An enzymatic assay for intracellular lamivudine triphosphate to enable objective antiretroviral adherence monitoring

Caitlyn Kwong

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Committee:

Ayokunle Olanrewaju

Kenneth Mugwanya

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ABSTRACT

Accessibly Measuring Lamivudine Triphosphate in Dried Blood Spots for HIV Drug Adherence Monitoring in Low-Resource Settings

Caitlyn Kwong

Chair of the Supervisory Committee:

Ayokunle Olanrewaju

Department of Bioengineering

Antiretroviral therapy (ART) and pre-exposure prophylaxis (PrEP) have overcome significant obstacles in the treatment and prevention of human immunodeficiency virus (HIV). However, poor adherence can lead to adverse patient outcomes, including drug resistance and treatment failure. Adherence measurement is therefore vital for efficacious HIV treatment to ensure dose optimizations and timely interventions. The REverse transcriptase ACTivity with CRISPR-enabled readout (REACTR) assay measures drug levels based on the inhibition of the HIV reverse transcriptase (RT) for deoxyribonucleic acid (DNA) synthesis. Using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) complexes and fluorescence reporters, synthesis can be tracked via enzyme activity in real-time through fluorescence output, with high HIV RT activity producing higher fluorescence. A noticeable gap in the development of REACTR is the lack of testing with other classes of antiretroviral drugs such as lamivudine triphosphate (3TC-TP). To allow for compatibility of 3TC-TP, a new DNA template has been developed to provide an adequate drug inhibition curve, taking length, nucleotide composition, and secondary structures into consideration. Distinguishability of drug concentrations was improved utilizing these new templates, showcasing the tunability of REACTR. For further optimization and exploration of REACTR components, components were tuned to showcase a preference for an imbalance in deoxynucleotide triphosphates (dNTP) at a rate of 1 dCTP per 20 dTAGTP. Early dried blood spot testing has showed adequate results in detecting spiked clinically relevant 3TC-TP concentrations with high specificity and sensitivity. Future developments will move to enable lateral flow readouts and test clinical samples.

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1 INTRODUCTION

1.1 BACKGROUND AND SIGNIFICANCE

Human immunodeficiency virus (HIV) continues to be a significant global health challenge since it's first documentation in 1981 [1]. As of 2024, 40.8 million people were living with HIV across the globe with 1.3 million new infections that year [2]. [3]. Spread through contact with bodily fluids of a person with HIV, including unprotected sex, through sharing needles, or mother-to child, HIV is a virus that weakens a person's immune system, making them susceptible to infection and disease. Left untreated, HIV leads to acquired immunodeficiency syndrome (AIDS). At this stage, the body's immune system is severely impaired and cannot fight infections. An estimated 630,000 people globally died from an AIDS-related illness in 2024 [2]. The average survival for those with AIDS not taking medication is about three years. There is no effective cure for HIV, it is a for life virus.

Though there is no cure, antiretroviral therapy (ART) has transformed an HIV diagnosis from a death sentence, to a manageable chronic condition. Additionally, pre-exposure prophylaxis (PrEP) protects partners or people at risk of HIV from transmission as a precautionary measure. PrEP and ART have played a crucial role in treating HIV to become a manageable virus. In 2024, 31.6 million people were accessing ART, contributing to the large reduction of AIDS-related deaths since its peak in 2004 by 70% [2]. In the United States, most people living with HIV do not progress to AIDS thanks to antiretroviral therapy [3].

ART reduces the amount of HIV in the blood, called viral load. Viral suppression is when a person's viral load is low enough to be undetectable. The Prevention Access Campaign launched U=U (Undetectable = Untransmittable) in 2016 as a global health movement implementing the idea that if someone living with HIV on treatment has an undetectable viral load, they cannot transmit HIV to their partners [4]. This campaign looked to advocate universal access to testing services and improve treatment adherence.

HIV treatment adherence plays a large part in a person living with HIV's quality of life. Once diagnosed, it's important to discuss and receive treatment as soon as possible to prevent both the spread of HIV and progression to AIDS. HIV treatment adherence means that a person has started treatment, is taking their medications as prescribed, and maintains their medical appointments (>95%) [5], [6], [7]. By maintaining good adherence, a patient's viral load is significantly decreased and reduces their risk of drug resistance, leading to decreased risk of IV transmission, better overall health and improved quality of life. Poor adherence (<95%) can include missed or late doses, discontinuations, and partial dosing, which can increase the risk of drug resistance, treatment failure, and transmission to others. Since HIV does not have a cure, it requires lifelong treatment and regular monitoring by health care providers. Consequently, this can lead to pill fatigue and decrease in adherence [8]. Some factors affecting adherence can be a patient's schedule, side effects, transportation/access to treatment/appointments, housing, support system, financial circumstances, etc. [5], [9]. Adherence continues to be difficult to achieve and monitor accurately in resource limited settings.

The Joint United Nations Programme on HIV/AIDS (UNAIDS) adopted the 95-95-95 HIV testing, treatment, and viral suppression targets in hopes of combatting this challenge. That is, at least 95% of people living with HIV know their HIV status, of which 95% of people who know their status are on treatment, of which 95% of people on treatment are virally suppressed. The goals of 95-95-95 were originally meant for a deadline in 2025, with some regions achieving the goals in 2023 (Botswana, Rwanda, Eswatini, Zimbabwe, and the United Republic of Tanzania) but globally falling short. In 2024 globally, 87% of all people living with HIV knew their status, of which 89% were accessing treatment, of which 94% were virally suppressed [2]. There were many factors as to why some regions performed better than others, such as financial investments and legal/political frameworks to protect human rights.

Another gap is in pediatric populations. An oftentimes neglected population when discussing HIV/AIDs and the importance of PrEP and ART are children ages 0-14. Children account for 3% of all people living with HIV, 9% of new HIV infections and 12% of all AIDs-related deaths [10]. Adherence for children is especially difficult as their options of medication formulation are more limited. Their dosage is impacted by their age and weight to decrease the chance of side effects and drug toxicities. Children ages 0-14 are least likely to receive HIV treatment due to decreased implementation guidelines, lack of age-appropriate formulations, less

attention/funding, and inadequate support systems [11]. To improve adherence is an individualized process, often, the child's adherence is dependent on a caregiver, introducing potential psychosocial, behavioral, and structural barriers based on the child and caregiver's environment. This means children are especially at risk of treatment failure, further emphasizing the importance of an accessible means of routine adherence monitoring such that immediate intervention can be taken in the case of low adherence.

Current methods of measurement for drug level feedback are inadequate for point-of-care testing. The gold standard for quantifying NRTIs is via liquid chromatography tandem mass spectrometry (LC-MS/MS), which is highly sensitive and specific. However, LC-MS/MS requires extensive training and sophisticated instrumentation to maintain high quality control and performance, making it a time-consuming (~1-2 weeks) and costly process [12]. This makes it an inadequate method of testing in low-resource settings where the burden of HIV is the greatest. Other commonly used method include self-reporting, where the patients reports their adherence in the form of a questionnaire or interview, and pill monitoring, such as pill counting or monitoring pharmacy-based refills [11]. These methods have the lowest cost with minimal patient burden, as it requires no direct patient sample extraction. However, this subjective method also has a high susceptibility to recall bias, social bias, and inaccurate memory leading to estimates that are 10-20% higher [13]. Therefore, it is evident that there is a need for an inexpensive, simpler, and objective method of adherence monitoring.

1.2 INNOVATION

HIV attacks and replicates in the target human cell through a life cycle, with reverse transcription via reverse transcriptase (RT) being one stage [14]. RT uses a primer and template to convert a viral single stranded RNA into a double stranded DNA (dsDNA). This genetic material can then be integrated into the target cell's genome to replicate and assemble into more HIV to take over more cells. This process is rapidly repeated until the human immune cells are compromised.

There have been various HIV medications that target various stages of HIV. Those that target the reverse transcription stage are classified as nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reserve transcriptase inhibitors (NNRTIs) [15]. Both classes halt the synthesis of RNA into dsDNA before it can be integrated into the cell. NNRTIs inhibit the RT enzyme directly, whereas NRTIs stop synthesis of RNA into DNA. By measuring the synthesis of dsDNA we can develop an understanding of RT activity and thus drug activity.

A strong potential in rapid and inexpensive nucleic acid detection is in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas12a systems [16], [17], [18]. CRISPR-Cas is a natural mechanism found in bacteria and archaea which target and degrade foreign nucleic acids as a form of adaptive immunity. CRISPR-Cas12a (Cpf1) uses guide RNAs (crRNA) for crRNA-directed cleavage indiscriminately.

The REverse-transcriptase ACTivity-crispR (REACTR) assay has been prior developed by the Olanrewaju Lab [19]. This assay utilizes the CRISPR-Cas12a enzyme to target DNA at specific locations and its ability to collaterally cleave quenched reporters to emit fluorescence, making it an ideal system for real-time measurements (Figure #). At high drug concentrations, termination of synthesis occurs due to impedance from the drug, resulting in shorter sequences before the CRISPR recognition sequence is synthesized. At low concentrations, HIV RT is able to synthesize the full length of a DNA, resulting in synthesis of the CRISPR recognition sequence and higher CRISPR-Cas12a activity. This activity causes collateral cleavage of reporters from quenchers to emit fluorescence that can then be measured using any fluorescence reader in real time to infer drug concentrations.

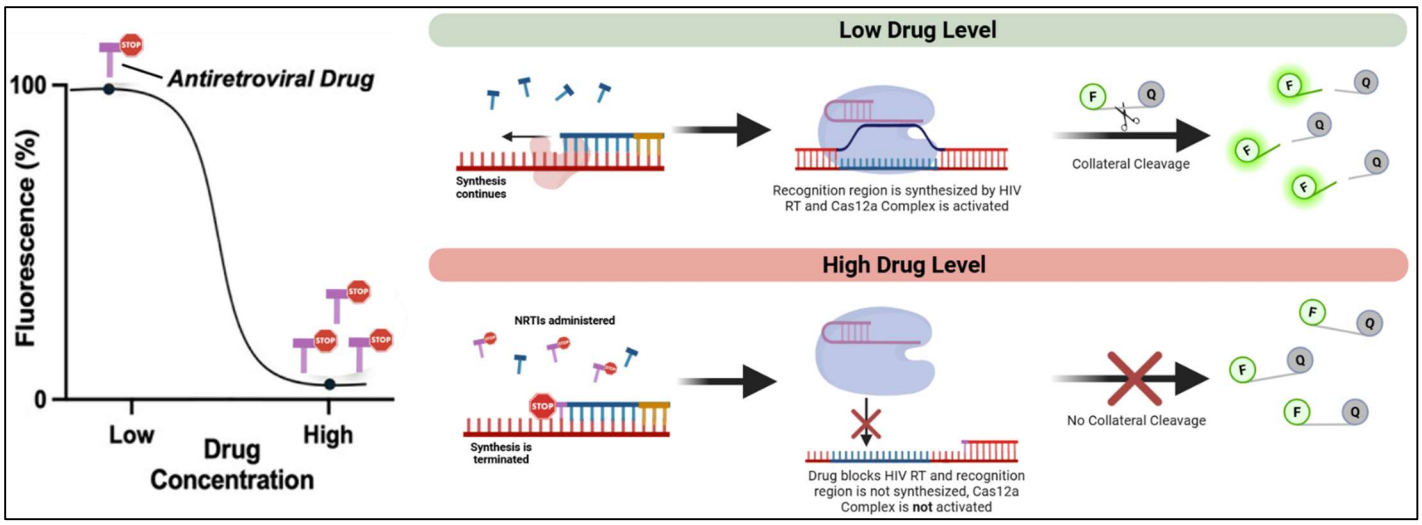


Figure 1. REACTR Assay workflow, showcasing low (top) and high (bottom) drug concentrations and their corresponding activity levels and fluorescence output.

There are now various ART regimens that can be administered based on an individual's needs, with most containing two NRTIs as the initial regimen backbone. This assay has been previously validated in lab with NRTIs such as TFV diphosphate (TFV-DP) and zidovudine triphosphate (AZT-TP), as well as ganciclovir triphosphate (GCV-TP) [19], [20]. These drugs, however, have a slightly difference mechanism of inhibition than others. Though there have been great strides in the development of REACTR, there is a lack of drug diversity in the drugs studied. One being lamivudine (3TC). 3TC is one of the preferred backbones for ART regimens in pediatric use, as it is typically well tolerated with few adverse effects [21]. On the other hand, tenofovir disoproxil fumarate (TDF), a newer formulation of TFV, is only approved for children at least two years old due to potential renal and bone toxicity. Tenofovir alafenamide (TAF) is preferred to TDF due to its lower risk of toxicity, but is only offer in FDC tablets to be swallowed whole, a challenge for small children. Zidovudine (AZT) is recommended for pediatric use but is used in conjunction with 3TC. Not only is 3TC vital for pediatric use, but is also used in various combination therapies for adults or for those who have treatment failure with other drugs.

3TC is an analog for cytidine, whereas the drugs detailed, AZT and TFV, are both thymidine analogs. Compared to TFV, something to note is the absorption of 3TC. 3TC is rapidly absorbed after oral administration, reaching maximum concentrations between 0.5 and 1.5 hours after dosage with 3TC-TP having an intracellular half-life of 15 to 16 hours [22], [23]. This is drastically different from the half-life of TFV-DP at 17 days [24]. Integrating 3TC into the assay allows for measurements of short-term and long-term adherence to NRTIs.

1.3 Proposal

The structure of this study was conducted based on the recently developed REACTR Assay [20], [25], [26]. Past studies prove validity of REACTR with measuring tenofovir-diphosphate (TFV-DP), ganciclovir triphosphate (GCV-TP), and was further validated by self-producing replicable data in the lab prior to working with 3TC-TP. The model for REACTR was also further confirmed with a MATLAB code provided by mentor Maya Singh, based on reaction kinetics and concentrations of reagents.

The general template structure shown in Figure 2 has been prior validated for work with REACTR [20], [27]. This template is composed of five primary sections. Poly T tails of 10-Ts have been shown to increase Cas12a-crRNA fluorescent signals, potentially due to increased activation and trans-cleavage activity [28]. Protospacer adjacent motif (PAM) regions are short sequences used to mark target sites for binding and the CRISPR Recognition Region is where the crRNA binds to for activation of Cas12a activity [28], [29]. Preservation of established template regions, particularly that of the CRISPR recognition region, is vital to the functionality of the REACTR Assay as well as the primer region for the HIV RT to interact with for synthesis to occur.

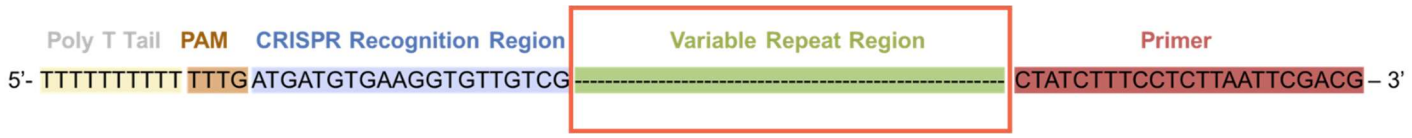


Figure 2. General structure of REACTR template strand, composed of poly T tail, PAM, CRISPR recognition region, variable repeat region, and primer. Variable repeat region is edited to account for drug specificity and then optimized.

To test design iterations, the OligoAnalyzer™ Tool by Integrated DNA Technologies was used to evaluate max ΔG Hairpin, max ΔG Self Dimer sequence, max self-dimer, and where self-binding occurs. The parameters were set for qPCR with a target type of DNA. The oligo concentration was set to 0.05 µM, 0 mM Na^{+}, 10 mM Mg^{++}, and 0.005 mM dNTPs. After inputting the designed template, data was recorded in Table S1. Templates were then purchased from Integrated DNA Technologies for testing implementation.
The REACTR assay will be adapted to measure 3TC-TP in dried blood spots (DBSs). The REACTR Assay has been prior adapted to measure GCV-TP in DBS and was thus applied to 3TC-TP with further optimization [20]. By using DBS from healthy blood donors not receiving any antiretroviral therapies, 3TC-TP can be spiked into blood elutions at varying concentrations of interest.

2 Materials and Methods

The developed enzymatic assay uses the SpectraMax iD3 Molecular Devices Plate Reader for fluorescence measurements. 5X HIV RT Buffer was made with 25 0mM Tris-HCl at pH 8 (77-86-1, Sigma-Aldrich), 250 mM Potassium Chloride (60142-100ML-F, Sigma-Aldrich), 75 mM Magnesium Chloride ($MgCl_2$) (7786-30-3, Sigma Aldrich), 35 mM dithiothreitol (DTT) (3483-12-3, Sigma-Aldrich), and 0.3% Triton X-100 (9036-19-5, Sigma-Aldrich). 1X HIV RT Buffer was made by diluting 5X HIV RT Buffer in water. All reagents were suspended in Tris-EDTA (TE buffer) in working aliquots before further dilution with deionized water (DI H_2O) and/or HIV RT Buffer.

2.1 Buffer REACTR Workflow

The Cas12a-crRNA complex was created by combining 1X HIV RT, 25 nM LbCas12a (M0653T, New England Biolabs), 25 nM crRNA (Integrated DNA Technologies, Sequence provided in Table S2)

Lamivudine-5'-triphosphate (3TC-TP) (Sierra Bioresearch, Lot No. LM2558) was diluted into nuclease-free water at various concentrations from 10^{-4} to 10^{-12} M. The Cas12a-crRNA complex was then created by combining 2.5E-2 µM LbCas12a (M0653T, New England Biolabs) and 2.5E-2 µM crRNA (Integrated DNA Technologies) in HIV RT buffer containing 50 mM Tris-HCl (77-86-1, Sigma-Aldrich) at pH 8, 50 mM Potassium Chloride (KCl) (60142-100 ML-F, Sigma-Aldrich), 10 mM Magnesium Chloride (MgCl2) (7786-30-3, Sigma-Aldrich), 2 mM dithiothreitol (DTT) (3483-12-3, Sigma-Aldrich), and 0.06% Triton X-100 (9036-19-5, Sigma-Aldrich). The complex was then incubated at 25°C for 15 minutes. HIV RT enzyme (LS005009, Worthington Biochemical) was then diluted in 1X RT Buffer to a final concentration of 0.053 units/µL. The Master Mix used may vary based on the conditions set, but was made up of 1 M MgCl2 and 1 M DTT, deoxynucleotides (dNTP) (N0447L, New England Biolabs), 20 µM RT primer, 2 µM DNA template, and 20 µM reporters (Integrated DNA Technologies), 5X RT buffer, and nuclease-free water. In a black opaque 96-well non-binding plate (3650, Corning), the reagents were added in the following order and quantity: 8 µL master mix, 10 µL cas12a-crRNA, 10 µL 3TC-TP, and 2 µL HIV RT. Ensure that this order is followed to allow the drug to integrate before HIV RT interferes. Positive and negative controls were also conducted. Positive controls contained no drug and instead had 10 µL of nuclease-free water, showcasing the maximum functionality of HIV RT with absolutely no drug. Negative controls also had nuclease-free water instead of the drug, with the addition of having 2 µL 1X RT Buffer instead of 2 µL HIV RT, showing a minimal to no signal when the system is functioning properly. The plate was then shaken for 60 seconds and incubated at 37°C in a microplate reader (SpectraMax iD3, Molecular Devices) for 120 min with fluorescence reads at 2-minute increments.

2.2 Dried Blood Spot REACTR Workflow

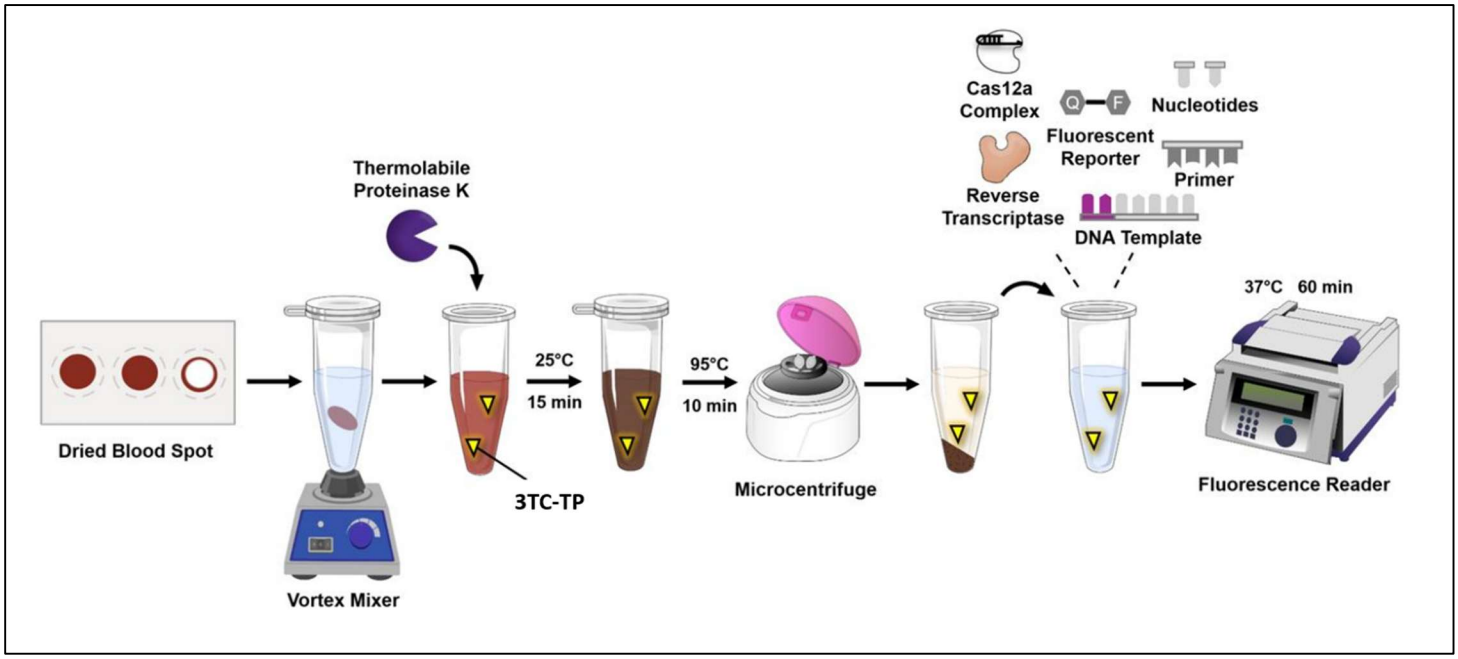


Figure 3. DBS workflow for downstream REACTR integration. Figure adapted from Chernoske et al., 2025

A dried blood spot (DBS) (BioIVT, 25 µL spotted onto cytiva Whatman™ 903 Proteinsaver cards) is vortexed with DI water to a 50% blood concentration for elution. 3TC-TP dilutions are added to the blood elutions along with DI water and 2.5% thermolabile proteinase K (TPK) per blood elution mix. TPK is added for denaturing of blood proteins to reduce non-specific inhibition of HIV RT by peripheral blood matrix components, including hemoglobin and immunoglobulins [20]. The blood elution mix is added with standard REACTR mixes (Master Mix, Cas12a-crRNA, and 5.3% HIV RT). Final drug concentrations after addition of REACTR mixes should convey concentrations of interest.

2.3 Data Analysis

The fluorescence levels were then plotted primarily in two ways. The first method showed the relationship between time and fluorescence levels between the different conditions. The second method showcased the enzyme inhibition curve, which was generated at the 60-minute time point and normalized using the positive and negative controls as maximum and minimum values. The graphs produced were fit to the logistic regression curves in GraphPad Prism 10. Data analysis includes the half-maximal inhibitory concentration (IC50), which measures the concentration at which the assay determines to inhibit dsDNA synthesis by 50%. The clinical range of interest is 1.26e-9 to 7.55e-9 M, meaning the IC50 should ideally fall within this range [30].

3 Template Design and Development

3.1 Decreasing Self-Binding

Using OligoAnalyzer, a variety of templates were placed into OligoAnalyzer to determine max ΔG Hairpin, max ΔG Self Dimer sequence, max self-dimer, and where self-binding occurs. The section of interest was the variable repeat region. This region was adjusted using various of patterns of nucleotides at varying lengths, with all patterns containing a guanine composition of ≥50%. An increase in guanine provides more opportunities for 3TC-TP to bind, but an abundance could risk the formation of secondary structures such as G-quadruplexes, which would disrupt RT activity due to template design rather than drug interaction [31]. Templates must also be stable to withstand

temperatures up to 37°C and be stable for synthesis with HIV RT, this can be determined based on the provided melting temperature and experimentation with buffer. The values produced can be seen in Table S11.1.1.

Templates with a longer length, 117 to 200 nucleotides, could provide increased opportunities for drug binding, but also posed a higher risk of hair pinning. They had a lower ΔG Hairpin (max) and typically had a higher ratio of ΔG/Self Dimer. These templates would have an excess of secondary structures, rendering the template unable to synthesize properly with the HIV RT. As much template as possible should remain available as a full single strand for reverse transcription. Although shorter templates were considered for faster synthesis, too short a template does not provide enough nucleotides for drug binding causing there would be reduced assay sensitivity. The template

Most templates had some degree of self-binding and dimerization with most binding at the primer location, often primer to primer, which would not pose a strong point of concern. However, templates that bind to the CRISPR Recognition Region were discarded as hindrance of the recognition region would greatly reduce the likelihood of the Cas12a-crRNA complex binding and thus decrease the effectiveness of the assay.

3.2 GGGC80NT

GGGC80nt contains the pattern GGGC repeated 5.75 times for a total of 80 nucleotides with the Poly T Tail, PAM, CRISPR Recognition Region and Primer. It exhibited the highest ΔG Hairpin (max) at about -2.3 and a higher ΔG/Self Dimer ratio of about 26.499. Upon further inspection, their proposed secondary structures did not seem to cause a conflict or self-binding within the CRISPR recognition region highlighted in blue in the table below. The self-dimer binds primer to primer and was not a point of concern.

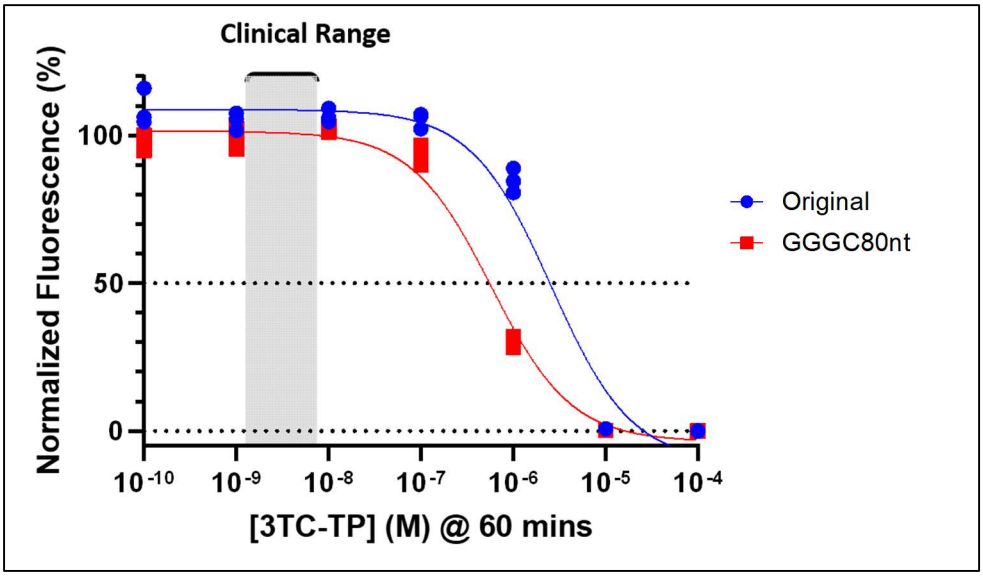


Figure 4. Original Template's 3TC-TP inhibition curve (Blue) IC_{50}: 2.577e-6 M (1.806e-6 to 3.681e-6) vs. GGGC80nt's inhibition curve (Red) IC_{50}: 5.770e-7 M (4.500e-7 to 7.364e-7 M)

REACTR was conducted with 5 nM template and 500 nM dNTP. Assessment of GGGC80nt was compared to a prior confirmed template for use with TFV-DP measurements. The GGGC80nt had a 50% inhibitory concentration (IC_{50}) (95% Confidence Interval [CI]) of 5.770e-7 M (4.500e-7, 7.364e-7 M), while the original template had 2.577e-6 M (1.806e-6, 3.681e-6). This shift left of the curve suggests that the newly developed template is better optimized for 3TC-TP concentration measurements as 3TC-TP's estimated clinical range is much lower than that of TFV-DP [32]. This showcases the tunability of REACTR for various antiviral drugs, as prior validated [20], [27]. Further optimization with GGGC80nt was conducted to further shift the drug inhibition curve left, however, hypothesized restrictions due to nonspecific synthesis of HIV RT led to further template design. Buffer optimizations of GGGC80nt are detailed in Section 4.

3.3 GTGT80NT

While GGGC80nt proved the need for a guanine-based repeat region, GGGC80nt showed limitations in optimization. After optimization, REACTR was conducted with 1 nM template and 50 nM dNTP. Though thymine was initially avoided in the repeat region due to concerns regarding future use with other antiretroviral drugs and its potential for cross reactivity, HIV RT is hypothesized to show a preference for synthesizing thymine heavy templates. This was further suggested by work in buffer, in which GTGT80nt has an IC_{50} of 4.574e-9 M (2.331e-9, 1.302e-8), a large shift left compared to the GGGC80nt with an IC_{50} of 8.705e-8 M (2.220e-9, 1.370e-5). Though further confirmation of HIV RT's synthesis preference is being evaluated, GTGT80nt was used as the primary template of choice for dried blood spot experiments.

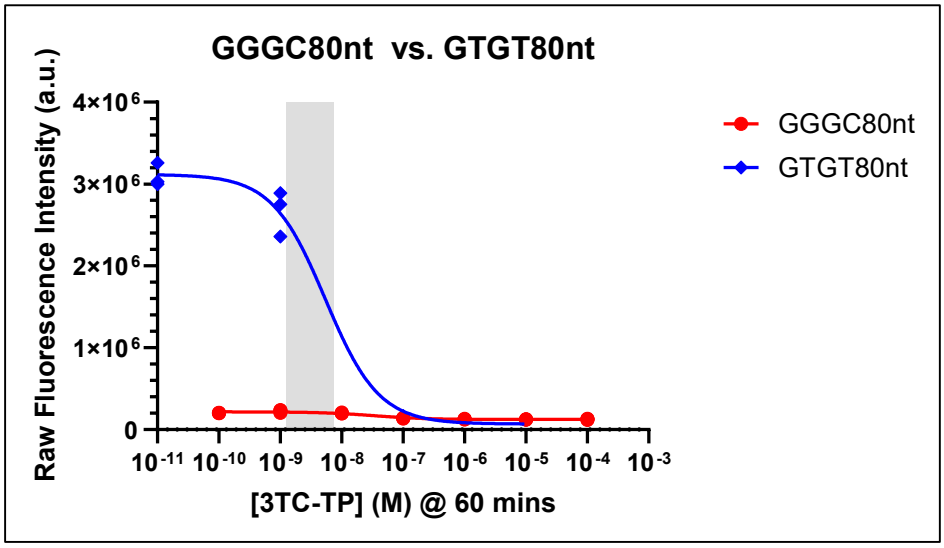


Figure 5. GGGC80nt's raw fluorescence 3TC-TP inhibition at 60 mins (red) vs. GTGT80nt (blue)

4 BUFFER OPTIMIZATION

The concentration range of interest for 3TC-TP was adjusted from Thompson et. al., in which 3TC-TP was quantified via LC-MS assays from 6 mm Whatman dried blood spots in healthy volunteers receiving TDF/3TC [32]. The range of interest is 7.55E-09 to 1.26E-09 M with 3.57801E-9 M representing the concentration of 3TC-TP 24 hours post-cessation (C_{48}).

4.1 TEMPLATE AND DEOXYNUCLEOTIDE TRIPHOSPHATES

Prior in lab experiments confirmed the ideal dNTP to template ratio to be 50 nM dNTP per 1 nM Template. As the concentration of dNTP and template increased together, the raw fluorescence collected increased. To determine the minimum concentration of dNTP and template needed, various template concentration's inhibition curves were analyzed concurrently (Figure 5a).

At 30 minutes, 1 nM Template: 50 nM dNTP was unable to produce a drug inhibition curve (Supplemental 13.4d). Though the %CV of all conditions was <10%, the IC_{50} was 8.05e-8 M with a wide CI of 2.22e-9 to 1.37e-5 M. This shows 30 minutes to be an inefficient timepoint of measurement under 1 nM template conditions. Improvement is seen at 60 minutes, with a IC_{50} of 3.37e-9 M, CI = 1.874e-8 to 6.101e-8 (Figure 5c, 5d). Raw fluorescence signals provided in Supplemental 13.4b. Normalization at the 30 minutes time interval provided variable data that was inconclusive (Supplemental 13.4d).

Increasing to 2 nM Template: 100 nM dNTP provided improved results. At 30 minutes, the %CV of all conditions was <10%, with the no drug controls at 2.407%. The IC_{50} was 3.91e-8 M, CI = 8.42e-9 to 1.828e-7 M (Supplemental 13.4c). At 60 minutes, the no drug controls had a %CV of 1.718 and an IC_{50} of 3.941e-8 M, CI = 3.079e-8 to 5.033e-8 M. By increasing the template/dNTP concentration, the overall fluorescence intensity

was increased. Raw fluorescence signals provided in Supplemental 13.4a. After normalization, 60 minutes showed to produce the most replicable data in both 2 nM template and 1 nM template (Figure 5c).

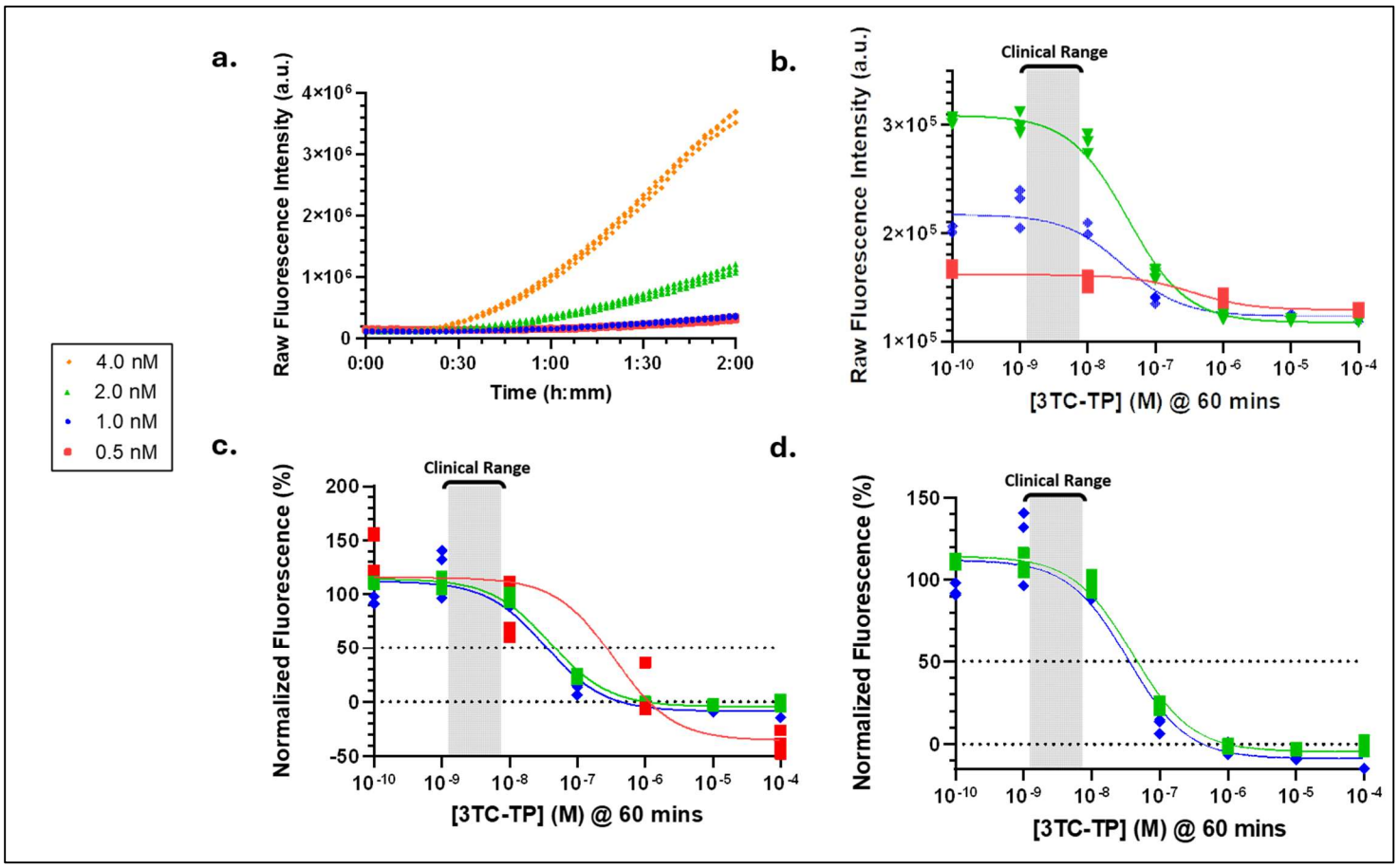


Figure 5. (a) Fluorescence of conditions over 2 hours with measurements taken at 2 minute intervals. (b) Raw fluorescence levels after 60 minutes of 2.0 nM, 1.0 nM, and 0.5 nM (c) Normalized fluorescence at 60 minutes at 2.0 nM, 1.0 nM, and 0.5 nM. (d) Normalized fluorescence of only1.0 nM and 2.0 nM template conditions. They do not have significantly different inhibition curves.

Due to the time limitation of 60 minutes, further dNTP adjustments were conducted. To promote deoxycytidine triphosphate (dCTP) competition with 3TC-TP, dCTP was decreased keeping deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), and deoxyguanosine triphosphate (dGTP) at 1 µM causing an imbalance between different dNTP groups. Future discussion groups dTTP, dATP, and dGTP as dTAGTP. This was done at a ratio of 20 dTAGTP per 1 dCTP. By increasing dTAGTP, there was a drastic increase in raw fluorescence intensity. There was a slight shift right in IC_{50}, with 1 µM dTAGTP, 50 nM dCTP, and 1 nM template, resulting in a value of 2.614e-8 M (1.608e-8, 4.259e-8 M).

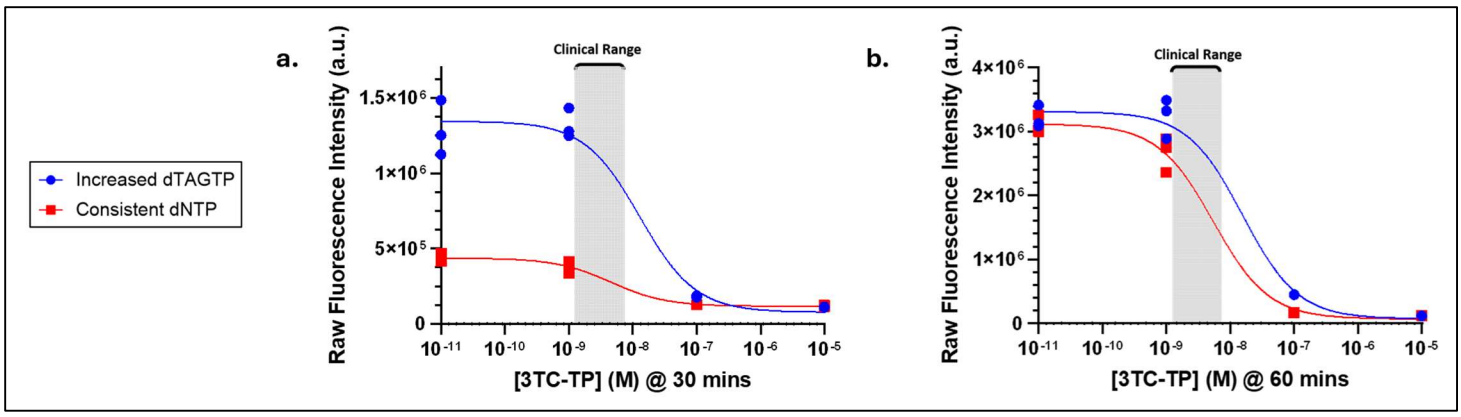


Figure 6. (a) Raw fluorescence intensity of decreased dTAGTP (blue) and standard 50nM dNTP (red). (b) Raw fluorescence of both conditions.

4.2 Read Height and Reaction Volume

To determine the effects of reaction volume, reagent volumes were adjusted from the standard 40 µL total volume. All concentrations of reagents were maintained at 1x HIV RT Buffer, 50 nM dNTP, 1 nM GGGC80nt, 50 nM primer, and 500 nM reporter. To ensure that read height does not need to be adjusted depending on the volume of the reaction, read heights 1-5 mm were tested with 2x volume after a full 2 hour incubation period to determine the impact of the Plate Reader setting "Read Height" on fluorescence readout. All reads averaged an IC_{50} of 3.17E-08 M with a standard deviation of 5.41E-10 (Figure 7a).

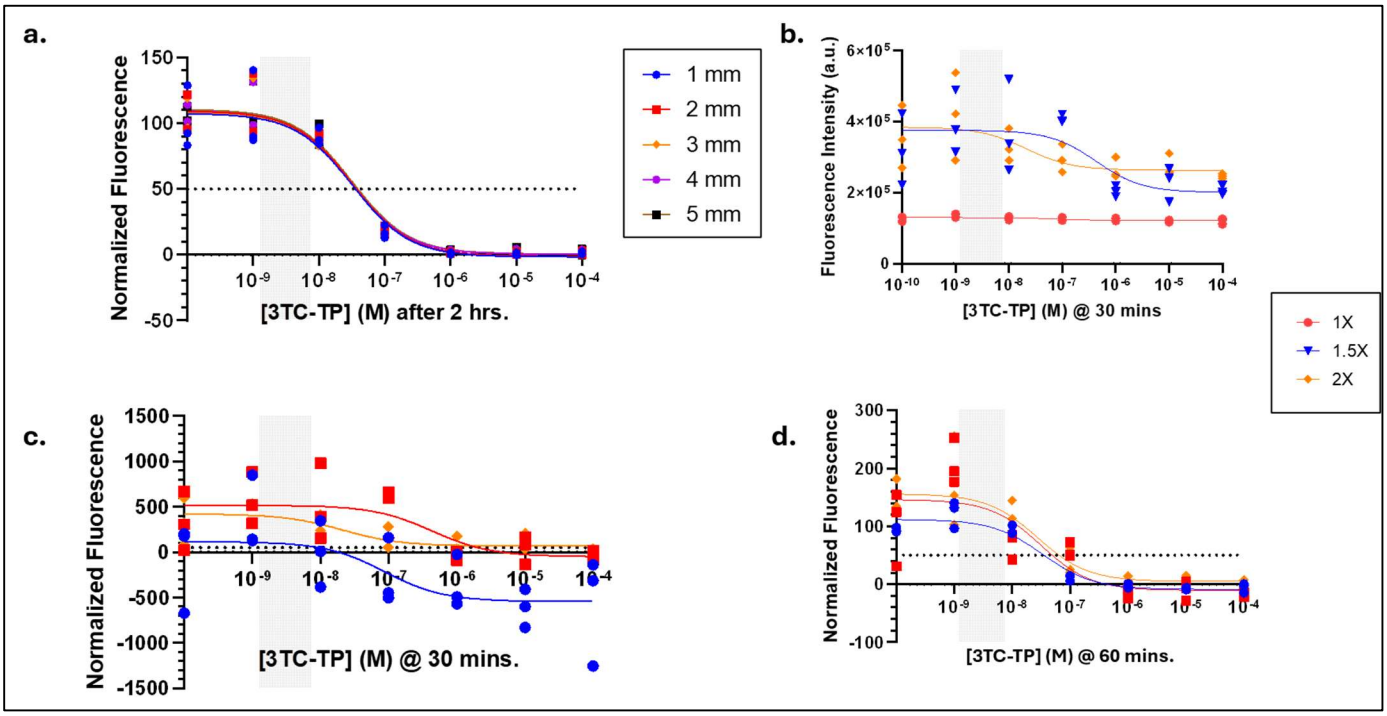


Figure 8. (a) Normalized fluorescence after 2 hours of varying read heights do not show significant differences in fluorescence reads. (b) Raw fluorescence reads of various volumes at 30 mins. (c) Normalized fluorescence reads of varying reaction volumes at 30 mins. (d) Normalized fluorescence reads of varying reaction volumes at 60 mins.

Reaction volumes 1x, 1.5x, and 2x for a total of 40 µL, 60µL, and 80 µL were tested. Increased reaction volume exhibited an increase in raw fluorescence intensity, with 1.5x having an average no drug fluorescence

of about 246516.67 and 2x having an average of 274100. These are almost both double the fluorescence provided by the average no drug fluorescence of 1x volume at 129931.33. Despite this, higher volumes also had much higher %CV and incomparable data due to poor normalization at 30 minutes. 60 minutes exhibited better normalization of all volume sizes, with 1x having the lowest %CV values.

The hypothesis that increased total reaction volume would increase raw fluorescence and sensitivity due to increased reactions not fully supported by data gathered. Read heights 1-5 mm were found to generate nearly normalized identical values, meaning that read height is not a variable that needs to be taken into consideration. Though increased reaction volume caused a significant increase in raw fluorescence read, data produced by higher volumes also had higher %CV and trouble normalizing due to its unreproducible data. Thus, 1x volume for a total of 40 µL was continued as the primary protocol.

4.3 Impact of Cas12a-crRNA, Reporter, and Primer Concentrations

To understand the impact of other reagents and their reaction kinetics with the template and dNTPs, various conditions were analyzed under no drug conditions with either HIV RT added (Positive) or no HIV RT added (Negative). All reactions were run with 0.5 nM GTGT80nt, with “decrease” indicating a halve from the original 1nM GTGT80nt protocol. That is, instead of 25 mM Cas12a-crRNA, 10 nM primer, and 500 nM reporter, a decrease would be 12.5 mM Cas12a-crRNA, 5 nM primer, and 250 nM reporter. All condition’s raw data can be found in Supplemental #.

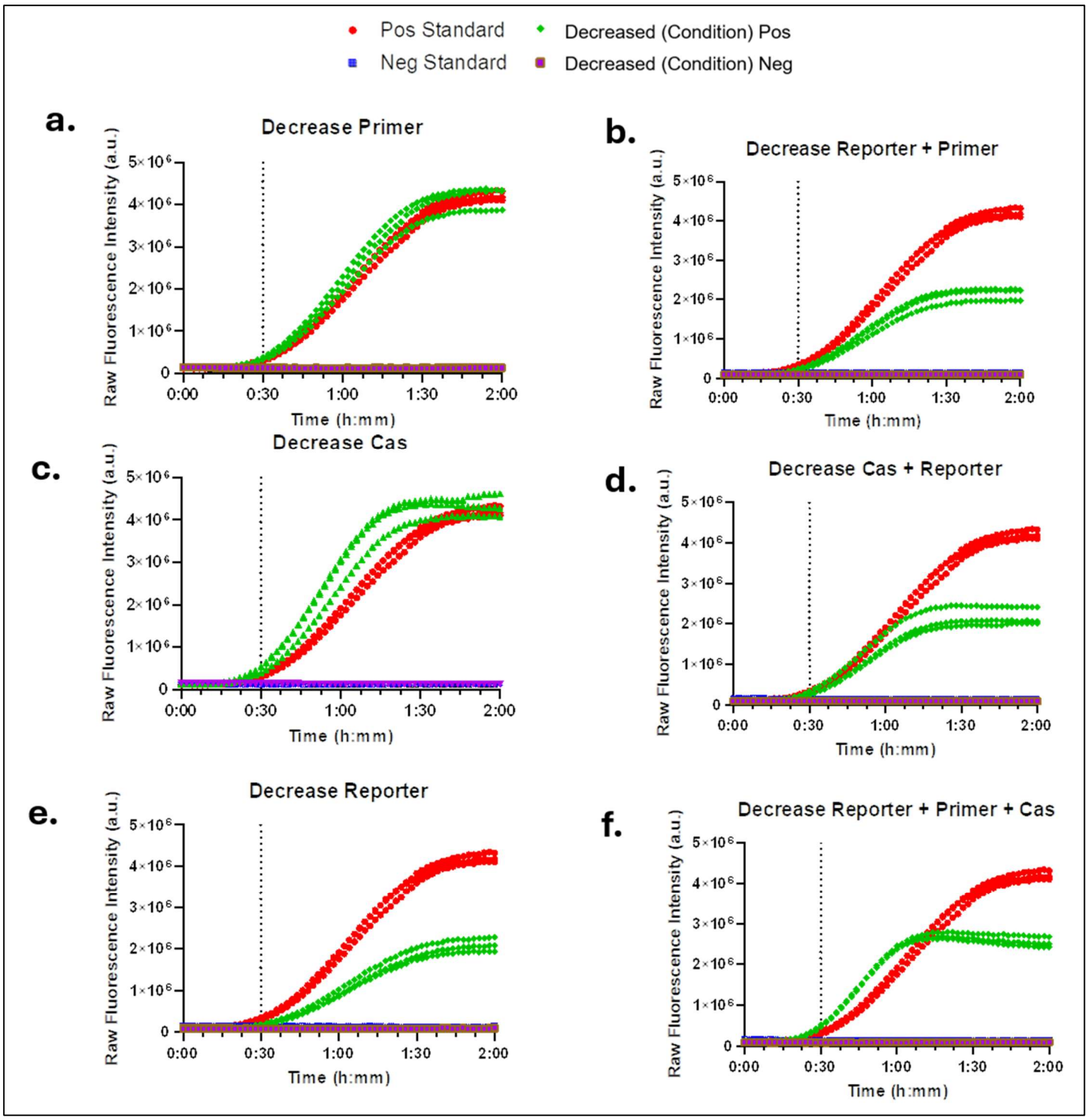


Figure 9. Real time fluorescence over 2 hours of positive and negative controls. Default conditions are shown in red and blue, adjusted conditions are shown in green and purple. (a) 25 nM primer (b) 25 nM primer and 250 nM reporter (c) 12.5 mM CRISPR-Cas (d) 12.5 mM CRISPR-Cas and 250 nM reporter (e) 250 nM reporter (f) 250 nM reporter, 12.5 CRISPR-Cas, and 25 nM primer

Most notably, the main source of decreased fluorescence was the concentration of reporters. Most conditions with half the concentration of reporters had about half the maximum fluorescence. Other conditions with only Cas12a-crRNA and only primer concentrations decreased did not show significant changes in fluorescence or reaction time.

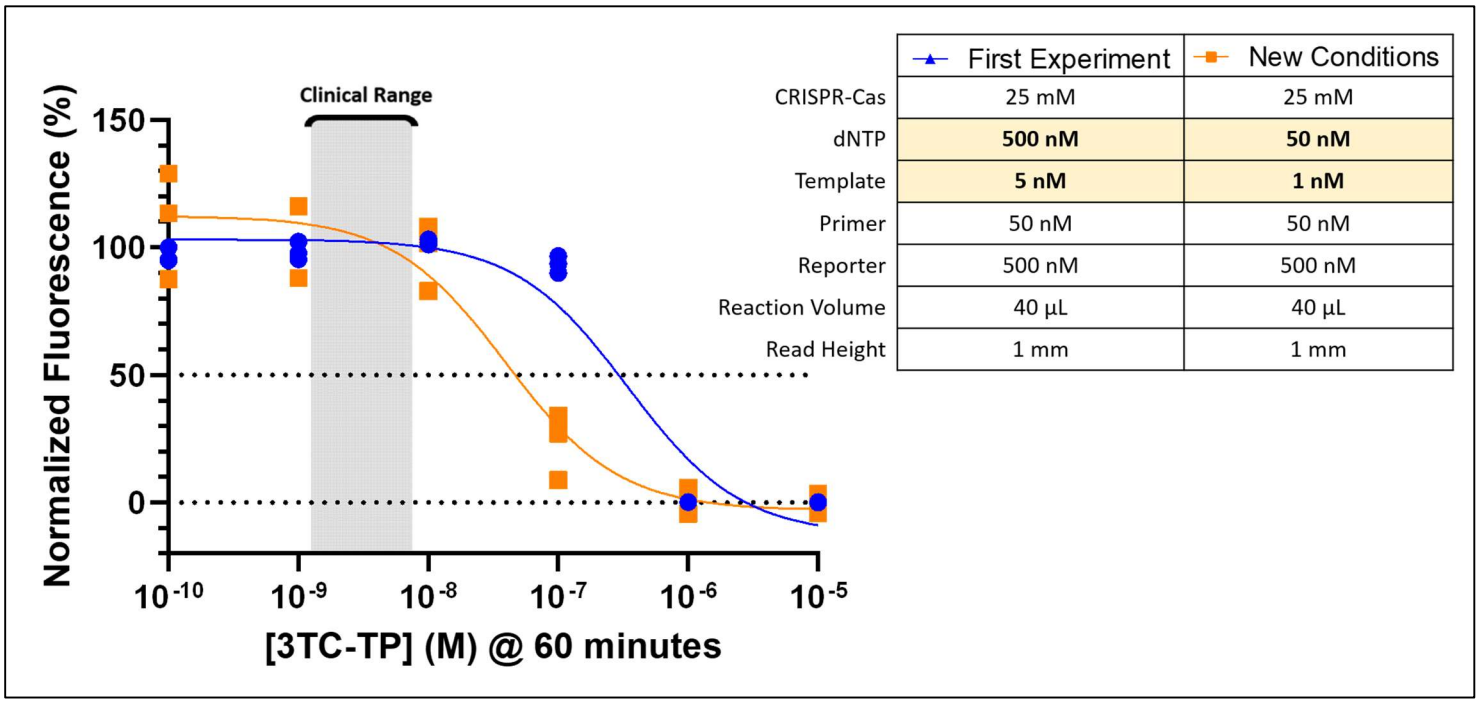
5 Buffer Discussion

Conditions considered and final choice conditions are detailed below dried blood spot testing are detailed below.

	Conditions Tested	Final Condition
Reaction Volume	1x, 1.5x, 2x	1x
Ratio of Template:dNTP	1:50, 1:10, 1:7	50:1
Template Concentration (nM)	0.5, 1, 2, 4	1
Nucleotide Ratios dCTP:dTAGTP	1:1, 1:20	1:20

Table 1. Conditions considered and final choices detailed for dried blood spot testing.

The tunability of the inhibition curve is seen in the progression from the first experiment to the final new conditions. This leftwards shift of the inhibition curve towards the clinical range using GGGC80nt showcases that the inhibition curve can be tuned to clinical ranges. Further adjustments are still needed to optimize the inhibition curve for 3TC-TP.



	First Experiment	New Conditions
CRISPR-Cas	25 mM	25 mM
dNTP	**500 nM**	**50 nM**
Template	**5 nM**	**1 nM**
Primer	50 nM	50 nM
Reporter	500 nM	500 nM
Reaction Volume	40 µL	40 µL
Read Height	1 mm	1 mm

Figure 10. Normalized fluorescence of first GGGC80nt experiment pre-optimization (blue) vs. post-optimization (orange). IC50 of pre-optimized experiments was 3.445e-7 M and post optimization was 3.977e-8 M.

6 Dried Blood Spot Optimization

The first dried blood spot (DBS) test was conducted with 40 µL reaction volume, 1 µM dTAGTP, 50 nM dCTP, and 1 nM GTGT80nt as established in buffer. The IC_{50} of this is 5.397e-9 M (3.207e-9, 9.357e-9) (Figure #). The average %CV across all concentrations was 9.73% with lower concentrations of drug exhibiting higher %CV. The Receiver Operating Characteristic (ROC) was also analyzed for sensitivity and specificity. At fluorescence levels >236726, there is a reported 100.0% (56.55,100) sensitivity and 100% (43.85,100) specificity. To determine if REACTR can be a viable measurement of 3TC-TP dosage within the past 24 hours, concentrations (n=3) were separated into two categories: Above C_{48} and Below C_{48} (Figure #).

The initial proposed conditions from buffer (40 µL reaction volume, 1 µM dTAGTP, 50 nM dCTP, and 1 nM GTGT80nt) showed to provide sufficient differentiation of spiked DBS samples, suggesting a good starting point for further optimization. Though drug measurement in buffer provides higher fluorescence, DBS exhibits a

closer IC_{50} to the clinical point of interest at C_{48}. Increased difference between concentrations 5E-9 M and 2.5E-9 M would indicate further optimization.

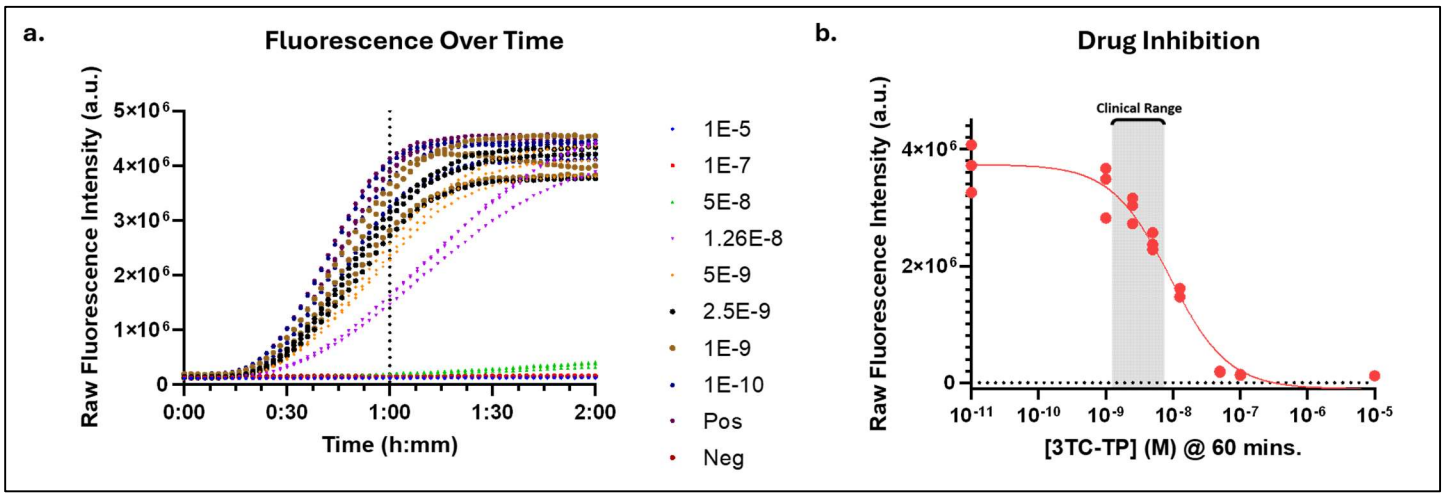


Figure 11. (a) Fluorescence over time (b) Drug inhibition at 60 minutes

7 Dried Blood Spot Discussion

To determine whether REACTR with DBS could be used as a binary indicator of 3TC-TP dosage within the past 24 hours, the concentration 24 hours post-cessation provided by Thompson et al. was converted to molarity in 7 mm DBS amounting to 3.58E-9 M. The receiver operating characteristic (ROC) presented 100% sensitivity and 100% specificity at 2689312 fluorescence. This represents the threshold between above and below the 24-hour post-cessation dosage. Those that output above a 2689312 raw fluorescence would indicate a low drug level and those below would indicate a high drug level.

A binary indicator such as this can showcase whether a patient has or has not taken their 3TC within the past day. This could be used as an early indicator of stopping treatment or whether an intervention has had an immediate effect in drug levels for a patient. This then allows for a clinician to take the proper steps to alter the treatment or provide immediate adherence counseling where needed. Early indications of treatment interruptions help clinicians to provide near-real-time, actionable feedback to prevent treatment failure.

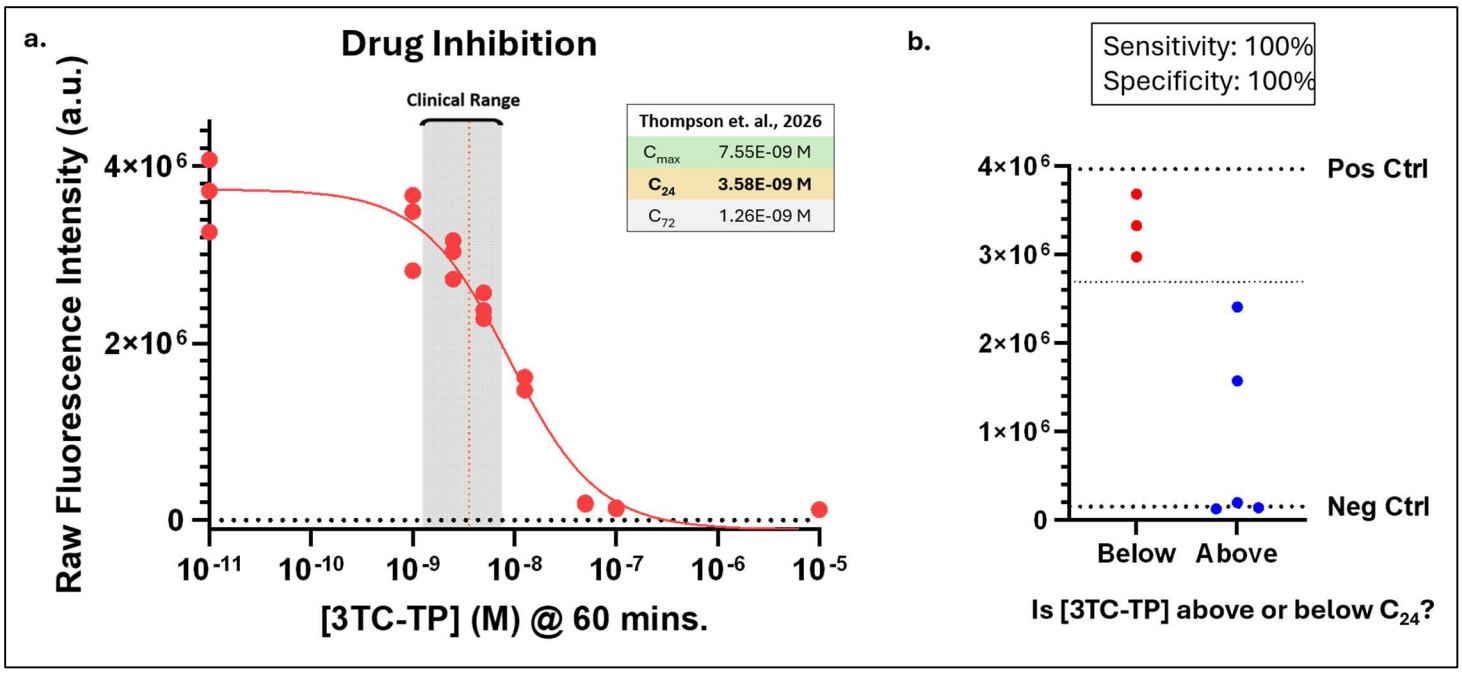


Figure 12. (a) Drug inhibition at 60 minutes (b) Binary table of raw fluorescence intensity of varying concentrations of 3TC-TP in dried blood spots based on C_{24} thresholds established in Thompson et al., 2026.

8 Conclusion

This project filled the gap in knowledge in understanding how various NRTI drugs beyond the previously drugs could be integrated with REACTR. It demonstrates the importance of template design for detection of different analog drugs, such as 3TC-TP, and how chain termination can be controlled and regulated via template structure and reagent concentrations to allow for selective detection of a specific drug. The discovery of the different templates to be used for drug level feedback with REACTR will be vital for future applications of 3TC adherence monitoring, as well as other cytosine analog drugs.

It should be noted that fluorescence levels overall did show a decrease compared to the original template, however, this is not a point of concern, as the main goal is differentiation of concentrations relative to each other. The REACTR assay still proves to be sensitive despite exhibiting an overall decrease in fluorescence and further investigation into the role of nucleotide sequence and HIV RT synthesis is ongoing. By expanding upon knowledge regarding how dNTP concentrations could affect the REACTR assay's sensitivity for varying drug concentrations, this project showcases the ability to fine-tune levels of synthesis and hence fluorescence output to adjust the shape and sensitivity of inhibition curves for clinical needs.

9 Future Work

Future work with REACTR and 3TC-TP will require further optimization to increase selectivity for 3TC-TP to increase specificity and sensitivity within the desired clinical range for DBS measurements.

Further analysis of the impact of dCTP:dTAGTP concentrations should be explored, adjusting for various ratios as well as confirming the impact on the inhibition curve. As seen in Figure 6, there appears to be a rightwards shift after increasing dTAGTP. Future work would require confirmation of this phenomenon at increased concentration conditions. Due to an increase in overall fluorescence, this could be attributed to different aliquots of reagents and experimental differences between the period at which these two experiments took place.

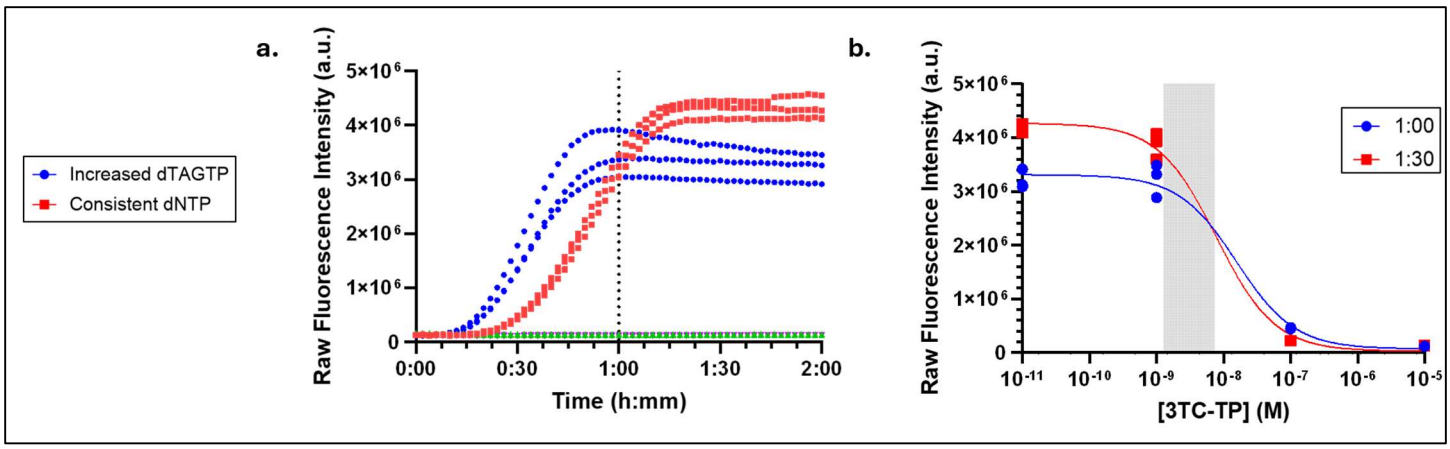


Figure 13. (a) Raw fluorescence over time. (b) Raw fluorescence of increased dTAGTP at 1:00 hr. and consistent dNTP at 1:30 hr.

Another question to investigate, includes the seemingly leftwards shift of the DBS inhibition curve compared to buffer conditions. DBS had a IC50 of 8.931e-9 M, while buffer conditions had an IC50 of 3.925e-8 M. When analyzing real time fluorescence over the 2-hour incubation period, the DBS had a higher overall fluorescence. This is not yet explained, though could be attributed to different aliquots of reagents and experimental differences between the period at which these two experiments took place.

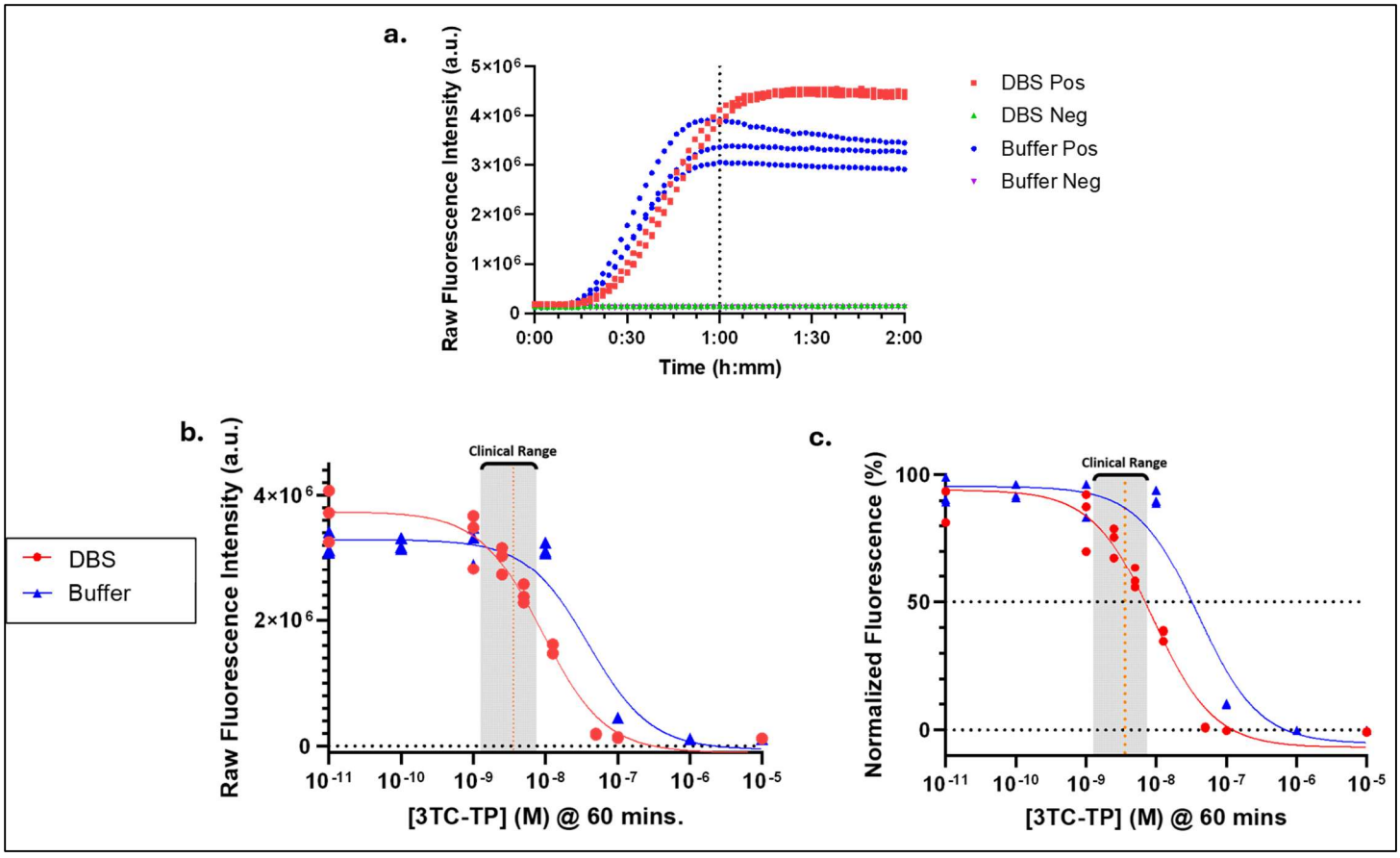


Figure 14. (a) Raw fluorescence over time of DBS vs. Buffer under same conditions (b) Raw fluorescence at 60 minutes with DBS inhibition curve (red) vs. buffer inhibition curve (blue) (c) Normalized fluorescence at 60 minutes

Understanding of HIV RT and its potential preference for nucleotide synthesis is still ongoing. This understanding is vital to developing template stands to increase sensitivity and specificity, and decrease time needed for an adequate readout.

REACTR should be compatible with any laboratory fluorescence reader but should be validated with other machines such as the T8-ISO-RA [Axxin]. This instrument is an example of a portable device with fluorescence read capabilities in real-time and has been proven to provide comparable data to that of a plate reader. Other methods of increasing accessibility include lateral flow assays. In the Olanrewaju Lab, significant strides have been made in the integration of REACTR into lateral flow assays for more point-of-care use. Clinical testing with dried blood spots provided by collaborators can be used to assess the results of REACTR compared to the gold standard LC-MS/MS method.

10 IMPACT

The global impact of this work supports global HIV management efforts, particularly that aligning with the UNAIDS' 95-95-95 targets. By contributing to the development of a means of drug level feedback in low-resource and pediatric settings, more people taking ART or PrEP will be able to achieve adequate viral suppression. The economic impact of this work aims to decrease the financial burden of adherence testing by removing the need for expensive equipment and increasing accessibility for low-income regions. This assay can be compatible with standard plate readers that are able to measure fluorescence at wavelengths of 592 nm and maintain a temperature of 37°C as well as with most lab equipment, such as a thermal cycler, and can be integrated into diagnostic workflows using existing lab infrastructure. Adequate inhibition curves can be developed after 30 minutes. This shows a possibility for an even shorter amount of time for a clinician to receive results regarding a patient's drug level feedback. With a better understanding of their drug adherence status, people living with HIV and taking ART will be better informed of their potential risk of spreading HIV, and those taking PrEP to prevent HIV will feel more confident in their protection. With this more informed drug level feedback, patients will be more equipped to change their treatment when necessary and prevent transmission and progression of HIV.

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