background fluorescence contribution of intercalating dye interacting with unpolymerized nucleic acid template. At high nucleotide analog concentrations, very little (if any) dsDNA is formed and most of the fluorescence output comes from unpolymerized ssDNA template (**Fig.1B**). In RESTRICT, this background varies with nucleotide analog concentration. Each dsDNA fragment has a corresponding ssDNA fragment with length equal to the difference between the total length of the nucleic acid template and the dsDNA fragment (**Fig.1B**). Thus, the background fluorescence from the ssDNA fragments can be calculated as,

$$F_{ssDNA-frag} = C_t \cdot \frac{K_{dye}}{11} \cdot \sum_{i=1}^{n-1} \left(P_{dNTP,i-1} \cdot P_{NA} \cdot [L_t - L_i]\right) \tag{6}$$

where $[\boldsymbol{L_t} - \boldsymbol{L_i}]$ is the length of the unpolymerized single-stranded portion of a fragment product that was terminated at base $\boldsymbol{i}$, and $\frac{\boldsymbol{K_{dye}}}{\boldsymbol{11}}$ accounts for the decreased fluorescence associated with PicoGreen™ binding to ssDNA rather than dsDNA.[34,35]

Contributions of bound and unbound primer: DNA templates used in RESTRICT assay all had a common 20 nt primer binding domain, while the chain terminating domain varied in sequence and length from 45 to 180 nt. Given that the primer binding domain constitutes up to 31% of the total template length, it is important to account for fluorescence that arises from the primer binding domain. Assuming the reaction goes to completion with all available templates bound to a primer, the fluorescence from dsDNA due to bound primers can be calculated as,

$$F_{bound-primer} = C_{template} \cdot K_{dye} \cdot L_{primer} \tag{7}$$

where $\boldsymbol{C_{template}}$ is the template concentration and $\boldsymbol{L_{primer}}$ is the primer length.

There is also background fluorescence from unbound primer in solution. To ensure that all templates bound to a primer, we used excess primer in the RESTRICT assay (ten times the template concentration). The fluorescence due to unbound ssDNA primer can be calculated as,

$$F_{free-primer} = \left(C_{primer} - C_t\right) \cdot \frac{K_{dye}}{\mathrm{D}} \cdot L_{primer} \tag{8}$$

where $\left(\boldsymbol{C_{primer}} - \boldsymbol{C_t}\right)$ is the difference between primer and template concentration, and the factor D is the fold reduction in fluorescence when intercalating dye is bound to ssDNA rather than dsDNA (D = 11 for PicoGreen®).[34,35]

Total Fluorescence from RESTRICT Assay Products: We can calculate the total fluorescence, $\boldsymbol{F_{total}}$, at the end of the RESTRICT assay by combining equations (1) – (8),

$$F_{total} = F_{full} + F_{dsDNA-frag} + F_{ssDNA-frag} + F_{bound-primer} + F_{free-primer} \tag{9}$$

Using this, we can estimate RESTRICT assay performance as we vary assay parameters such as nucleotide concentration, template length, and template sequence.

Experimental Section

RESTRICT Assay Reagents and Workflow: RESTRICT reactions were carried out in buffer containing 60 mM Tris (77-86-1, Sigma, St. Louis, MO), 30 mM KCl (7447-40-7, Sigma, St. Louis, MO), 8 mM $MgCl_2$ (7786-30-3, Sigma, St. Louis, MO), and 10 mM dithiothreitol (20-265, Sigma, St. Louis, MO) buffered to pH 8.0 using HCl (7647-01-0, Acros Organics, Fair Lawn, NJ). Recombinant RT enzyme was obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 RT Catalog #3555 from Dr. Stuart Le Grice and Dr. Jennifer T. Miller.[36] The nucleotide analogs TFV-DP (166403-66-3 sodium salt), azidothymidine-5'-triphosphate (AZT-TP) (B1331-007254 triethylammonium salt), and FTC-TP (1188407-46-6 triethylammonium salt) were obtained from BOC Sciences Inc (Shirley, NY).

RESTRICT master mixes contained 2.5 nM final concentration of DNA template, 500 nM of each deoxynucleotide triphosphates (dNTPs) (D7295, Sigma, St. Louis, MO), and 25 nM of 16S rRNA forward primer AGA GTT TGA TCC TGG CTC AG (51-01-19-06, Integrated DNA Technologies, Coralville, IA). DNA templates had 20 nucleotide (nt) primer binding domains on their 3' ends complementary to the 16S rRNA primer and chain terminating domains designed according to the target nucleotide analog that ranged in length from 65 to 200 nt (see **Table 1**).

Table 1. Sequences of the chain terminating domains of DNA templates used in the RESTRICT assay.

Short name	**Chain Terminating Domain Sequence**
180 nt TTCA	TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA

180 nt GGCA	GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA GGCA
135 nt TTCA	TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTC
90 nt TTCA	TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TT
45 nt TTCA	TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA TTCA T
90 nt TCAA	TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TCAA TC
90 nt TCA	TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA
90 nt TTTCA	TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA TTTCA

RESTRICT assays were initiated by adding 5 µL RT (100 nM final concentration in the assay) to 35 µL of master mix in a 384-well microplate (3575, Corning, Corning, NY), incubated at 37˚C for 30 min in a microplate reader (SpectraMax iD3, Molecular Devices, San Jose, CA). Reactions were stopped by adding PicoGreen® dye (P7581, ThermoFisher Scientific, Waltham, MA) diluted 1:400 in 1xTE (10128-588, VWR, Radnor, PA) and incubating for 1 min prior to fluorescence readout. Three replicate reactions were tested unless otherwise stated.

Comparison between Probabilistic Model and Experimental Data: We performed a curve fit to determine K_{aff} values that minimized the difference between experimental and theoretical normalized fluorescence values for TFV-DP, FTC-TP, and AZT-TP using the 180 nt TTCA, 180 nt GGCA, and 90 nt TCAA templates, respectively. RESTRICT experiments were carried out at 500 nM dNTP over a broad range of drug concentrations (10^{-10} to 10^{-5} M). We varied K_{aff} in the theoretical model from 0.1 to 0.9 in 0.1 increments and chose the value that minimized the sum of the square of the difference between experiment and theory for all data points tested for a given drug-template pair.

We performed RESTRICT assays with the 180 nt TTCA template at dNTP concentrations of 0.10, 0.30, 1.56, and 6.25 µM using serial dilutions of TFV-DP with concentrations ranging from 10^{-10} M to 10^{-5} M in 10-fold increments to determine the effect of nucleotide concentration on RESTRICT. We computed theoretical fluorescence intensities within the same range of dNTP concentrations using the optimal K_{aff} value determined at 500 nM dNTP and compared experimental and theoretical IC_{50} values.

We performed RESTRICT assays at 500 nM dNTP using the 45 nt TTCA, 90 nt TTCA, 135 nt TTCA, and 180 nt TTCA templates (**Table 1**) to determine the effect of template length on RESTRICT. Similarly, we performed RESTRICT assays at 500 nM dNTP using the 90 nt TCAA, 90 nt TCA, 90 nt TTCA, and 90 nt TTTCA templates to determine the effect of template sequence on RESTRICT. In both cases, we tested serial dilutions of TFV-DP with concentrations ranging from 10^{-10} M to 10^{-5} M in 10-fold increments and compared experimental and theoretical IC_{50}.

Selective Detection of Nucleotide Analogs: We performed RESTRICT assays at 500 nM dNTP with serial dilutions of AZT-TP and FTC-TP in buffer at concentrations ranging from 10^{-11} to 10^{-5} M of each drug. RESTRICT assays with AZT-TP and FTC-TP were carried out with 90 nt TCAA and 180 nt GGCA templates, respectively. K_{aff} was determined empirically to minimize the difference between experimental and theoretical IC_{50} for each drug and template combination.

We used 180 nt GGCA DNA templates (excluding adenosine bases) for selective detection of FTC-TP (cytidine analog) by Watson-Crick-Franklin base pairing without cross-reactivity with TFV-DP (adenosine analog). Similarly, we used 180 nt TTCA DNA templates (excluding guanosine bases) for selective TFV-DP detection without FTC-TC binding. We spiked 10 µM of both TFV-DP and FTC-TP into RESTRICT assays with each DNA template and measured endpoint fluorescence. We normalized fluorescence output using “no-enzyme” and “no drug” controls.

Data Analysis and Statistics: RESTRICT data were normalized using fluorescence from negative controls (no RT enzyme) as 0% and fluorescence from positive controls (no nucleotide analog) as 100%. We also normalized the fluorescence intensity of theoretical calculations of fluorescence from the probabilistic model of RT inhibition to facilitate comparison with experimental results. Fluorescence intensities from experimental data and theoretical calculations were fit to four-parameter logistic regression curves using GraphPad Prism 9 (GraphPad Software Inc.) to obtain the 50% inhibition concentration (IC_{50}). Data obtained from specificity experiments with TFV-DP, FTC-TP, 180 nt TTCA, and 180 nt GGCA templates were compared using unpaired t-tests after normalization.

Results and Discussion

Determination of Nucleotide Analog Affinity Coefficient: The nucleotide analog affinity coefficient, K_{aff}, indicates the relative affinity of RT enzyme for a particular nucleotide analog compared with the endogenous nucleotide that it mimics. Given the strong preferences exhibited by HIV-1 RT for different nucleotide analogs based on the nature (RNA or DNA) and sequence of nucleic acid templates,[32,33] we determined K_{aff} empirically for each DNA template and nucleotide analog tested. We chose Kaff values that minimized the sum of the difference between experiment and theory for all data points for each template-drug pair. K_{aff} values of 0.3, 0.2, and 0.2 provided the minimum difference between experiment and theory for TFV-DP, FTC-TP, and AZT-TP using the 180 nt TTCA, 180 nt GGCA, and 90 nt TCAA templates, respectively. These K_{aff} values were used in subsequent experiments.

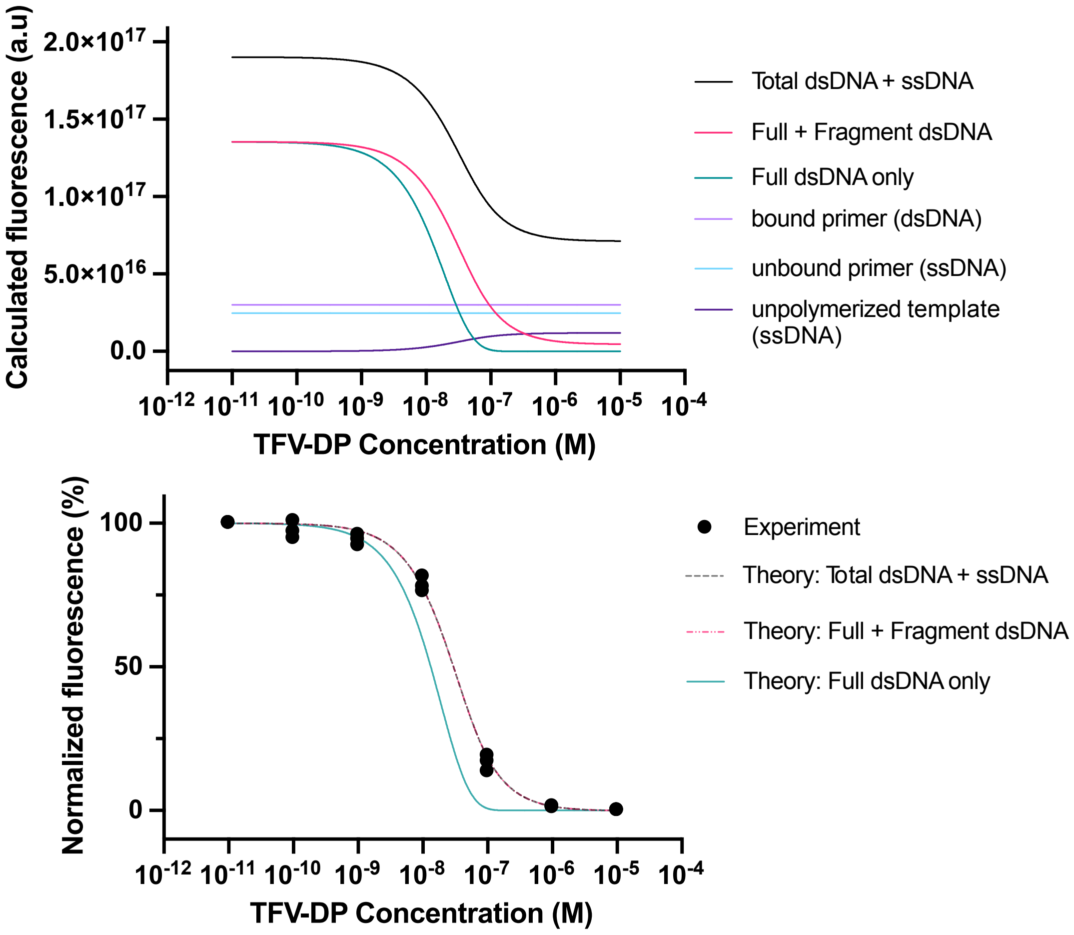

Fig. 2. Contributions of different model components to RESTRICT assay results. (A) Fluorescence from the theoretical model for unpolymerized ssDNA, unbound primer, bound primer, full dsDNA product, as well as both full and fragment dsDNA product calculated using equations (1) to (9). **(B)** Accounting for both full and fragment dsDNA improves the agreement between normalized theoretical and experimental data (500 nM dNTP, 180 nt TTCA template, N = 3).

Contributions of RESTRICT Assay Products to Endpoint Fluorescence: We can account for the contributions of different assay inputs and outputs to the total and normalized fluorescence from the RESTRICT assay. Accounting for background fluorescence from primers and unpolymerized template increases the total fluorescence from the RESTRICT assay (**Fig.2A**). Our focus in this study was to investigate the parameters that influence the shape and position of normalized RESTRICT curves on the concentration axis rather than obtaining exact calculations of the absolute endpoint fluorescence. Consequently, we normalized fluorescence intensities to compare theoretical and experimental data (**Fig.2B).**

Accounting for both full and fragment dsDNA improves the agreement between experiment and theory (**Fig.2B**). Experimental RESTRICT data were obtained using the 180 nt TTCA template, 500 nM dNTP, and serial dilutions of TFV-DP. When only full-length dsDNA was included in the theoretical model, the average difference between experimental and theoretical values was 36.68 ± 19.98%. Meanwhile when both full-length and fragment dsDNA were included, the average difference between theory and experiment decreased to 6.36 ± 4.04%. Including background fluorescence from primers and unpolymerized template did not change the shape of the normalized RESTRICT curves or improve the agreement between normalized experimental and theoretical data.

Varying dNTP Concentration, Template Length, and Template Sequence: We examined the effect of dNTP concentration, template length, and template sequence on RESTRICT assay performance in theory and experiment (**Fig.3**). As dNTP concentration increases, we observe less inhibition (and consequently higher fluorescence) as a function of TFV-DP concentrations since higher dNTP concentrations make TFV-DP insertion less likely and more TFV-DP is required to have a similar inhibitory effect (**Fig.3A**). This has the effect of shifting the inhibition curves to the right. **Fig.3B** plots the IC_{50} value as an exponential function of dNTP concentration and shows good agreement between experimental data and theoretical calculations.

Fig.3C examines the effect of template length on RESTRICT by keeping dNTP concentration constant at 500 nM and varying the length of the chain terminating domain of the TTCA template. The data and predictions show that as template length increases, RESTRICT curves shift to the left as lower TFV-DP concentrations now have an increased inhibitory effect, since longer DNA templates provide additional opportunities for TFV-DP insertion. **Fig.3D** shows an exponential decay in IC_{50} value with increasing template length.

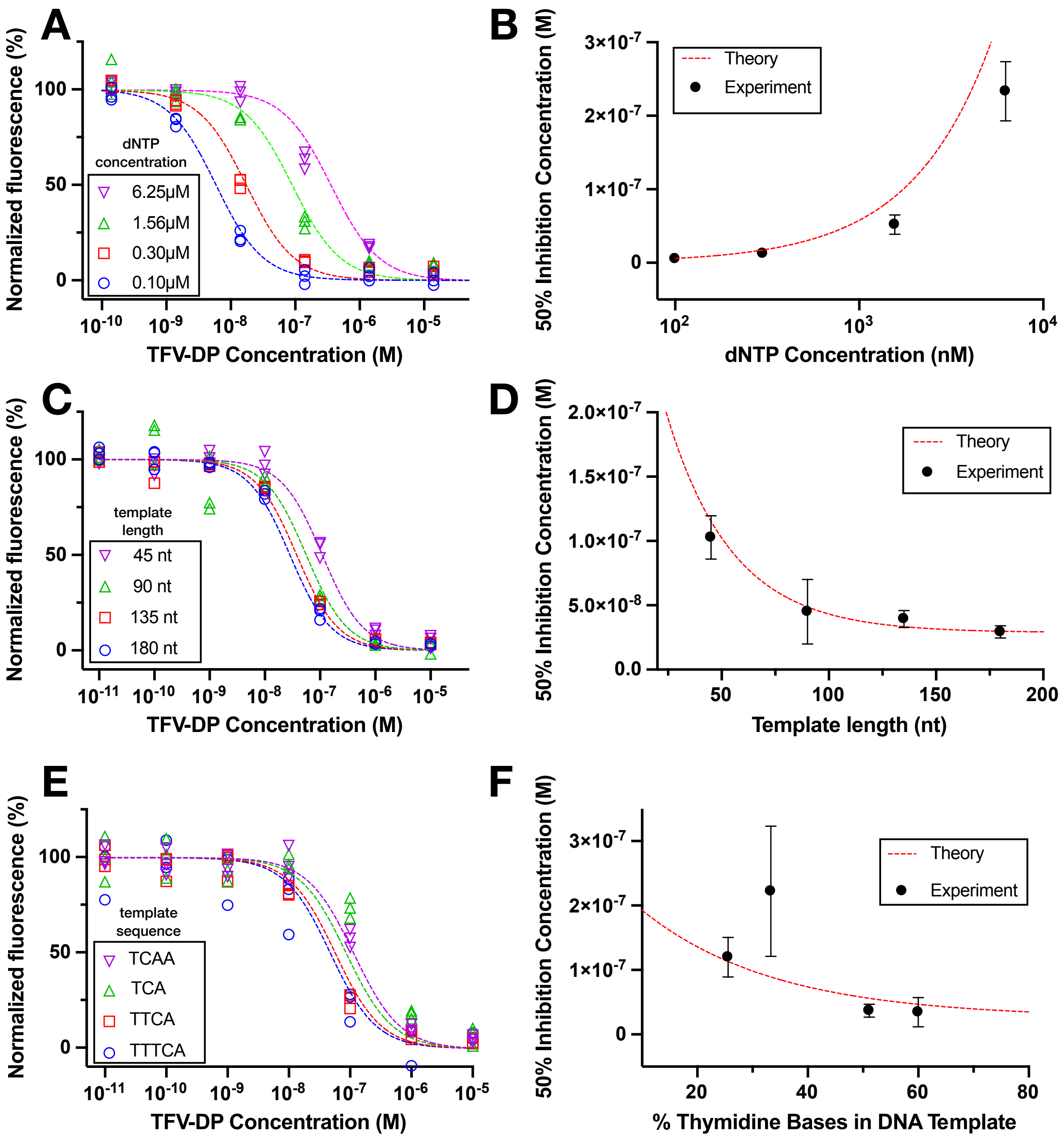

Figure 3: Effect of nucleotide concentration, template length, and template sequence on detection of TFV-DP using the RESTRICT assay. (A) As dNTP concentration increases, RESTRICT curves shift to higher TFV-DP concentrations. (B) As dNTP concentration increases, IC_{50} increases. (C) As template length increases, RESTRICT curves shift to lower TFV-DP concentrations. (D) As template length increases, IC_{50} decreases. (E) As the thymidine base content of DNA templates

increases, RESTRICT curves shift to lower TFV-DP concentrations. (F) As thymidine content increases, IC_{50} decreases. Symbols indicate experiments and dashed lines indicate theory. N = 3. Error bars indicate 95% confidence intervals.

Fig.3E shows the effect of varying template sequence on RESTRICT inhibition curves. DNA template length was fixed at 90 nt and dNTP concentration was fixed at 500 nM, while the sequence of the chain terminating domain was varied to test the impact of thymidine content on TFV-DP detection. **Fig.3E** shows that as thymidine content increases RESTRICT curves shift left towards lower TFV-DP concentrations since there are increased opportunities for TFV-DP incorporation (like the effect of increased template length). **Fig.3F** confirms this trend in both the experimental and theoretical IC_{50} values. Although the TCA template (33% thymidine content in the chain terminating domain) deviates from this trend, all the other templates tested confirmed the expected trend of lower IC_{50} as thymidine content increases given the greater number of TFV-DP insertion sites in the chain terminating domain.

Taken together, **Fig.3** shows that the probabilistic model for RESTRICT can be used to inform systematic assay design and choose appropriate assay parameters to shift RESTRICT curves to desired concentration ranges for TFV-DP detection. The RESTRICT assay can be shifted towards more sensitive measurement of nucleotide analogs at low concentrations by decreasing the dNTP concentration, increasing the template length, and incorporating a higher fraction of nucleotides that the drug of interest will bind to during polymerization.

Detection of Multiple Nucleotide Analogs: RESTRICT assays account for the endogenous nucleotide that a drug mimics and Watson-Crick-Franklin base pairing between the template and the drug. DNA templates were designed to be thymidine-rich to enable competitive inhibition of DNA synthesis by TFV-DP (a deoxyadenosine analog). Meanwhile, guanosine-rich DNA templates were designed for detection of FTC-TP (a deoxycytidine analog).

Fig.4A shows a RESTRICT curve obtained using 500 nM dNTP, a 90 nt TCAA template, serial dilution dilutions of AZT-TP, and empirically derived $K_{aff} = 0.2$. The linear region of the RESTRICT curve overlaps with the clinical range for AZT-TP in peripheral blood mononuclear cells (PBMCs).[37] Meanwhile, **Fig.4B** shows a RESTRICT curve obtained using FTC-TP, 500 nM dNTP, and a 180 nt GGCA template ($K_{aff} = 0.2$). The linear region of the RESTRICT curve overlaps with the clinical range for FTC-TP in red blood cells (RBCs).[13] Taken together, **Fig.4** demonstrates that RESTRICT DNA templates can be designed to detect clinically relevant concentrations of different nucleotide analogs used in HIV treatment and prevention.

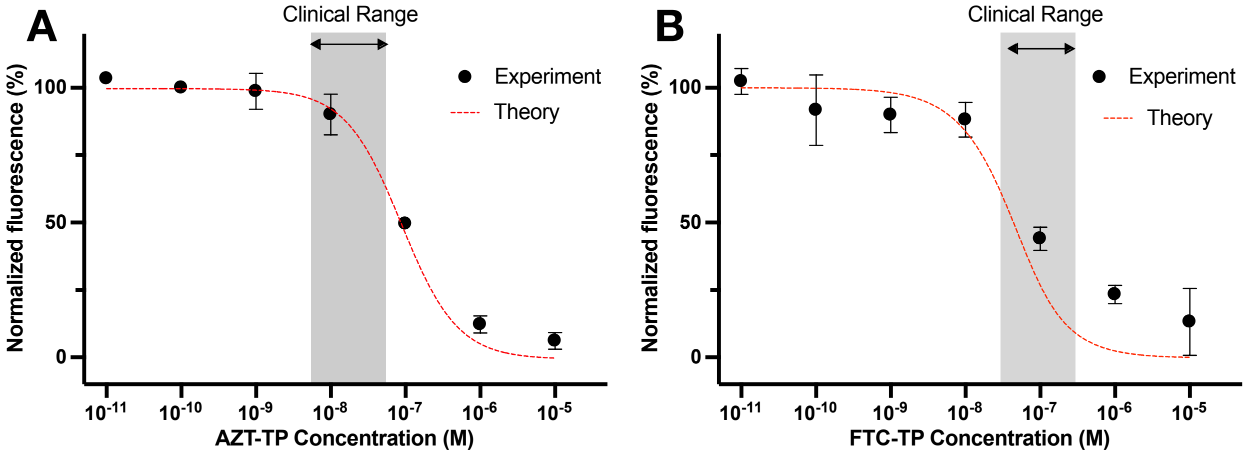

Figure 4: Detection of clinically relevant concentrations of AZT-TP and FTC-TP using RESTRICT. Symbols indicate experiments and dashed lines indicate theory. N = 3. Error bars indicate standard deviation. Grey shaded regions indicate clinical range for each drug.

Selective Detection of FTC-TP and TFV-DP: RESTRICT can measure specific nucleotide analogs without cross-reactivity with companion nucleotide analogs present in the same clinical sample. For example, TFV-DP and FTC-TP are common companion drugs used in ART and PrEP. Selective detection of nucleotide analogs is important to avoid conflating recent pill ingestion with long-term adherence.

We designed a guanosine-rich DNA template (180 nt GGCA) for selective detection of FTC-TP (deoxycytidine analog) and excluded thymidine bases to cross-reactivity TFV-DP (deoxyadenosine analog). Similarly, we designed a thymidine-rich DNA template that excluded guanosine bases (180 nt TTCA) for selective TFV-DP detection without cross-reactivity with FTC-TP. “No enzyme” controls produced no fluorescence since no DNA synthesis occurred, while “no drug” controls produced maximum fluorescence since there was no inhibition of DNA synthesis (**Fig.5**). RESTRICT assays with guanosine-rich DNA templates produced low fluorescence (4.0 ± 4.3%) with FTC-TP and high fluorescence (86.2 ± 26.3%) with TFV-DP, indicating selective FTC-TP detection without cross-reactivity with TFV-DP ($p = 0.0060$). Conversely, thymidine-rich DNA templates produced high fluorescence (105.7 ± 3.1%) with FTC-TP and low fluorescence (2.4 ± 1.5%) with TFV-DP indicating selective TFV-DP detection ($p < 0.0001$).

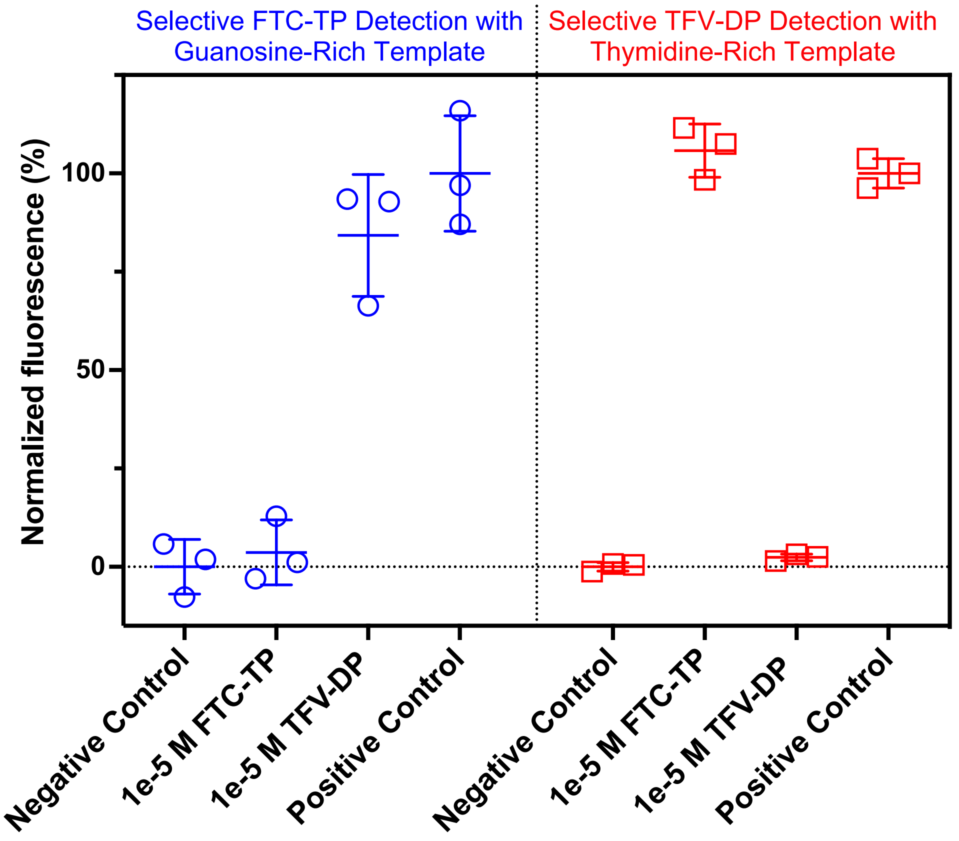

Figure 5: Detection of TFV-DP and FTC-TP using DNA templates designed for selective detection of each drug without cross-reactivity. TFV-DP (A analog) was detected with a thymidine-

rich DNA template that excluded G bases to prevent cross-reactivity with FTC-TP (C analog). Similarly, FTC-TP was detected with a guanosine-rich template that excluded T bases. N = 3. Error bars indicate standard deviation.

Benefits of Probabilistic Model for RESTRICT assays: We developed a probabilistic model that guides the design and optimization of RESTRICT assay parameters including dNTP concentration, template length, and template sequence. Although other models of the binding of nucleotide analogs to HIV-1 RT have previously been developed,[38,39] those models were mainly focused on the implications of nucleotide analog binding on treatment efficacy *in vivo* using biologically relevant HIV RNA templates. Conversely, our probabilistic model is focused on optimizing the performance of our *in vitro* diagnostic assay for measuring drug concentrations in clinical samples. The model is useful for systematic design of RESTRICT assays so that the linear region of the assay overlaps with the clinical range of the nucleotide analog of interest. To that end, we use synthetic DNA templates (more inhibitor-resistant than RNA templates) designed specifically designed to maximize the likelihood of RT inhibition for different nucleotide analogs. RESTRICT assays detect multiple nucleotide analogs by modifying the sequence of the template DNA and including/excluding specific nucleotides to enable Watson-Crick-Franklin base pairing of target drugs without cross reactivity with companion drugs.

Potential for Integration into Point-of-Care Format: RESTRICT assays are completed in less than one hour using readily available nucleic acid analysis reagents and a fluorescence reader.[30] RESTRICT assays are user-friendly and could be integrated into a POC format for use directly at the point of need (e.g., patient’s home, doctor’s office, or event setting) by using freeze-dried reagents and a low-cost fluorescence reader. We have previously shown that blood dilution to lyse red blood cells is a simple and effective sample preparation strategy to release TFV-DP from red blood cells and minimize non-specific RT inhibition by blood matrix components in whole blood samples from PrEP clients.[31]

Limitations of RESTRICT assay: RESTRICT currently focuses on nucleotide analogs (like TFV-DP and FTC-TP) that accumulate in RBCs and allow simple drug release by blood dilution. Extending RESTRICT to drugs that accumulate primarily in PBMCs rather than RBCs (e.g., AZT-TP) would require additional sample preparation to isolate and lyse PBMCs. Another limitation of RESTRICT is that the empirically derived K_{aff} parameter in our probabilistic model depends on the type (DNA or RNA) and sequence of nucleic acid template and the choice of RT enzyme used. Given the template preferences observed in RT activity assays,[32,33] we expect that K_{aff} might need to be determined empirically for each template-drug pair.

Conclusions

Our results demonstrate that RESTRICT assays can detect multiple nucleotide analog drugs used in HIV treatment and prevention. The activity-based approach to measuring nucleotide analogs in clinical samples presented here could be extended to detect other drugs used in infectious and non-communicable disease management. Nucleotide analogs and polymerase inhibitors are used to treat hepatitis B,[40] herpes,[41] tuberculosis,[42] and cancer.[43,44] Assays like RESTRICT could support therapeutic monitoring and precision dosing of nucleotide analogs for a variety of diseases to ensure efficacy and safety. As awareness of the clinical need and utility of medication adherence and therapeutic drug monitoring increases, we anticipate that activity-based diagnostic assays will play a key role in the development of POC diagnostics in this burgeoning translational research area with global and public health impact.

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Author contributions

Conceptualization: AOO, ATB, BPS, DS, JDP, PKD

Methodology: AOO, AHG

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Competing interests

AOO, BPS, DS, ATB, PKD, and JDP are listed as inventors on a patent filed based on this work. (PCT/US2020/037609).

Data and materials availability

All data and code used in the analyses are available at: doi.org/10.5281/zenodo.5140303

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