# Towards Rapid Measurement of Non-Nucleoside Reverse Transcriptase Inhibitors in a Portable, Low-Cost Platform

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## Abstract

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While antiretrovirals (ARV) for HIV prevention and treatment have become very effective over the last 40 years of development, many people living with HIV (PLWH) encounter inadequately suppressed viral loads and treatment failure. Providing drug-level feedback (DLF) can identify those with subtherapeutic ARV levels quickly to promote interventions to improve outcomes. Most DLF approaches measure drug levels via liquid chromatography-tandem mass spectrometry (LC-MS/MS), which can incur high costs and lengthy delays that make it unsuitable for near point-of-care settings. In response, we developed a rapid enzymatic assay for the measurement of non-nucleoside reverse transcriptase inhibitors (NNRTIs) based on inhibition of DNA synthesis using an inexpensive, portable instrument. This assay is entitled the **REverse transcriptase ACTivity (REACT) assay.** This work demonstrates proof-of-concept measurement of multiple NNRTIs in buffer and spiked plasma samples. We also demonstrate the

feasibility of using a portable reader to support routine drug measurement at or near the point of care, providing prompt feedback to facilitate rapid interventions.

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I especially want to thank my family, who never wavered in their support and kept the wind at my back. To my father, who saw me start this journey but could not be here to see me finish it, you told me to soar, and this is me jumping off the cliff.

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# 1. Summary of Dissertation Aims

Antiretroviral therapy (ART) and pre-exposure prophylaxis (PrEP) are cornerstones of HIV treatment and prevention, offering diverse options tailored to the needs of various populations. Despite advancements in antiretroviral (ARV) medications, maintaining adequate drug levels remains crucial for their efficacy (1,2). Insufficient drug levels arise for several reasons including: inconsistent medication adherence, treatment interruptions, and interindividual pharmacokinetic variations (3,4). Key populations in HIV treatment and prevention, including elderly and pediatric individuals, pregnant people, those with comorbidities (e.g., tuberculosis), and people taking multiple concurrent drug regimens may have additional complications achieving adequate drug levels (5,6). Sufficient drug levels are vital for ARV efficacy. Persistent low-level ARV exposure heightens the risk of drug resistance, endangering both individual and population-level HIV treatment and prevention outcomes (4,7–10).

Incorporating drug-level feedback (DLF) into routine clinical practice is associated with increased therapy adherence, reduced HIV-related hospitalization duration, and reduced overall illness cost (3). It also facilitates timely identification of people with inadequate drug exposure, especially those at risk due to drug interactions, comorbidities, or noncompliance (11–13). However, the gold standard for HIV ARV measurement, LC-MS/MS, is unsuitable for routine DLF due to its cost, infrastructure requirements, and lengthy turnaround times, potentially delaying appropriate counseling and intervention.

We recently developed the REverSe Transcriptase Chain Termination (RESTRICT) assay as a rapid and less-instrumented alternative to LC-MS/MS for measuring ARV drug levels. RESTRICT measures nucleotide reverse transcriptase inhibitors (NRTIs) – the backbone of HIV treatment and prevention regimens – based on the drugs' termination of DNA synthesis by the

HIV reverse transcriptase (RT) enzyme. When NRTI levels are high, DNA synthesis is terminated and an intercalating DNA dye produces low fluorescence. Meanwhile when NRTI levels are low, DNA synthesis is permitted, and an intercalating dye provides high fluorescence. Our initial demonstrations with RESTRICT focused on measuring tenofovir diphosphate (TFV-DP), an NRTI used in all oral PrEP regimens and most first-line ART regimens. We demonstrated excellent agreement between RESTRICT fluorescence and LC-MS/MS measurements at TFV-DP concentrations that indicate moderate (≥ 4 doses/week) and perfect (≥7 doses/week) PrEP adherence (14).

However, RESTRICT has so far been limited to NRTIs, representing only one class of RT inhibitors. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) have been used for decades in HIV treatment regimens. NNRTIs are also included in recently approved long-acting and extended-release regimens like monthly vaginal rings and bi-monthly injections. These existing and emerging ART and PrEP regimens have demonstrated a unique need for DLF. For example, women receiving monthly vaginal rings containing the NNRTI dapivirine (DPV) expressed a desire for real-time DLF and their healthcare providers concurred that it helped facilitate candid conversations about the underlying reasons for non-adherence (15). Meanwhile, DLF is recommended by the French National Agency for Research on AIDS and Viral Hepatitis Emerging Infectious Diseases for bi-monthly HIV treatment injections containing the NNRTI rilpivirine (RPV), and the integrase strand transfer inhibitor cabotegravir (CAB) for people with high BMI (≥30 kg/m$^2$), missed or delayed injections, known NNRTI resistance viral subtype (A1/A6), or those taking concurrent drug regimens known to alter RPV plasma concentrations, to minimize the risk of treatment failure (16).

Here we expand upon the capabilities of the RESTRICT assay to measure RT inhibition by evaluating its efficacy with NNRTIs. NNRTIs inhibit DNA synthesis by binding to an allosteric site on the RT enzyme, inducing a conformational change that inhibits its ability to synthesize dsDNA (17). We have renamed this expanded assay the **RE**verse transcriptase **ACT**ivity (**REACT**) assay.

**The goal of this thesis is to develop a rapid DLF test for NNRTI measurement in a portable, low-cost platform.** Research for this thesis will include the following:

### *Aim 1. Optimize REACT for NNRTIs in Buffer*

Building upon the RESTRICT assay for NRTIs, DNA synthesis reagents will be modified to optimize readout for NNRTIs. Enzymatic inhibition assay reagents tested include nucleotide (dNTP), primer, and template concentrations. To validate if these parameters are compatible with a range of NNRTIs from established and emerging treatments rilpivirine (RPV), dapivirine (DPV), doravirine (DOR), nevirapine (NVP), and etravirine (ETV) will also be tested to optimize performance. Additionally, the ability of REACT to distinguish between the inhibition of an NNRTI compared to an NRTI will be determined.

### *Aim 2. Validate REACT Performance in Plasma*

NNRTIs are found primarily in plasma, which is a complex biological matrix that may contain inhibitory compounds. We evaluate REACT performance in 1:4 diluted plasma samples spiked with DOR to evaluate its performance in a more complex sample matrix using simple sample preparation.

*Aim 3. Characterize REACT Compatibility on Portable Reader*

To demonstrate the potential of REACT as a portable point-of-care DLF test we aim to translate fluorescent measurements from a traditional plate reader to a portable reader. The Harmony, a portable heater and fluorescence reader, was previously developed and validated for point-of-care COVID-19 diagnostics(18). The fluorescence readout of REACT from the Harmony portable reader and plate reader will be correlated to determine if there are comparable fluorescence detection capabilities. Both spiked buffer and diluted plasma samples will be tested.

## 2. HIV as a Global Public Health Issue

HIV was first classified as a disease in 1981 when the U.S. Centers for Disease Control and Prevention (CDC) published a report on clusters of rare illnesses among gay men in Los Angeles and New York(19). Since then, the World Health Organization (WHO) estimates that it has claimed the lives of 42.3 million people and transmission remains ongoing globally. As of the end of 2023, 39.9 million people were living with HIV. In response to this ongoing crisis, the Joint United Nations Programme on HIV/AIDS (UNAIDS) established the 95-95-95 target as part of the broader UNAIDS strategy to end the HIV/AIDS epidemic as a public health threat by 2030: 95% of all people living with HIV (PLWH) should know their HIV status, 95% of those diagnosed with HIV should receive antiretroviral therapy (ART), and 95% of those receiving ART should achieve viral suppression. As of 2023, we have globally reached 86%, 89%, and 93%, respectively, in relation to these goals(20).

While many countries, particularly in high-income regions like Western Europe and North America, are close to or have already met the 95-95-95 goals, progress has been uneven. As of end of 2023, 65% of PLWH are in the WHO African Region(20). Some low- and middle-income

countries, especially in parts of sub-Saharan Africa, have made significant advances, but others still face challenges due to limited access to healthcare, stigma, and other social barriers. Additionally, specific populations such as adolescents, marginalized groups, and key populations like sex workers and men who have sex with men often lag behind in testing, treatment, and viral suppression(21).

## 2.1 Antiretroviral Therapy

The advent of antiretroviral therapy (ART) has been a transformative breakthrough in the fight against HIV. ART has fundamentally changed the course of HIV treatment, turning what was once seen as a death sentence into a manageable chronic condition, allowing people with the virus to lead long, healthy lives by suppressing viral replication and preventing the progression to AIDS(22,23).

Antiretrovirals are classified into different classes, each strategically targeting specific stages of the HIV replication cycle. Entry inhibitors prevent HIV from infiltrating target cells by obstructing crucial interactions or fusion events between the virus and host cell membranes. Reverse transcriptase inhibitors impede the enzyme responsible for converting viral RNA into DNA, either by mimicking DNA building blocks (nucleoside reverse transcriptase inhibitors - NRTIs) or directly binding to reverse transcriptase (non-nucleoside reverse transcriptase inhibitors - NNRTIs). Integrase strand transfer inhibitors (INSTIs) prevent the integration of viral DNA into the host cell genome, hindering viral replication. Protease inhibitors (PIs) disrupt the cleavage of viral polyproteins during late viral maturation, thereby inhibiting the production of mature infectious virions(22,24).

By disrupting various stages of the HIV life cycle, such as viral entry, reverse transcription, integration, and maturation, these antiretroviral drugs work synergistically to exert comprehensive control over viral replication. Combination therapy involving drugs from multiple classes is often employed to optimize treatment outcomes and mitigate the risk of drug resistance to any one class(4). By targeting multiple facets of the replication process, they collectively suppress the ability of the virus to proliferate within the body, leading to a reduction in viral load. This reduction in viral load helps to alleviate the burden on the immune system, diminishes the risk of transmission, and improves overall health(22). When taken consistently and correctly, ART can reduce the viral load to undetectable levels, improve quality of life, extend life expectancy, and eliminate the risk of transmitting HIV through sexual contact(25–27).

In addition to treating those who are already infected, ART has given rise to PrEP (Pre-exposure Prophylaxis), a preventative treatment for people who do not have HIV but are at high risk of contracting the virus. PrEP involves taking antiretroviral drugs daily or on-demand to prevent HIV infection. When taken consistently, PrEP is highly effective, reducing the risk of HIV acquisition by over 99% in sexual transmission(28). Together, ART and PrEP represent the two key pharmacological arms of HIV prevention and treatment: ART is life-saving for people living with HIV by stopping viral replication and reducing transmission risk, while PrEP empowers individuals at risk of HIV to protect themselves from infection. Both therapies have revolutionized the global response to HIV, providing effective tools to manage and prevent the virus across diverse populations(23).

## 2.2 Reverse Transcriptase Inhibitors as Backbone of ART

Reverse transcriptase inhibitors have long been essential components for HIV treatment, playing a crucial role in preventing the virus from converting its RNA into DNA, a critical step in its replication cycle. Currently, the recommended ART regimen consists of a "2+1" method where the patient should start on two NRTIs followed by either an NNRTI, a PI (with ritonavir boosting), or an integrase inhibitor(23). While the field is currently moving toward integrase strand transfer inhibitors instead of NNRTI or PI-based regimens, reverse transcriptase inhibitors remain core elements in HIV management(29,30). There are two approved classes of ARV that target reverse transcription: nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). While both classes target the activity of the enzyme reverse transcriptase, the mechanism of action differs(24).

### *2.2.1 NRTIs*

NRTIs were the first class of HIV drug approved by the FDA. NRTIs inhibit replication by acting as nucleoside analogs (Figure 1A). As analogs, NRTIs are structurally similar enough to endogenous nucleotides that the RT enzyme can incorporate them into the viral DNA strand being synthesized. However, NRTIs are missing the 3'hydroxyl group at the 2′-deoxyribosyl moiety, preventing the formation of a 3'-5'-phosphodiester bond in growing DNA chains (22,30). This chain termination halts DNA synthesis and therefore replication (Figure 1B). NRTIs are extremely prevalent in both prevention and treatment. NRTIs are administered as prodrugs, which require host cell entry and phosphorylation to activate the inhibitory effect (22,31). Two NRTIs, tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF), are converted to tenofovir diphosphate (TFV-DP), which is used in all approved oral PrEP regimens and recommended as a first-line NRTI in ART regimens (23,31,32). Other prevalent NRTIs include abacavir (ABC),

didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), zalcitabine (ddC), and zidovudine (AZT) (22).

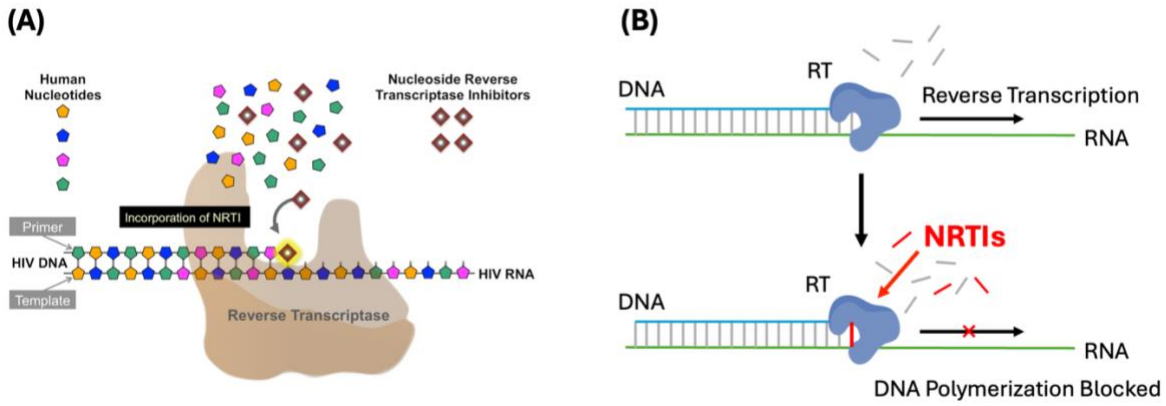


**Figure 1.** NRTIs inhibit reverse transcription through early chain termination. **(A)** NRTIs act as nucleotide analogs that can be incorporated by the RT enzyme. Figure adapted from (33). **(B)** Once NRTIs are incorporated, no additional nucleotides can be incorporated, blocking DNA polymerization.

### *2.2.2 NNRTIs*

While NRTIs inhibit RT activity by initiating chain termination after incorporation of a nucleotide analog, NNRTIs inhibit RT activity by binding to a hydrophobic pocket on the enzyme, changing the conformation of the substrate-binding site, making it unable to incorporate nucleotides for dsDNA synthesis (Figure 2) (17,22,34). NNRTIs have played a critical role in ART due to their potency, ease of use, and favorable safety profile. Established NNRTIs such as efavirenz and nevirapine have significantly contributed to the effectiveness of HIV treatment regimens (12).

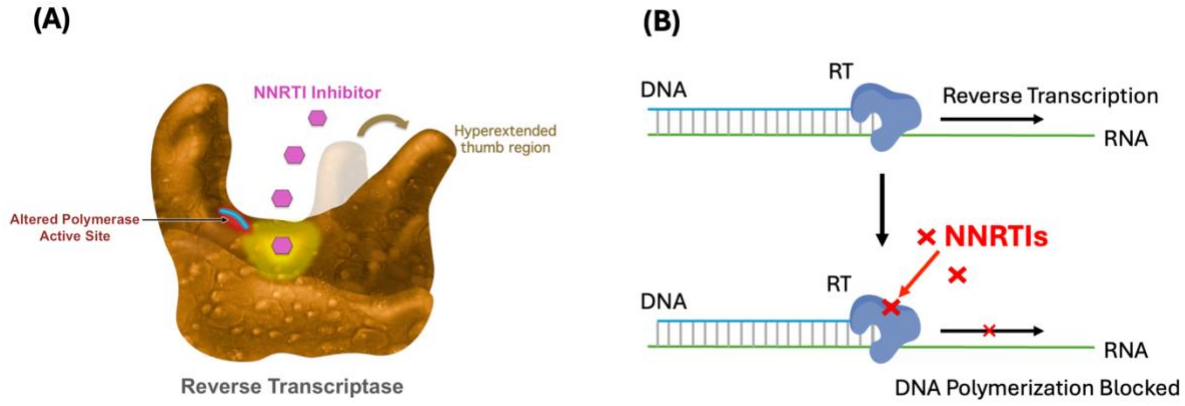


**Figure 2.** NNRTIs inhibit reverse transcription by inactivating the RT enzyme. **(A)** When NNRTIs bind to RT, it causes a conformational shape change, altering the active site. Figure adapted from (33). **(B)** A bound NNRTI inactivates RT function, inhibiting further DNA polymerization.

However, the extensive use of NNRTIs, particularly in resource-limited settings, in combination with their generally low genetic barrier to resistance, has facilitated the emergence and transmission of resistant strains. According to the most recent WHO HIV Drug Resistance Report, 10% of adults beginning HIV treatment exhibit resistance to NNRTIs, with those previously exposed to antiretrovirals being three times more likely to develop resistance to this drug class (35–37). NNRTI resistance can be rapidly selected for since the allosteric binding site is not crucial to RT function and is not directly involved in substrate binding or viral DNA synthesis(24). Consequently, point mutations can occur within the binding site that hinder NNRTI binding but do not interfere with RT`s ability for viral DNA synthesis. First generation NNRTIs, such as EFV and NVP, show a significant loss of activity with single-point mutations such as Y181C, P236L, and K103N, making them particularly susceptible to resistance (24).

Early NNRTIs have also been associated with notable adverse side effects. Those on EFV-based regimens have exhibited relatively high rates of neuropsychiatric and central nervous system

side effects, while those who discontinued NVP-based regimens due to side effects cited severe rash and hepatoxicity concerns, affecting overall tolerability (24,38). The challenges brought on by the rapid emergence of resistance and adverse side effect have prompted the development of new NNRTIS with improved efficacy, tolerability, and resistance profiles (24).

## 2.3 Emerging NNRTI Regimens

Novel NNRTI regimens such as long-acting injectable (LAI) rilpivirine and the dapivirine vaginal ring represent innovative advancements in HIV treatment and prevention. These formulations aim to increase adherence, reduce the frequency of dosing, and expand options for individuals living with or at risk for HIV.

Rilpivirine is traditionally available as an oral NNRTI, but the development of a long-acting injectable form offers a promising alternative for people living with HIV (11,39). This formulation is combined with long-acting cabotegravir (an integrase inhibitor) in a monthly or bi-monthly injectable regimen, under the brand name Cabenuva® (11,40). Approved by the FDA in 2021, Cabenuva® is the first long-acting injectable treatment for HIV. This regimen offers the convenience of infrequent dosing (once a month or every two months), which can improve adherence and reduce the burden of daily pills, particularly for individuals who struggle with oral medication adherence or prefer an injectable option (11,41).

The dapivirine vaginal ring is an NNRTI-based HIV prevention tool designed for women, particularly in regions where women are disproportionately affected by the HIV epidemic (42). Approved by regulatory authorities in certain regions (e.g., South Africa) and recommended by the WHO, this monthly vaginal ring slowly releases dapivirine to prevent HIV transmission during sexual intercourse (42,43). This discreet, long-acting method of HIV prevention offers women

more control over their protection, especially in situations where negotiating condom use may be difficult (44). It provides an alternative to oral pre-exposure prophylaxis (PrEP) like Truvada, which requires daily adherence (42).

These novel NNRTI-based regimens feature long-acting or extended-release formulations, reducing the need for daily medication, which can help individuals stick to their treatment or prevention regimen more consistently (45). Both the LAI rilpivirine (and cabotegravir) and the dapivirine ring offer more discreet methods of HIV treatment or prevention, which may reduce stigma and increase uptake, particularly in vulnerable populations. These regimens give individuals living with or at risk for HIV more choices, tailoring treatment or prevention to their preferences and lifestyles. These innovations highlight the ongoing effort to make HIV treatment and prevention more accessible, adaptable, and effective, particularly for populations that face challenges with daily medication adherence.

## 2.4 Other Emerging RT Inhibitors Beyond NNRTIs

### *2.4.1 Islatravir*

Islatravir (ISL) is a first-in-class nucleoside reverse transcriptase translocation inhibitor (NRTTI) that inhibits reverse transcription using multiple mechanisms of action (11). ISL is phosphorylated intracellularly into the active metabolite islatravir-triphosphate (ISL-TP) (11). During reverse transcription, ISL-TP is incorporated into the growing DNA chain as a nucleotide analog. The unique 4'-ethynyl group prevents the ability of RT to translocate along the DNA template to add the next nucleotide, effectively stalling reverse transcription and causing chain termination (11,46). Unlike traditional nucleoside analogs, ISL retains a 3'-hydroxyl group, and in some instances, an additional dNTP can be incorporated into the viral DNA chain prior to the

stalling of DNA synthesis, but the incorporation disrupts further extension due to steric and structural effects (46). The 3'-hydroxyl group also promotes high binding affinity for reverse transcriptase, contributing to the high potency of ISL. Additionally, the 2-fluoro group protects ISL-TP from deamination by adenosine deaminase, which extends the half-life of the drug (11,46). After oral administration the plasma half-life of ISL is 50-60 hours, and the intracellular half-life of ISL-TP is 130-210 hours (11).

The unique mechanism of action, long half-life, and high potency make ISL an attractive candidate for long-acting regimens. However, in late 2021, some trials involving ISL were temporarily paused due to concerns about dose-dependent declines in total lymphocyte and CD4 T-cell counts (47,48). Adjusted dosing regimens are now being studied to address these safety concerns while maintaining efficacy (47,48). Preliminary data from an ongoing Phase 2 clinical trial (NCT05052996) evaluating oral weekly ISL in combination with lenacapavir (LEN) in virologically suppressed PLWH demonstrates that oral weekly ISL and LEN maintained high rates of virological suppression (94.2%) at week 24 with no differences in CD4 or absolute lymphocyte count changes compared to the control group on Biktarvy® (daily oral regimen of bictegravir, emtricitabine, and TAF)(49). Additionally, clinical trials with a lower dose of ISL (0.25 mg) in combination with DOR (100 mg) have reported maintained viral suppression through week 96 among people who were switched from 3TC-containing regimens (48,50).

While ISL has a high genetic barrier to resistance, the emergence of resistance mutations through in vitro assays and the identification of naturally occurring polymorphisms in different subtypes raise concerns for widespread use of ISL(48). However, DOR has a complementary resistance profile, indicating that the use if ISL and DOR in a two-drug regimen may impose a high barrier to resistance (48).

The clinical holds placed on the long-acting formulation of ISL clinical trials due to CD4 cell decline highlights the continued utility of DLF in the context of the development of novel classes of ARV and long-acting formulations. The long-acting formulations of ISL required drug concentrations that were too high and caused dose-dependent adverse effects(47,48). In this case monitoring drug levels provides information on risk levels of adverse effects and can be used to optimize dosing schedules that balance efficacy and safety. Additionally, the long half-life, which allows for longer times between dosing, can also create situations where drug levels are present for considerable periods of time(11). This could be problematic in the case of missed doses or switching regimens where prolonged low levels of drug can breed resistance (11).

### *2.4.2 Long-acting TFV formulations*

As mentioned previously, the NRTI tenofovir (TFV) is used ubiquitously in both PrEP and ART globally, and many groups have been interested in formulating controlled, sustained delivery of TFV-based ARVs for long-acting strategies that do not require daily oral dosing. The prodrug TFV alafenamide (TAF) has been identified a promising candidate for long-acting delivery due to its high potency and lower daily dose requirements (47). Compared to the other available TFV prodrug, TDF, TAF has ten times greater potency and does not suffer the same renal toxicity and bone mineral density loss adverse effects (47,51). Multiple strategies are being explored to develop TAF implants for long-acting delivery, including biodegradable polycaprolactone (PCL) implants, non-degradable nanofluidic implants that can be refilled transcutaneously, titanium osmotic mini-pumps, and silicone reservoir implants (47,52–54).

The ongoing CAPRISA-018 clinical trial is the first human clinical trial evaluating the use of TAF implants for HIV prevention. CAPRISA-018 is an ongoing phase I/II trial to evaluate the

safety, acceptability, tolerability and pharmacokinetics of a TAF free base subdermal silicone implant containing 110 mg of TAF with a target release rate of 0.25 mg/day (55). In the phase 1 trial, to assess initial safety six healthy, adult South African women at low risk for HIV received a single TAF implant for 4 weeks. Thereafter, 30 women were randomized 1:1 to receive either one or two TAF implants and assigned blinded in a 4:1 active to placebo ratio (n=24 and 6, respectively), for 48 weeks. Pharmacokinetic assessments of TAF plasma concentrations and TFV-DP levels in peripheral blood mononuclear cells (PBMCs) were performed in addition to implant safety and tolerability evaluations throughout implant placement and 4 weeks post-removal (55,56).

Preliminary results reported expected safety profiles but sub-optimal tolerability. Early removal prior to 48 weeks occurred in 37% (n=11) participants at a median of 19 weeks post-insertion primarily due to injection site reactions. Of these removals, 55% were participant-initiated and the remainder clinician-initiated. One third (10/30) of TAF implants and 17% (1/6) of placebo implants were removed early, as were 22% (4/18) of single and 50% (6/12) of double implants. Additionally, implant drug release rates did not reach the target PBMC TFV-DP concentrations of 36 fmol/million cells in most participants. The median TFV-DP concentration were 3.9 fmol/million cells for those with 1 active implant and 14.8 fmol/million cells with two active implants. Overall only 15% of samples reached or exceeded the target concentration(56). Ultimately, additional implants or increased drug release rates would be needed to achieve target drug levels and would need to be balanced with effects on adverse events and tolerability.

These challenges showcase a role for DLF as a tool that could be utilized for optimizing clinical trials that must balance sustained sufficient drug exposure with potential side effects. The gap between the expected drug release rates and the observed drug levels also showcases how DLF

has utility in not only ensuring drug exposure is sufficient but also may help in determining the gap in knowledge that led to unexpected results. It can also help determine the cause of intolerability, and whether that can be attributed to the physical impacts of the implant or the effects of the placement, dose, and release rate of the drug.

For example, Su et al., reported concerning local toxicity in NZW rabbits and rhesus macaques when delivering TAF hemifumarate from a polyurethane implant(57). The issues were greater in the medicated group compared to the placebo group suggesting drug-related effect, although two of four macaques in both with both placebo and medicated implants placed contralaterally for 90 days also showed extensive inflammation in the placebo groups. The local TFV and TFV-DP concentrations measured near the implant were low or below the limit of quantification in macaques, and the local tissue TFV-DP concentrations at the implant site in rabbits were highly variable. Because the toxicity in macaques was worse than rabbits, but far lower local TFV and TFV-DP concentrations were measured, it is difficult to attribute the toxicity to either TFV or TFV-DP (54,57).

Gunawardana et al., 2022 posits that possible mechanical trauma from implantation and the physical characteristics of the device, together with the chemical composition of the polyurethane shell, generated an inflamed local environment –as observed in some of the placebo groups– that was further aggravated by TAF or one of its metabolites in combination with fumaric acid, since the hemifumarate salt is used (54). DLF may help pinpoint the cause of adverse effects that limit the use of long-acting implants and whether drug dosing or implant design need to be optimized to overcome these challenges.

Several research groups are also exploring the use of vaginal rings for sustained release of TFV (58–61). Liu et al., reports on a phase 1 randomized pharmacokinetic and safety study of a

90-day TFV vaginal ring. While the ring was well-tolerated, monitoring TFV-DP levels in cervical tissues showed acceptable concentrations through day 56, but concentrations declined thereafter (62). Further studies are needed to characterize optimal drug release and duration of use, in which DLF can be utilized. DLF can be used for precision dosing and ensuring complete coverage near the end of implant lifespan, especially in these early investigative stages of testing implants to determine lifespan of implant.

These studies demonstrate the relevance of DLF use in the development and validation of novel long-acting strategies. As novel extended-release methods are being investigated, complex factors contribute to their success or failure. Unexplained variability and disparate results across research groups remain issues (54). DLF can help evaluate the efficacy of these methods, analyze the cause of barriers to implementation, optimize dosing, and provide clinically important information about protection levels both while using long-acting products as well as inform next steps on initiating alternative regimens when participants choose to opt-out of these strategies.

## 3. Need for Drug-Level Feedback

ART and PrEP are fundamental to HIV treatment and prevention. As of 2023, 93% of people living with HIV (PLWH) taking antiretroviral (ARV) treatment achieved viral suppression (20), however, this estimate varies widely across region (63), sex (64), age group (65), and treatment regimen (23). Despite the impressive efficacy of current ARV treatments, many people have treatment regimens that fail, meaning viral load is not suppressed and allowed to proliferate causing further harm.

There are many reasons why ARVs can fail, but it is clear that maintaining adequate drug levels remains crucial for their efficacy (1,2). Studies have indicated a relationship between ARV

levels and therapeutic efficacy, toxicity, and risk of developing resistance (2,66,67). Sufficient drug levels are vital for therapeutic efficacy and inhibiting viral replication, while consistent low-level exposure heightens the risk of drug resistance, endangering both individual and public health outcomes as drug-resistant HIV strains proliferate (4).

Drug-level feedback (DLF) entails monitoring the concentration of antiretroviral medications in clinical samples and adjusting treatment accordingly to achieve and maintain optimal therapeutic levels. There is growing evidence that incorporating DLF into clinical trials and routine care is associated with increased adherence to ART and improved HIV-related health outcomes(3,15,68). For example, a retrospective cohort study in Italy found that integrating therapeutic drug monitoring (DLF) into routine care for people living with HIV (PLWH) reduced hospitalization time and treatment costs (3). They reported that adherence to ART was significantly higher in PLWH that received DLF compared those who had not received feedback (94% versus 78%, respectively) (3).

DLF serves as a valuable tool in clinical settings, enabling prompt identification of patients with inadequate drug exposure, particularly those at risk due to drug interactions, comorbidities, or noncompliance (12,69). Early initiation of clinical interventions can decrease the risk of death, HIV stage progression, and AIDS-related illnesses (70). DLF helps tailor dosages to individual patient needs, considering factors such as age, weight, metabolism, and health status. Personalized dosing can lead to optimal viral suppression, reducing the likelihood of virologic failure. Consequently, there is a pressing need for tools that can accurately measure drug levels for DLF, to ensure both therapeutic success and prevention of drug resistance.

## 3.1 Potential Causes of Subtherapeutic Drug Levels

Insufficient drug levels arise for several reasons including inconsistent medication adherence, treatment interruptions, and interindividual pharmacokinetic variations (3,4). Key populations in HIV treatment and prevention, including elderly and pediatric individuals, pregnant people, those with comorbidities (e.g., tuberculosis), and people taking multiple concurrent drug regimens may have additional complications achieving adequate drug levels (3,6). As such, there is a need for interventions to ensure adequate drug levels to achieve viral suppression.

### *3.1.1 Inadequate Adherence*

Adherence to ART delivers regular dosing of ARV, which is essential for maintaining drug levels. An adherence to ART of 95% is required as an appropriate level to achieve maximal viral suppression, although newer treatment regimens may provide more flexibility (71–73). Non-adherence is related to the development of ART resistance (74), progression to AIDS (75) and death (76). A study by Haberer et al. posited that providing patients with feedback on their drug levels can improve adherence by making them more aware of the importance of consistent medication intake (77). Non-adherence to ART is a widely studied, complex, and multifactorial problem. Many causes of sub-optimal adherence can contribute to unsuccessful treatment outcomes.

It remains challenging for some people to take oral pills daily. Some ART regimens suffer from “pill burden,” which is the impact of the effort to remember and take a pill every day that may interfere with regular adherence. Taking medications daily can be difficult due to forgetfulness, a change in schedule, business, or travel(78). Supply chain issues, lack of healthcare infrastructure, and pharmacy-related problems can impede consistent adherence. Many

socioeconomic barriers, including financial constraints, lack of access to healthcare, and unstable housing, have also been cited by PLWH (78,79).

Psychosocial issues such as depression, substance abuse, and lack of social support can negatively impact adherence (78,80,81). Being suspicious of the medical establishment and a distrust or dislike of the healthcare providers can be an additional impediment(82). Some PLWH cited not wanting to risk disclosing or being reminded of their HIV status when taking treatment (82). Rintamaki et al. reported those who were concerned about HIV stigma were 3.3 times more likely to be non-adherent than those who were not as concerned (83).

ARV drugs, while effective, can also have significant adverse effects (AEs), especially if drug concentrations exceed therapeutic doses. DLF allows for the monitoring of drug concentrations to mitigate these effects. AEs of ARV have been noted as a predictor of nonadherence (84). A review from Al Dakkak et al. states that adherence is 37% lower in PLWH experiencing AEs than in PLWH that did not experience AEs (85). Chesney et al. report that 19-25% of PLWH are nonadherent due to side effects (78). Treatment-related AEs include temporary events such as nausea, vomiting and diarrhea, as well as events of a longer duration such as lipodystrophy (85). The specific AEs reported to have a significant negative impact on ARV adherence included fatigue, cough, anxiety, confusion, taste disturbances, loss of appetite and nausea (85). DLF can identify patients at risk of toxicity due to high drug concentrations, enabling clinicians to adjust dosages before severe side effects occur (86). This proactive approach enhances patient safety and improves the overall tolerability of ART regimens.

### *3.1.2 Comorbidities*

PLWH often experience a higher prevalence of comorbidities compared to the general population, a trend that is expected to intensify as this population ages. A modeling study projected that by 2030, approximately 70% of PLWH in the United States will have multiple comorbid conditions, up from 63% in 2020 (87). The high prevalence of renal and liver diseases that affect drug metabolism and excretion is of particular concern for ARV regimens, often due to co-infections with hepatitis B virus (HBV) or hepatitis C virus (HCV), or chronic kidney disease. For example, PLWH have a 2- to 20- fold greater risk of end stage renal disease compared with the general population (88–92). In the US, approximately 1 in 10 PLWH also have HBV, and approximately 1 in 5 PLWH have HCV (93,94).

Comorbidities that cause hepatic dysfunction impair the liver's ability to process and metabolize ARV drugs (95). Liver damage, such as cirrhosis, significantly impacts the metabolism and elimination of HIV antiretroviral drugs (96). Cirrhosis alters liver structure, disrupting the enzymes and transporters responsible for drug metabolism. This can lead to variations in drug levels in the bloodstream, with potential risks of either toxicity or subtherapeutic dosing (96).

The liver plays a crucial role in drug metabolism, and when it becomes compromised due to conditions like cirrhosis, its ability to process and clear drugs diminishes. This is due to both a reduction in the liver's blood flow and a decrease in the function and number of hepatocytes, the cells that carry out metabolic processes (97). As a result, drugs that are normally metabolized by the liver, particularly those dependent on specific cytochrome P450 (CYP) enzymes, may accumulate in the body, leading to higher systemic concentrations (96).

Cirrhosis also affects the production of drug-binding proteins, reducing the amount of bound drug and increasing the unbound fraction in the bloodstream (96,97). This unbound fraction is pharmacologically active and can increase the risk of drug toxicity (98). Additionally, the impaired liver function in cirrhosis can reduce the first-pass metabolism of orally administered drugs, leading to higher bioavailability and further complicating drug management in patients with liver damage (99). The European AIDS Clinical Society states hepatic dysfunction is a good indication for drug level monitoring since clinical experience of proposed dose adjustments are limited (100).

Regazzi et al. studied the pharmacokinetics of nelfinavir (NFV) and its metabolite M8 in HIV-Hepatitis C coinfected subjects and determined that the NFV absorption rate was significantly lower in cirrhotic individuals leading to a longer time to reach maximum concentration in serum and proposed that DLF could be useful in treatment of HIV-Hepatitis C coinfected individuals, especially those with cirrhosis (101).

Drugs used to treat these additional comorbidities can also influence ARV levels, which is described in the next section.

### *3.1.3 Drug-Drug Interactions*

PLWH are more likely to use concomitant medications compared to the general population, and this trend is likely to be amplified by the growing aging population and the presence of additional comorbidities (102,103). These factors raise concerns about the potential for drug-drug interactions (DDIs) that can significantly alter the absorption, metabolism, and overall exposure of these medications. These interactions have the potential to either increase or decrease drug levels, potentially resulting in toxicity or reduced therapeutic efficacy (103,104).

Reports of the prevalence of potential DDIs in PLWH range from 21%-63%, which vary from study location (102,103). In one study of women living with HIV in Iran, the prevalence of DDIs experience was reported to be 21.4%, and that these DDIs were associated with a significant decrease in patients' adherence to ART (103). DDIs can not only influence drug levels, but also spur adverse symptoms that create an obstacle for adherence and may affect desire to continue treatment (103). Overall, DDIs have been associated with decreased efficacy, side effects, and suboptimal adherence (102,105).

Most DDIs are caused by the induction or inhibition of metabolic pathways (103). DDIs affecting ARV levels often involve the cytochrome P450 (CYP) enzyme system, particularly CYP3A4, which is responsible for the metabolism of many ARVs (102–104). Other drugs that inhibit or induce these enzymes can either increase ARV concentrations, heightening the risk of adverse effects, or decrease them, leading to subtherapeutic levels and possible treatment failure (104).

For example, a common DDI that must be navigated in PLWH is the use of rifamycin antibiotics such as rifampicin for tuberculosis (TB) treatment. Coinfection of HIV and TB is extremely prevalent. PLWH are 16 times more likely to fall ill with TB than people without HIV, and TB remains the leading cause of death among PLWH (106). In 2023, approximately 161,000 individuals with HIV died due to TB-related complications (106). The first line treatment for TB is rifamycin antibiotics, which are known to have considerable potential for DDIs as potent inducers for CYP450 enzymes (107). As a result, the use of rifampicin can enhance the metabolism of ARVs, primarily PIs and NNRTIs (107–109). For instance, when rifampicin is co-administered with efavirenz, adjustments to the efavirenz dose are often necessary to maintain therapeutic levels(38,109). Rifamycin antibiotics may also influence other drug classes such as INSTIs, the

CCR5 antagonist maraviroc, the gp120 attachment inhibitor fostemsavir, and the capsid inhibitor lenacapavir (107). As such several ARV drugs are not recommended for use with rifampin (107). As first-line HIV regimens now recommend the use of an INSTI instead of PI or NNRTI, DDIs with TB drugs can mostly be avoided through prescription management. However, rifampicin has also been shown to influence dolutegravir levels, which is recommended as part of first line HIV treatment, and requires increase in ARV doses (107). In the case of limited ARV availability and TB options, DLF and virological monitoring may be recommended if the optimal regimen to avoid DDIs is not possible (108).

Additionally, there is a paucity of data as to optimal dosages when more than two drugs affecting the P450 metabolic system are administered concomitantly (95). Drugs that inhibit this same drug metabolizing enzymes, such as certain antifungals or antibiotics, can decrease the metabolism of ARVs, resulting in elevated drug levels that exceed normal values and increase the risk of toxicity (110). Polypharmacy (the use of five or more medications simultaneously) is likely to become more prevalent, which in addition to the presence of comorbidities, creates increasingly complex clinical scenarios where traditional drug-checkers cannot predict outcomes (102,103,111). In these cases, DLF is a useful tool in investigating the effects of polypharmacy and ensure appropriate treatment.

Furthermore, certain drugs and supplements, such as proton pump inhibitors or products containing polyvalent cations (e.g., iron supplements), can impair the absorption of specific ARVs (112,113). The use of antacids can be concerning with drugs like atazanavir and rilpivirine, which require gastric acidity for optimal absorption (113). The presence of traditional medicines and supplements, which patients might not disclose, further complicates the scenario (5,112,114). For instance, St. John's wort, a common herbal supplement, can significantly reduce ARV levels by

inducing CYP3A4, leading to subtherapeutic drug concentrations of ARV metabolized through that pathway (112). In such situations, DLF provides a way to detect and quantify these deviations, enabling clinicians to adjust doses appropriately to maintain ARV concentrations within the desired range.

### *3.1.4 Metabolism and Genetic Factors*

In addition to changes in metabolic enzymes induced by physiological damage or DDIs, underlying biological metabolic and genetic factors can influence enzymatic drug metabolism. The metabolism of ARV drugs, primarily occurring in the liver, is mediated by enzymes such as cytochrome P450 (CYP450). Genetic polymorphisms in CYP450 enzymes can lead to significant interindividual differences in ARV metabolism (110). The CYP2B6 gene, responsible for metabolizing drugs such as efavirenz (EFV) and nevirapine (NVP), is highly polymorphic, with over 100 known single nucleotide polymorphisms (SNPs) (115). These genetic variations can result in different metabolic phenotypes. For instance, the 516G>T and 983T>C SNPs are associated with a significant loss of CYP2B6 function, leading to reduced drug clearance and prolonged half-life, which can elevate plasma drug levels beyond the therapeutic range, increasing the risk of toxicity. Conversely, SNPs such as 785A>G are associated with increased enzyme activity, leading to faster drug metabolism, potentially resulting in subtherapeutic drug levels and increased risk of treatment failure. In conjunction with the effects of individual SNPs, there is an added layer of complexity when considering the combinations of these SNPs into different haplotypes, which can result in varying degrees of slow or fast EFV/NVP metabolizer phenotypes(115).

Depending on an individual's unique genetic risk factors, certain ARVs may lead to an increased risk of side effects or a lack of therapeutic response (116). One of the earliest examples of precision medicine in HIV treatment was genotyping to predict the risk of abacavir (ABC) hypersensitivity reaction, a potentially life-threatening immune-mediated reaction. This reaction is strong linked to the presence of the HLA-B57:01* allele. Around 6% of people taking the NRTI ABC experience this reaction in the absence of genetic testing, which can become life-threatening with continued treatment (117). Due to the necessity of requiring HLA-testing to avoid ABC-associated hypersensitivity, DLF is now recommended as part of first line HIV regimens in certain clinical scenarios, such as where there is concern about renal or bone-associated adverse events what preclude the use of TDF or TAF (118).

Genetic variation also has influence on other first-line ARVs such as dolutegravir and lamivudine. Dolutegravir is extensively metabolized by the UGT1A1 allele. Those found to be carriers of a reduced-function UGT1A1 allele were observed to have decreased oral clearance, and individuals carrying UGT1A1*6, UGT1A1*28 reduced-function alleles were more likely to report neuropsychiatric adverse events than those with normal alleles. Other ARVs, such as atazanavir, indinavir, and raltegravir are also influenced by this allele(119). Lamivudine is affected by expressing organic cation transporter (OCT) 1 and OCT2 involved in renal excretion. Intrinsic clearance of lamivudine decreased significantly in the presence of OCT1 variants P283L and P341L, and OCT2 variants T199I, T201M, and A270S (120). These specific variants are commonly found in Asian populations (119,121).

These genetic and metabolic factors contribute to inter-patient variability in drug response, emphasizing the need for individualized treatment approaches. In cases where these factors cause

drug levels to fall outside the normal therapeutic range, DLF becomes a useful tool to adjust dosages and ensure optimal treatment outcomes.

## 3.2 Special Populations

### *3.2.1 Pregnant Women*

ART is recommended for pregnant women with HIV to prevent mother-to-child transmission (72). Pregnant women with HIV have been proposed as a special population that may benefit from DLF (13,95,104,122–124), although specific guidelines and recommendations differ by health authority organization (122).

During pregnancy, significant physiological changes can affect the pharmacokinetics of ARV drugs, leading to altered drug levels. These changes include increased blood volume and cardiac output, which dilute drug concentrations, and modifications in body composition, such as increased body fat and total body water, affecting drug distribution. Altered gastrointestinal transit due to hormonal changes can impact drug absorption, while increased renal blood flow and glomerular filtration rate enhance renal clearance, reducing plasma levels of renally excreted ARVs. Additionally, hepatic enzyme activity, particularly cytochrome P450 (CYP450) enzymes, is altered during pregnancy, often increasing the metabolism of certain ARVs, such as PIs, and necessitating dose adjustments to maintain therapeutic levels. For example, drugs like lopinavir/ritonavir may require increased doses due to enhanced metabolism (125).

Caswell et al., performed a retrospective review of the use of DLF for pregnant women with HIV receiving lopinavir-based ART across five centers in the United Kingdom. Of the 73 identified women, 11% had plasma lopinavir concentrations below the minimum recommended level for wild-type HIV (1,000 ng/mL). Initial DLF results led to dose adjustments in 10% of cases

and adherence reviews in 11%. Repeat DLF was performed in 29% of women, often in response to low initial concentrations, dose changes, or concerns about toxicities (123).

While there is limited data on the use and effectiveness of DLF for pregnant women, the data showing changes in drug pharmacokinetics due to physiological changes brought on by pregnancy and the high level of interindividual variability of ARV concentrations reported suggest this population could benefit from DLF. DLF during pregnancy can ensure that drug levels remain within the therapeutic range, preventing virological failure, resistance, and toxicity, thereby ensuring effective treatment for both the mother and fetus (122,125,126).

### *3.2.2 Children*

Dosing ARVs in pediatric patients is complex in part due to the unpredictability of plasma concentrations based on the administered dose (95,127). Pediatric pharmacokinetics are different compared to adult patients and are influenced by a child's age, body size, and developmental stage. For example, the stomach pH, bowel length, motility and mucosal integrity of pediatric patients are different from adults, and this may affect the absorption of drugs. Enzymes relevant to drug metabolism are also expressed more prominently at different ages which may affect drug metabolism, and the water-fat-muscle composition of pediatric patients varies depending on their age which may affect drug distribution (95,127–129). Pediatric pharmacokinetic parameters are subject to high interpatient variation, leading to a potential risk of underdosing or overdosing when drugs are used in real life (127).

Several reports have posed children as a special population that could benefit from DLF because of the large interindividual variations in plasma concentrations and children's increased vulnerability to drug-related AEs (13,67,95,127). A five-and-a-half-year retrospective analysis

reviewed DLF requests for pediatric patients in a South African tertiary hospital to evaluate the utility of DLF in pediatric HIV treatment (129). At Tygerberg Children's Hospital, DLF was primarily requested for lopinavir, especially in cases of suspected non-adherence, interactions with rifampicin, and neonatal safety concerns. A resulting concentration within the therapeutic range excluded pharmacokinetic causes of the clinical problem and prompted investigation of other causes of treatment failure like non-adherence or drug resistance. Efavirenz DLF was most often requested for suspected toxicity, resulting in a 100% positive predicted value (129). DLF can also help generate much needed pharmacokinetic data from a population with a paucity of dosing information to further assist in optimizing pediatric ARV dosing.

### *3.2.3 Elderly*

As HIV diagnosis and effective antiretroviral treatment become more ubiquitous, PLWH can live long lives approaching the life expectancy of the general population. As people are living longer, there is unprecedented level of elderly PLWH (defined by the World Health Organization as >65 years old) (130). However, managing HIV in the elderly presents unique challenges due to age-related comorbidities, polypharmacy, and physiological changes that affect the pharmacokinetics and pharmacodynamics of medications (130–132).

Age-related physiological changes, such as reduced gastric acid secretion, delayed gastric emptying, and altered body composition (e.g., reduced body water, increased body fat), can impair drug absorption, distribution, metabolism, and excretion (130,132). One of the most significant effects of aging is a 30% to 40% reduction in hepatic clearance, primarily due to a decline in liver mass and blood flow rather than changes in hepatic enzyme activity (133). Liver mass decreases by 10% to 15% per decade in women and by 20% in men after the age of 65 (132). Moreover, a

progressive decline in renal function, reflected by a reduced glomerular filtration rate, impairs the clearance of renally eliminated drugs (132).

Older patients often experience age-related comorbidities, which can lead to complex polypharmacy and an increased risk of drug-drug interactions. Polypharmacy is most commonly defined as the concurrent use of five or more medications, which has been linked to an increased risk of adverse outcomes (130). This makes managing HIV treatment in elderly individuals particularly challenging, as multiple medications can interact with ART, potentially affecting drug efficacy and safety. Additionally, there is generally limited data on how aging affects the pharmacokinetics of ARV drugs since elderly individuals are frequently excluded from clinical trials (130).

Given the significant physiological changes, increasingly complex clinical presentations, and increased vulnerability to side effects of elder PLWH, ensuring adequate drug concentrations becomes even more important. There is a clear opportunity for DLF to be used to optimize treatment outcomes.

## 3.3 Resistance

One of the most critical challenges in HIV treatment is the development of drug resistance. Resistance arises when ARV drugs are no longer as effective in suppressing the virus, leading to the need for higher dosages or switching to different treatment regimens (134). Acquired drug resistance typically results from intermittent or low drug concentrations, allowing resistant viral strains to proliferate (4,8,135). This resistance can extend to other ARVs in the same or similar drug classes (4). Given the limited number of ARV combinations available, especially in resource-limited settings, this poses a significant public health risk. Furthermore, if a person with a resistant

virus transmits it to others, those individuals are similarly restricted in the ARVs they can use, potentially worsening outcomes both individually and for public health systems.

Since low ARV levels are a known precursor to resistance, DLF can identify individuals who are at risk by monitoring drug concentrations in the body. DLF can provide valuable information about the presence or risk of resistance, especially when used in conjunction with viral load testing and previous adherence history (1,134,136,137). DLF can be used to investigate the cause of treatment failure and allow clinicians to tailor interventions based on the specific root causes. For example, if drug levels are low and viral loads are high, low adherence may be the cause of negative outcomes. This may prompt clinicians to implement interventions such as behavioral counselling or direct the person to adherence support programs like daily SMS reminders (4,134,138). If drug levels are high and viral load is also high, resistance is a likely culprit and can prompt genotypic resistance testing and changes to ARV dosing or regimens (1,134,139).

DLF has been used to assess risk of resistance in previous studies (140). McCluskey et al. evaluated the utility of a POC urine tenofovir assay to objectively assess adherence and predict HIV drug resistance in people failing first-line ART in adults with virological failure in Uganda and South Africa. The urine lateral flow assay was able to predict drug resistance with a positive predictive value of 91% and a negative predictive value of 43% (141).

Hermans et al. performed a retrospective analysis of South African adults experiencing treatment failure on ritonavir boosted-lopinavir (LPV/r) second-line ART (134). PI-exposure testing was performed through LC/MS measurements on remnant plasma or dried blood spot samples as well as using a low-cost immunoassay. This was done in conjunction with drug resistance testing. LPV levels had a negative predicative failure of 95% for the presence of major

LPV/r resistance. The authors also reported if PI-exposure testing would have been used to screen for eligibility of drug resistance testing in this study, approximately half of requested resistance tests could have been avoided (134). This highlights how DLF can help prevent unnecessary and costly resistance testing. DLF can also prevent unnecessary switching of treatments where options are already limited (2,136,142).

## 3.4 Dynamic Choice

There are many choices of effective PrEP and ART treatments, and the number of options will continue to grow as novel interventions are discovered and approved. Studies have found that people switch between products in response to changes in prevention needs and preferences (50,143–145). Evidence from decades of family planning studies demonstrate that product switching is a prevalent behavior, often resulting in improved user satisfaction and increased rates of contraception use (143). Recognizing the dynamic nature of HIV prevention needs, strategies that enable flexibility and product switching over time could strengthen coverage and help lower HIV incidence rates (143,144). Dynamic choice emphasizes adapting treatment options to a person's evolving circumstances, clinical needs, and preferences over time, recognizing that these factors are not static but continuously influenced by personal and external factors (143).

A recent study evaluated the effect of a dynamic choice model of HIV prevention on biomedical prevention coverage and HIV incidence in rural Uganda and Kenya. This study demonstrated that offering a range of prevention options—including daily oral PrEP, post-exposure prophylaxis (PEP), and CAB long-acting injectables (CAB-LA)—increased biomedical prevention coverage fivefold compared to standard care. Participants were also able to switch between prevention products over time, with 28% of the intervention group using at least two

different products during the 48-week follow-up. Notably, 52% of participants initially opted for CAB-LA, underscoring the demand for long-acting options, though many later transitioned to oral PrEP or PEP, highlighting the importance of flexibility in care (144).

Dynamic choice models are person-centered, addressing the diverse and evolving needs of individuals. By allowing participants to adjust their prevention strategies based on personal circumstances, such as side effects, accessibility, or life transitions, these models help optimize adherence and expand protection. However, self-reported adherence, as used in this trial, presents challenges in reliably evaluating coverage (144). Incorporating DLF into dynamic choice models could provide actionable insights into adherence, particularly when individuals transition between products or face adherence challenges. Additionally, it could offer clarity on whether transient gaps in prevention coverage were due to behavioral or pharmacokinetic factors.

As the study findings highlight, a one-size-fits-all approach is insufficient for achieving widespread HIV prevention. Instead, dynamic, choice-driven strategies must be paired with tools like DLF to ensure adequate drug levels across various regimens, improve adherence, and address evolving prevention needs. This is particularly relevant with the introduction of long-acting and extended-release formulations, which offer convenience but also create complexities when individuals choose to switch regimens due to the long pharmacokinetic tail. Switching ARV regimens presents a key opportunity for DLF to guide transitions, ensuring that individuals maintain adequate drug coverage and avoid risks such as drug resistance or subtherapeutic levels.

# 4. Emerging Applications of DLF

## 4.1 Long-Acting Injectable Cabotegravir and Rilpivirine

Long-acting injectable (LAI) formulations of cabotegravir (CAB), an integrase strand transfer inhibitor (INSTI), and the NNRTI rilpivirine (RPV) represent a significant advancement in HIV treatment, addressing adherence challenges associated with daily oral regimens. These injectables, administered every one to two months, offer a convenient and discreet alternative, improving adherence and potentially enhancing viral suppression rates (11,146,147). However, several clinical and pharmacokinetic challenges must be addressed to maximize their effectiveness.

Phase 3 clinical trials, such as the ATLAS and FLAIR studies, have demonstrated the efficacy of LAI CAB and RPV in maintaining viral suppression. In the ATLAS trial, 93% of participants maintained viral suppression at 48 weeks, compared to 94% in the control group on daily oral ART(148). Similarly, the FLAIR trial reported viral suppression rates of 93.6% in the LAI group, indicating that these injectables are comparable to standard oral regimens(149). In addition to efficacy, high levels of patient satisfaction were reported, with many preferring the LAI regimen over daily pills due to convenience (150). The ATLAS-2M trial further demonstrated that extending the dosing interval to eight weeks did not compromise efficacy, with 94.3% of participants maintaining viral suppression at 48 weeks (148). These studies highlight the potential of LAI therapy to simplify treatment regimens and enhance adherence, particularly for individuals who struggle with daily pill intake.

Interindividual pharmacokinetic variability is a significant factor in the administration of LAI CAB and RPV. Figure 3 shows the range of interindividual CAB plasma concentrations from the HPTN 077 multicenter, double-blind, randomized, placebo-controlled phase 2a trial done at eight sites in Brazil, Malawi, South Africa, and the USA where participants (aged 18–65 years),

who did not have HIV and at low-risk for acquiring HIV, were randomly assigned to LAI CAB (800 mg given three times at 12 week intervals or 600 mg given five times, administered at one 4 week interval, and every 8 weeks thereafter) or placebo. The variability clearly shown in Figure 3 highlights how interindividual variability can lead some individuals to fall below certain drug-level thresholds at different times (146).

Factors such as body mass index (BMI), sex, and injection site can influence drug absorption and clearance rates. Higher BMI (>30) has been associated with lower drug concentrations due to increased volume of distribution, while pharmacokinetic differences between men and women can affect drug distribution and metabolism, necessitating sex-specific considerations in dosing (146,149). Variability in injection technique and site can also lead to differences in drug absorption. Even within the same individual, inconsistent intramuscular injection practices can lead to variations in drug absorption and efficacy. Differences in clinician expertise and clinic settings can exacerbate this issue (148).

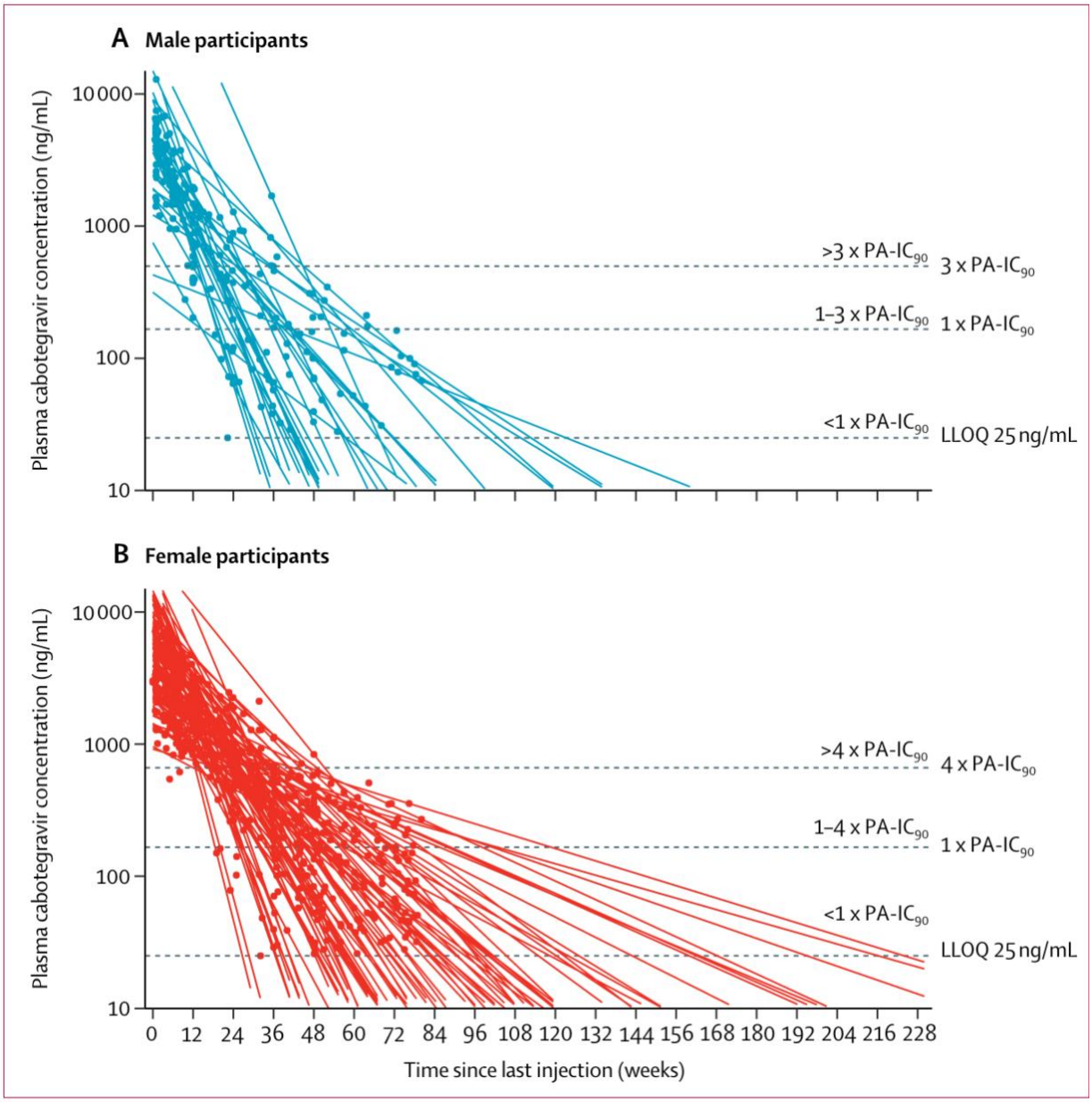


**Figure 3.** Interindividual variability in LAI CAB. Individual participant log-linear regression curves of plasma CAB concentrations in (A) Male participants and (B) Female participants by sex at birth. Dots represent individual participant values based on days since the last injection. LLOQ=lower limit of quantification. PA-IC90=protein-binding-adjusted 90% inhibitory concentration. Figures from reference (146).

As LAI use scales up globally, the variation observed in clinical trials is likely to increase in real-world conditions beyond a pristine clinical setting, where patient populations are more diverse and healthcare resources may be limited (146). The extended dosing intervals of LAI CAB and RPV address adherence challenges associated with daily oral ART. However, maintaining adherence to the injection schedule is still critical to avoid the risks associated with the pharmacokinetic tail. Ensuring equitable access to LAI therapies and addressing variability through personalized dosing and support mechanisms are critical for the successful implementation of these treatments.

Another significant concern with LAI formulations is the pharmacokinetic tail in the metabolic profile. This tail represents a period of prolonged drug underexposure following the last injection, during which drug levels fall below therapeutic thresholds. If a subsequent dose is not administered in a timely manner, this underexposure can lead to viral rebound, treatment failure, and the development of drug resistance across all drugs within the same ARV class, including oral formulations (41). Managing this risk is critical, especially in settings where access to timely healthcare may be inconsistent. These variations preclude a one-size-fits-all approach to LAI dosing, emphasizing the need for personalized treatment strategies to maintain therapeutic drug levels.

DLF platforms can play a pivotal role in mitigating the challenges associated with LAI formulations. Monitoring drug levels through feedback platforms can provide information allowing healthcare providers to tailor dosing schedules and enhance adherence support. Inter-individual variability and genetic factors that influence drug metabolism necessitate optimizing dosing strategies for diverse populations, and certain subpopulations that may require adjusted dosing regimens to achieve optimal drug level, such as those with high BMI or pregnant women.

This personalized approach can help maintain therapeutic drug levels, minimize the risk of resistance, and improve overall treatment outcomes (75). The French National Agency for Research on AIDS and Viral Hepatitis Emerging Infectious Diseases defined an Alert Threshold for those on LAI RPV and CAB, obtained through the 1st quartile of $C_{min}$ at week 8 of the pooled data analysis of the phase 3 trials. They recommend DLF for people with high BMI ($\geq$30 kg/m$^2$), missed or delayed injections, known NNRTI resistance viral subtype (A1/A6), or those taking concurrent drug regimens known to alter RPV plasma concentrations, to minimize the risk of treatment failure (16).

## 4.2 Dapivirine Vaginal Ring

The dapivirine (DPV) vaginal ring represents an innovative approach to HIV prevention, particularly empowering women in low-resource settings. This silicone ring, infused with the NNRTI DPV, provides sustained drug release over a month, offering a discreet and user-controlled method of HIV prevention. Clinical trials, such as the ASPIRE and Ring Study, have demonstrated the ring's safety and efficacy, with adherence being a critical factor in its success (42). Adherence data suggests that higher levels of consistent use correlate with better protective outcomes, underscoring the importance of adherence support mechanisms in such interventions. Phase III trials indicated that the DPV vaginal ring lowered HIV-1 incidence by around 30% compared to placebo, with even higher efficacy exceeding 50% among subgroups demonstrating stronger adherence to ring usage.

The MTN-034 Reversing the Epidemic in Africa with Choices in HIV Prevention (REACH) study provided DLF as part of a menu of tailored adherence interventions for adolescent girls and young women (AGYW) receiving either oral PrEP or the DPV ring in South Africa,

Uganda and Zimbabwe (15). At monthly counselling sessions, DLF was provided based on LC-MS/MS measurements of residual DPV from returned vaginal rings (or dried blood spots for oral PrEP users). REACH participants demonstrated higher adherence to both PrEP options compared to previous studies with AGYW, with 73%-89% of results indicating high adherence to the ring. However, the median time between return of used DPV ring and adherence counselling was 56 days, and DLF was only available at 55% of monthly visits, with >1 result available at 29% due to delays, highlighting the limitations of LC-MS/MS DLF measurements (15).

Participants also reported that repeated validation through DLF built self-efficacy and motivated persistence, and some participants admitted they would not have used the study products without it. Both the AGYW and their healthcare providers expressed the desire for DLF to facilitate candid discussions about underlying reasons for non-adherence and spur timely interventions (15).

The authors noted that routine DLF would be hard to replicate outside of the study due to cost and logistical challenges. In the HTPN 082 study, a previous study investigating oral PrEP adherence in AGYW, found that providing DLF had no effect on adherence. However, DLF was only provided at months 2 and 3, with the primary outcome as PrEP adherence at month 6. The authors suggest that less frequent DLF may not provide adequate feedback to support adherence and that alternative, lower-cost strategies are needed (15).

# 5. Current and Developing Technology for DLF

## 5.1 Subjective Adherence Monitoring

Traditional adherence measures, such as self-reported adherence, have been important in evaluating PrEP implementation. Self-reported adherence is the most commonly used method for assessing adherence, especially in real-world clinical settings. However, it has significant limitations, including social reluctance and recall biases. To improve accuracy, researchers and clinicians have tried combining self-reported adherence with pill counts and pharmacy refill data. While this combined approach can provide a more comprehensive picture, it also requires additional time, staff, and costs, and does not necessarily result in significantly higher accuracy (1).

## 5.2 Objective Drug Level Monitoring

Pharmacologic metrics of adherence and drug levels involve measuring drug concentrations in various biomatrices such as plasma (151), urine (152), peripheral blood mononuclear cells (PBMCs) (153), hair (154), and dried blood spots (DBS) (155). This is most commonly done using LC-MS/MS methods (1). These drug level measurements provide key information about ART in clinical settings.

The iPrEx trial, which was the first to demonstrate the efficacy of PrEP among men who have sex with men (MSM) and transgender women, demonstrated that treatment efficacy was much higher among participants with detectable drug levels in plasma and PBMCs (92% and 44%, respectively) (156). The VOICE and FEM-PrEP trials among women in sub-Saharan Africa, highlight how objective drug level measurements can provide crucial information to elucidate

clinical data that subjective adherence monitoring alone could not provide. Over 95% of participants reported adherence to the regimen, but random plasma tenofovir (TFV) levels were detectable in fewer than 30%. These findings highlight the importance of pharmacologic DLF in accurately assessing adherence in PrEP (157,158). DLF can also assist clinicians in interpreting if an elevated viral load is due to low adherence or drug resistance (139). Elevated viral loads in conjunction with low drug levels suggest non-adherence. However, a detectable drug level may indicate drug resistance (1).

DLF sample type and analysis method is dependent on the specific ARV and the time frame of adherence testing for recent or cumulative adherence (Figure 4). For example, many adherence and DLF monitoring tests target measurement of TFV, an extremely prevalent nucleotide reverse transcriptase inhibitor that is included in 100% of all oral PreP regimens and recommended in most first-line ART regimens (159,160). There are currently two different prodrug formulations, tenofovir disoproxil fumarate (TDF) and the more recent tenofovir alafenamide (TAF) (161). These prodrugs are converted to tenofovir (TFV) and then metabolized intracellularly by cellular kinases to the active form of the drug, TFV-diphosphate (TFV-DP) (32). TFV levels in urine and plasma may only reflect short-term adherence, which is vulnerable to the "white coat effect" where drug concentrations may reflect improved adherence for a short time immediately preceding clinic visits(162). TFV-DP on the other hand accumulates intracellularly in PBMCs and therefore can provide information on exposure over longer periods (7–14 days). While this provides longer-term adherence information that is less vulnerable to the white coat effect, processing, isolating and counting PBMCs is costly and technically challenging. TFV-DP also accumulates in red blood cells (RBCs), and exhibits a 17-day half-life in DBS (1).

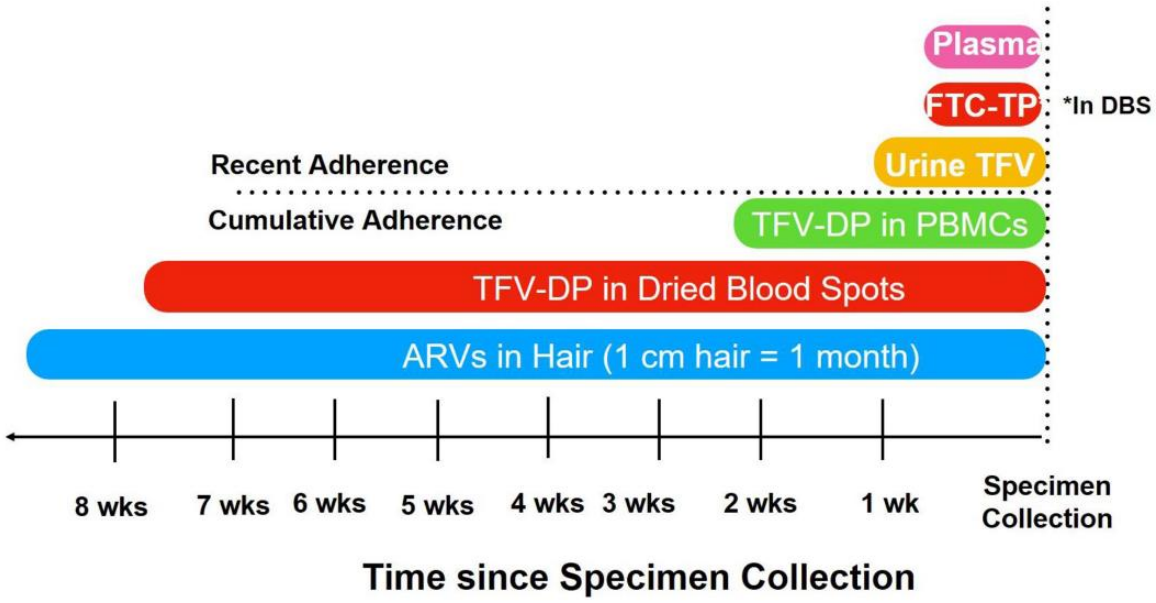


**Figure 4.** Pharmacologic measures of adherence by sample type and time frame ARV is detectable. Figure from reference (1).

TFV-DP in DBS has proven to be a robust predictor of PrEP efficacy among high-risk individuals taking TDF and TAF-based PrEP. Concentrations of 700 fmol/3 mm punch and 900 fmol/two 7 mm punches are associated with high protection against HIV for TDF and TAF, respectively (163–165). Jennings et al. demonstrated that TFV-DP levels in DBS strongly predicted future viral breakthrough in a clinical cohort of South African PLWH (136).

## 5.3 Gold Standard LC-MS/MS

LC-MS/MS is the gold standard for measuring ARV drug levels due to its high sensitivity, specificity, and accuracy(1). As mentioned previously, the ARV concentration in clinical samples such as DBS, hair, blood, PBMCs, plasma, and urine samples is most commonly measured using

LC-MS/MS. However, its widespread implementation is hindered by cost, centralized laboratory requirements, the need for trained personnel, and lengthy turnaround times (>1 month) (1,15,166). LC-MS/MS measurements can only be done in laboratories equipped with expensive equipment by people with specific training. This binds LC-MS/MS measurements to centralized laboratories, meaning that any clinical samples must be obtained, then shipped to the laboratory where they will run the test and then send the results back to the clinicians which can then be given to the patient. The centralization, time, and logistics required severely limit its use in low resource areas. In the MTN-034/REACH trial, discussed in more detail in section 4.2, DLF was provided through LC-MS/MS measurements of either TFV-DP in DBS or of residual DPV levels in returned intravaginal rings. The median time between DBS collection to DLF counselling about TFV-DP concentration was 42 days (IQR 28-70). DLF for dapivirine rings took 56 days (IQR 29-76) (15,167). These limitations render LC-MS/MS unsuitable for routine DLF testing, particularly in low- resource settings, thereby impeding the regular assessment of ARV concentrations and potentially postponing counseling and interventions (13,167).

## 5.4 Alternatives to LC-MS/MS

Immunoassays, including enzyme-linked immunosorbent assays (ELISAs), offer a more accessible alternative to LC-MS/MS. These tests are generally easier to perform and require less specialized equipment, making them suitable for use in various clinical settings. However, they tend to be less specific and can be affected by cross-reactivity, leading to potential inaccuracies (168).

The recent development of antibody-based detection for TFV levels in urine enables real-time measurement. These immunoassays have been adapted into a lateral flow immunoassay

(LFA) format, allowing for the detection of recent TFV dosing at the point of care, at a low cost, and without requiring specialized training. Pratt et al. were able to develop gold-nanoparticle-based competitive lateral flow strip assay for direct measurement of spiked TFV in urine samples with a sensitivity of 1 μg/mL. A significant limitation however is the 55-minute time required to obtain results (169).

Gandhi et al. have developed a rapid urine test for the detection of TFV on a lateral flow strip. Antibody-based lateral flow immunoassays are low-cost, enable drug detection to occur within minutes, and can be performed by non-trained personnel. Urine is a particularly suitable matrix for POC testing since its collection is noninvasive. Gandhi et al. demonstrated 100% specificity and 96% diagnostic sensitivity of the assay and high correlation with LC/MS measurements (170). A CDC implementation study evaluated the utility of the urine TFV assay deployed in 38 HIV clinics in Namibia for participants who did not achieve viral suppression despite enhanced adherence counseling for ≥ 3 months. 87% of participants were virally suppressed by month 6, strongly supporting how the implementation of rapid DLF can improve adherence and viral suppression outcomes. In the same study, 86% of participants and 91% of providers agreed/strongly agreed that the urine test should be in care, highlighting significant interest in a rapid DLF test (171,172).

Further, a randomized trial evaluated the impact of providing DLF using a urine-based tenofovir assay on long-term PrEP adherence among women in Kenya. Participants were assigned to either standard-of-care adherence counseling or counseling informed by real-time urine assay results, with follow-up every three months over a 12-month period. The primary outcome was long-term adherence, measured by hair TFV concentrations, with urine levels serving as a short-term adherence metric. Results showed that participants receiving urine-test counseling had

significantly better adherence at 12 months, with fewer having undetectable hair TFV levels compared to the standard-of-care group (21% vs 37%, respectively). Additionally, 72% of the urine-test counseling group had detectable urine TFV at 12 months, compared to 45% in the standard-of-care group. The intervention was well-received, with most participants finding the test acceptable. Incorporating a low-cost urine TFV assay into PrEP counseling improved adherence metrics over both the short and long term, suggesting DLF could be an effective tool to promote long-term adherence to PrEP (173).

Promising early results indicate the potential of this tool to improve virological suppression outcomes. However, the platform only determines if TFV levels are above or below a certain threshold (yes/no), may be vulnerable to the white-coat effect as urine TFV concentrations indicate short-term adherence levels, and is currently limited only to TFV detection (170,172,174,175).

## 5.5 Enzymatic Assays

Olanrewaju et al. recently developed the REverSe TRanscriptase Chain Termination (RESTRICT) assay as a rapid and less-instrumented alternative to LC-MS/MS for measuring NRTI drug levels for adherence monitoring (14,176,177). RESTRICT measures NRTIs based on the drugs' termination of DNA synthesis by the RT enzyme (Figure 5). Intercalating fluorescent dye is used to provide fluorescence measurements that can then be correlated to drug levels. When NRTI levels are high, DNA synthesis is terminated and the intercalating DNA dye produces low fluorescence. Meanwhile when NRTI levels are low, DNA synthesis is permitted, and an intercalating dye provides high fluorescence. RESTRICT focused on measuring tenofovir-diphosphate (TFV-DP). We demonstrated excellent agreement between RESTRICT fluorescence and LC-MS/MS measurements of TFV-DP concentrations that indicate moderate ($\geq$ 4 doses/week)

and perfect (≥7 doses/week) PrEP adherence (14). RESTRICT fluorescence readout correlated with LC-MS/MS measurements (r= -0.845 p< 0.0001).

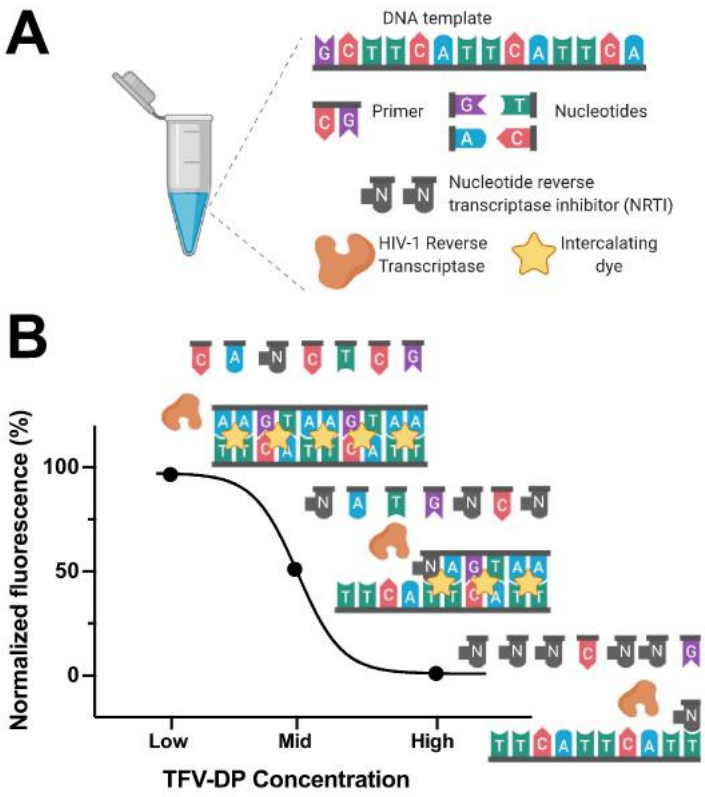


**Figure 5.** RESTRICT Assay can detect NRTI concentrations through fluorescence measurement of DNA synthesis. (A) RESTRICT requires nucleic acid templates, primers, nucleotides, HIV-1 reverse transcriptase enzyme (RT), and a sample containing NRTIs. Following incubation at 37°C for 30 minutes, the reaction is quenched by adding an intercalating dye. (B) Fluorescence readout measures double-stranded DNA (dsDNA) synthesis by RT correlated to NNRTI drug concentration. Figures from reference (176).

## 6. Expanding Access in Low Resource Settings

PLWH in low resource settings face additional barriers to successful diagnosis and treatment of HIV. In low-resource settings, where access to ARV drugs is often limited, optimizing the use of available treatment options is critical. Patients in resource-limited settings often begin ARV treatment with significantly advanced immunodeficiency and lower CD4 cell counts compared to those in developed countries, putting them at higher risk of inadequate drug concentrations due to their advanced disease status (178). Other factors such as malnutrition can affect the absorption and metabolism of ARVs, increasing the likelihood of subtherapeutic drug levels, which can lead to viral replication and resistance (179,180).

The scarcity of ARV combinations in these regions leaves healthcare providers with fewer alternatives when patients develop resistance or experience treatment failure(13,95). This shortage heightens the importance of maximizing dosing accuracy and ensuring adherence to prevent drug resistance from emerging. With fewer ARV combinations available, providers may need to rely on less-than-ideal regimens that may not offer optimal efficacy or have unfavorable side effect profiles. In such cases, drug level monitoring becomes an important tool. By closely tracking ARV levels, healthcare workers can ensure drugs are used effectively, avoiding both under-dosing, which can promote viral replication and resistance, and overdosing, which increases the risk of side effects. This approach is especially important when there are limited options to switch to in case of treatment failure (95).

Moreover, drug resistance testing, while essential in some cases, can be prohibitively expensive in low-resource settings (136,181). These tests require advanced laboratories, specialized equipment, and trained personnel, all of which are often unavailable in regions with limited healthcare infrastructure(181). As a result, many patients might not receive timely

resistance testing, leading to unnecessary switching of treatment regimens based on clinical judgment alone, which can be suboptimal and costly.

In this context, DLF may offer a cost-effective alternative that can help prevent unnecessary regimen changes. By monitoring drug levels, healthcare providers can identify poor adherence or drug interactions as the cause of treatment failure rather than drug resistance (1,134). This allows for interventions such as adherence counseling or dose adjustments without prematurely switching regimens or ordering expensive resistance tests. This strategy not only preserves the effectiveness of available ARVs but also reduces the need for costly and complex resistance testing, making HIV treatment more sustainable and accessible in low-resource settings.

Further complicating HIV treatment in low-resource settings is the high prevalence of comorbidities, such as tuberculosis and malaria, which require additional medications that may interact with ARVs. These drug interactions can alter ARV levels and effectiveness, increasing the risk of suboptimal dosing and treatment failure. For instance, rifampicin, a key drug in TB treatment, significantly lowers the blood levels of many commonly used ARVs, such as protease and integrase inhibitors, by accelerating their metabolism in the liver (182) (see section 3.1.3 on Drug-Drug Interactions). Monitoring ARV levels helps ensure that the presence of comorbidities and their treatments do not compromise the efficacy of HIV treatment. This feedback can allow healthcare providers to adjust doses in real-time, ensuring optimal drug exposure despite the challenges posed by multiple treatments.

This underscores the urgent need for portable and decentralized drug monitoring systems in low-resource settings that allow allows healthcare workers to optimize dosing, prevent

resistance, and manage treatment failure, even in the presence of co-occurring diseases and limited ARV options.

## 6.1 Need for Portability

Expanding access to DLF through portable testing platforms significantly improves the reach of its benefits. These platforms allow for more frequent and convenient monitoring, particularly where regular clinic visits are not feasible, thereby enhancing accessibility, timeliness, convenience, and cost-effectiveness of DLF (183). Timely results facilitate prompt clinical decisions and therapy adjustments crucial for enhancing patient outcomes. They offer convenience of use in various settings, including homes, workplaces, and public health clinics. Portable DLF can also reduce the need for sample transportation to centralized laboratories, cutting costs and enhancing efficiency(183). Rapid and accurate testing is particularly important in remote and underserved areas lacking advanced laboratory facilities, ensuring that crucial clinical information is available to guide optimal treatment effectively regardless of location.

## 6.2 Portable Readers in the COVID-19 Era

The COVID-19 pandemic catalyzed significant technological advancements driven by unprecedented investments in research and development. The urgency to combat the global health crisis funneled vast amounts of money, time, and resources into creating technologies that could deliver rapid, accurate, and accessible testing solutions. This surge in innovation has laid the groundwork for advancements in performing molecular tests on a portable platform (184).

There are many factors to consider when evaluating a portable diagnostic platform in addition to clinical sensitivity and specificity, including instrument size and weight, power requirements, time-to-result, integrated data analysis and data export options, the number of manual processing steps, and cost of equipment and consumables (184). The parameters to consider in the context of use for drug level measurement remain largely the same, with physical restraints remaining a key factor for portability, components such as programmable heating steps required depending on the assay used, and the compatibility of the readout format with the assay.

Platforms such as Cue Health and Lucira®, use isothermal nucleic acid amplification as the basis for amplifying and detecting the presence of SARS-CoV-2 RNA. Isothermal amplification methods received significant attention for COVID-19 detection due to their simplicity (typically requiring only a single step) and their adaptability to point-of-use devices as these technologies eliminate the need for precise thermal cycles(185). Also, since they can run at one consistent temperature, this is a main reason why the diagnostic amplification ability normally associated with lab-based PCR machines can now be found in a convenient, at-home test that is powered by 2 AA batteries (186).

For example Cue Health developed a COVID diagnostic system comprised of a single-use test cartridge, a lower nasal swab (Cue Sample Wand), and the Cue Health Monitoring System, which includes the Cue Cartridge Reader and a mobile application that delivers results directly to the user's device within approximately 20 minutes. However, the reader alone cost $199 or $394 for the reader and a pack of 3 disposable test cartridges(187). A 10-pack of cartridges sold separately cost $650. Despite being granted Emergency Use Authorization by the FDA in March

2021, it was revoked in 2024 due to an increased risk of false positives after the company made modifications to the test (188).

Lucira – now owned by Pfizer - gained the first FDA authorization for COVID-19 self-testing at home (189). The single-use kit includes a nasal swab, a vial for sample processing, and a battery-operated device that delivers results in approximately 30 minutes. Building upon this foundation, Lucira expanded its diagnostic capabilities to include influenza detection. In February 2023, the FDA granted EUA for the Lucira COVID-19 & Flu Home Test(190). This advancement enabled simultaneous detection and differentiation between SARS-CoV-2 and influenza A and B viruses within a single test, providing users with comprehensive diagnostic information (186). This showcases how a portable testing platform can be modified to perform additional testing modalities. Unlike the Cue Health platform that has a reusable reader and disposable testing cartridges, the Lucira COVID-19 & Flu Home Test is designed as a single-use tool, meaning that all components including the reader must be disposed of after use. The cost for a single test is $39 as of December 2024. Overall, the optimal portable reader will need to balance specific priorities relating to price, ease of use, and accuracy.

### *6.2.1 The Harmony Portable Reader*

The Harmony COVID-19 portable reader is a significant advancement in POC testing, combining affordability, portability, and ease of use with high accuracy and rapid results (18). The system utilizes reverse transcription loop-mediated isothermal amplification (RT-LAMP) to detect viral RNA with high sensitivity and specificity, enabling rapid SARS-CoV-2 detection using a smartphone interface and fluorescence readout, with a limit of detection down to 0.38 RNA copies/µl and results in as little as 17 minutes for high viral loads. Users collect a nasal swab, elute

it in a buffer, and transfer the eluate to a reaction tube. The tube is then inserted into the heater/detector, and the smartphone app guides the process (Figure 6).

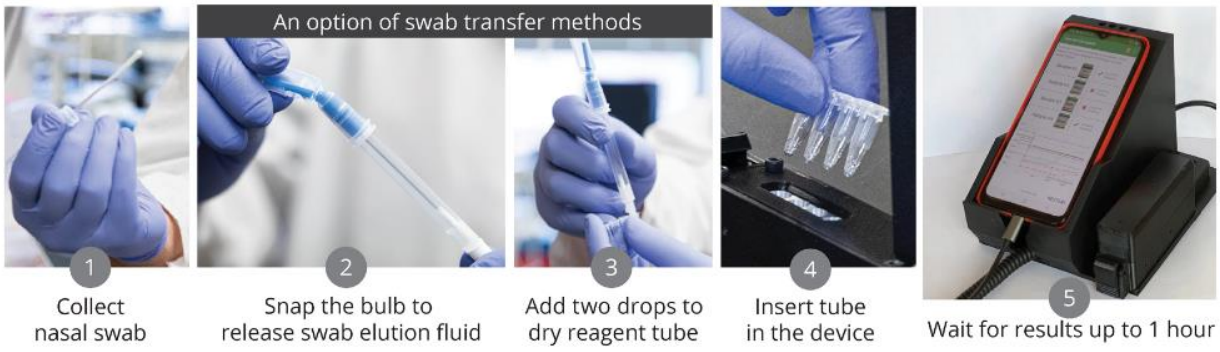


**Figure 6**. **Harmony COVID-19 workflow**. A nasal swab is collected and eluted in the rehydration buffer. The swab eluate is then transferred to the reaction tube to rehydrate and preloaded lyophilized RT-LAMP reagents. Four samples can be analyzed in parallel. Users follow the instructions on the cell phone app to record the sample identity, insert the tube in the test slot, and close the device. Figure from reference (18).

The Harmony COVID-19 system features a portable reader designed for simplicity, affordability, and ease of use. It includes a lightweight, portable heater/detector device controlled by a smartphone, making it ideal for POC testing. The device comprises an aluminum heater block with four wells, each capable of holding a reaction tube, and a spring-loaded heated lid to prevent condensation. A microcontroller manages the temperature, ensuring stability within three to four minutes after disturbances. Two LEDs provide excitation light, and emission filters and photodiodes on the sides of each sample well detect fluorescence, allowing for real-time analysis of the reaction. The entire setup, including the plastic housing and the heater/reader device, weighs less than 0.5 kg and has compact dimensions, allowing it to be easily transported and used in

various settings, including resource-limited environments. The device operates on low power (<15 W) and can be powered via USB, enhancing its portability and applicability in diverse locations without extensive infrastructure (18). The Harmony has many advantageous characteristics that could be utilized in ART DLF. The small size, programmable heating steps, and importantly and integrated fluorescence measurement capabilities make the Harmony an ideal choice for performing enzymatic assays that use fluorescence as a quantification method.

# 7. Summary of Completed Work

## 7.1 Aim 1. Optimize REACT for NNRTIs in Buffer

### *7.1.1 Introduction*

Building upon the RESTRICT assay for NRTIs, DNA synthesis reagents will be modified to optimize readout for NNRTIs. The optimal Master Mix concentrations determined by Olanrewaju et al. included 1 nM template, 10 nM primer, and 100 nM dNTPs. The goal of this aim is to optimize and validate if these parameters are compatible with a range of NNRTIs including RPV, DPV, ETV, DOR, and NVP.

The REACT assay measures DNA synthesis by HIV-RT as a function of the concentration of NNRTIs (Figure 7). NNRTIs inhibit DNA synthesis by binding to the RT enzyme, interfering with its ability to incorporate dNTPs into a template strand. At high drug concentrations, DNA synthesis is greatly inhibited leading to a low amount of dsDNA product. Low drug concentrations exert less inhibition allowing for more dsDNA synthesis. The REACT assay employs an intercalating dye to quantify the amount of dsDNA synthesized with fluorescence, and consequently correlates fluorescence to drug levels.

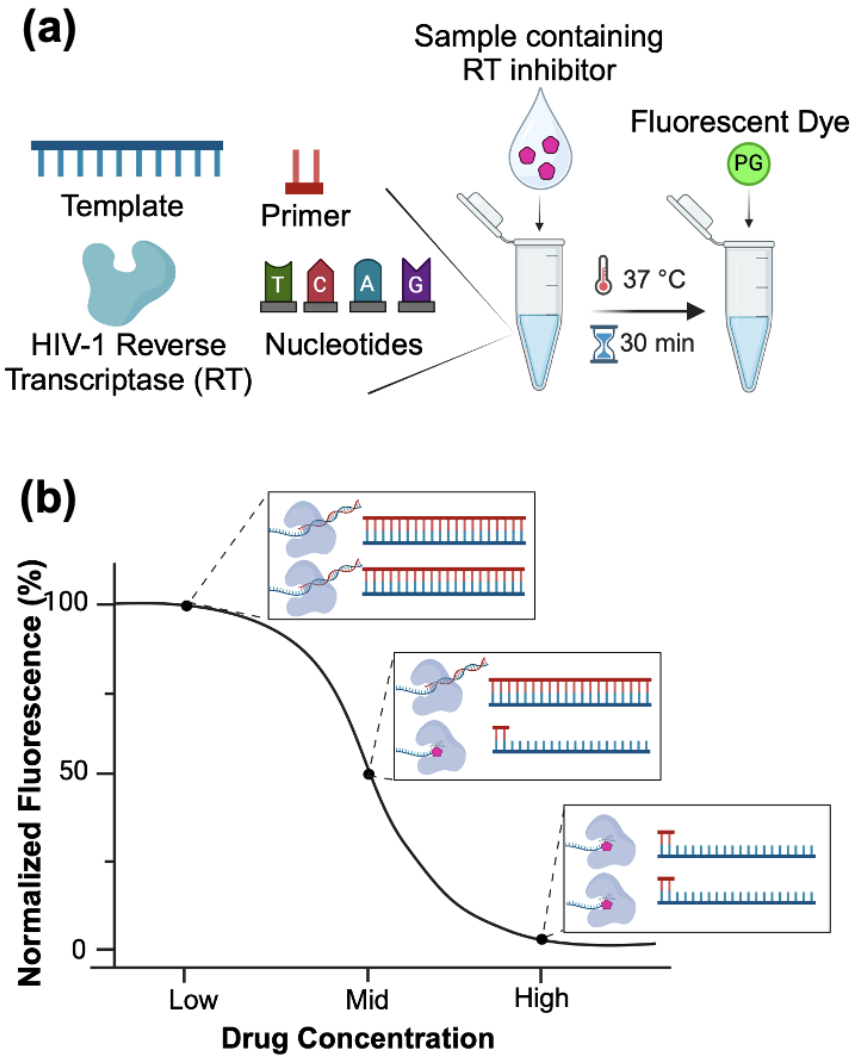


**Figure 7. Overview of REverse transcriptase ACTivity (REACT) assay for rapid HIV drug-level feedback**. **(a)** REACT measures HIV reverse transcriptase (RT) inhibitors in a sample by incubating it with DNA synthesis reagents (nucleic acid templates, primers, nucleotides, and recombinant HIV RT enzyme). Following incubation at 37°C for 30 minutes, the reaction is quenched by adding PicoGreen™ intercalating dye. **(b)** Fluorescence readout measures double-stranded DNA (dsDNA) synthesis by RT as a function of drug concentration

### *7.1.2 Materials and Methods*

REACT reactions were performed in an RT buffer containing 60 mM Tris (Invitrogen, AM9855G), 30 mM KCl (Sigma Aldrich, 101553), 8 mM M $gCl_2$ (Sigma Aldrich, 63069), 10 mM dithiothreitol (Sigma Aldrich, 20-265) adjusted to a pH 8.0 with 1M NaOH (Sigma Aldrich, S8263). The NNRTIs tested including RPV (NIH HIV Reagent Program), DOR (NIH HIV Reagent Program), NVP (NIH HIV Reagent Program), ETV (NIH HIV Reagent Program), and DPV (Adooq Bioscience, A12587), which were reconstituted in dimethyl sulfoxide (DMSO) (Fisher Bioreagents, BP231-100). The DNA template and corresponding primer were synthesized in silico as described in our past work (Custom DNA Oligo, Integrated DNA Technologies) and resuspended in 1xTE buffer pH=8 (Integrated DNA Technologies, 11-05-01-09). TFV-DP (BOC Sciences Inc., 166403-66-3) and 3TC-TP (Sierra Biosciences, Lot #3567) were diluted in water and serial dilutions performed.

<u>REACT for NNRTIs</u>

REACT was performed by combining 20 µL of Master Mix including 1000 nM dNTPs (unless otherwise stated), 10 nM template, and 100 nM primer in RT buffer, 10 µL of NNRTI in DMSO, and 10 µL of 0.053 units/mL RT (Worthington Biochemical, LS05009) for a total volume of 40 µL per reaction. NNRTI concentrations were tested in serial dilutions spanning eight orders of magnitude ($10^{-3}$ M to $10^{-10}$ M) to encompass possible clinically relevant concentrations, with the exception of NVP ($5x10^{-2}$ to $10^{-8}$ M). The diminished potency of NVP required testing of higher drug concentrations to exhibit a full sinusoidal curve due to potency and solubility limitations. Reactions were performed in black, non-binding, flat-bottom polystyrene 96-well plates with

nonbinding surfaces (Corning Incorporated, 3650). 10 µL of RT was added as the last reagent to initiate DNA synthesis and the 96-well plates were immediately transferred to a benchtop plate reader (SpectraMax iD3, Molecular Devices). After incubation for 30 minutes at 37°C at medium shaking (517 rpm), 40 µl of the fluorescent intercalating dye Quanit-iT™ PicGreen™ (PG) (Invitrogen, P7581) diluted 1:200 in 1xTE was added to quench the reaction and provide fluorescence readout. After additional 1 minute incubation, readout was performed on a plate reader unless otherwise stated.

"No enzyme" negative controls containing 10 µL of RT buffer instead of the RT enzyme, and the resulting fluorescence due to background signal and any possible dsDNA created by template-primer binding or passive interactions was taken into account to determine only the amount of dsDNA synthesized by RT. "No drug" positive controls containing 10 µL of DMSO instead of a drug dilution were used to normalize fluorescence readings. Normalized fluorescence values were fitted to four-parameter logistic regression curves using GraphPad Prism Software (GraphPad Software Inc.). Three replicates were performed unless otherwise stated.

dNTP optimization

To optimize REACT for NNRTIs, detection of RPV was compared to the detection of TFV-DP under differed Master Mix formulations. REACT was performed as described above using 1000 nM, 2500 nM, 5000 nM, and 10,000 nM dNTP concentrations with corresponding increases of template and primer concentration in a fixed 1:10:100 template:primer:dNTP ratio. Serial dilutions of TFV-DP (in RT buffer) or RPV (in DMSO) were tested across multiple orders of magnitude. TFV-DP concentrations spanned $1x10^{-4}$ to $10^{-10}$ M and RPV concentrations spanned $10^{-3}$ M to $10^{-10}$ M. Normalized values were compared to more easily compare the inhibition curves

at each dNTP concentration. Normalized fluorescence values were fitted to four-parameter logistic regression curves using GraphPad Prism Software (GraphPad Software Inc.). Three replicates were performed unless otherwise stated.

NRTI vs NNRTI Detection

To determine the ability of REACT to detect and differentiate between NRTIs and NNRTIs, 3 curves were performed.

For NRTI detection- REACT was performed using the previously determined optimal Master Mix concentrations for NRTIs(14,176,177): 1 nM template, 10 nM template, 100 nM dNTPs. Eight 3TC-TP concentrations ($10^{-3}$ M to $10^{-10}$ M) in RT buffer were tested along with buffer positive and negative controls. Buffer controls contained 20 µL of Master Mix, 10 µL of water, and either 10 µL of RT for positive controls or 10 µL of buffer for negative controls.

For NNRTI detection- REACT was performed as previously described for NNRTIs using 10 nM template, 100 nM primer, 1000 nM dNTPs. Eight DOR serial dilutions in DMSO ($10^{-3}$ M to $10^{-10}$ M) were tested along with DMSO positive and negative controls as described previously.

For NNRTI detection in the presence of an NRTI- REACT was performed using 10 nM template, 100 nM primer, 1000 nM dNTPs. The drug conditions tested included a serial dilution of DOR in DMSO ($10^{-3}$ M to $10^{-10}$ M) in addition to a fixed 3TC-TP concentration of $10^{-6}$ M which was added to each DOR concentration condition. Combined DMSO and Buffer positive and negative controls were run as well to account for any inhibition caused by the presence of DMSO from the NNRTI samples. Controls contained 20 µL of Master Mix, 5 µL of water, 5 µL of DMSO and 10µL of RT for positive controls or 10 µL of buffer for negative controls.

Fluorescence values were normalized for comparison to respective control conditions to account for inhibition of DMSO as well as the difference in Master Mix concentrations. Normalized fluorescence values were fitted to four-parameter logistic regression curves using GraphPad Prism Software if possible (GraphPad Software Inc.). Three replicates were performed unless otherwise stated.

### *7.1.3 Results and Discussion*

#### *7.1.3.1 Effect of dNTP concentration*

Previous work done to optimize the RESTRICT assay for NRTIs demonstrated the effect of increasing dNTP concentration. As dNTP concentration increases, the curve shifts along the ex-axis towards higher drug concentrations. Normalized data was compared to more easily compare the inhibition curves at each dNTP concentration. A 1:10:100 fixed ratio of template:primer:dNTP was used in each Master Mix. With increasing dNTP concentrations, the template and primer concentrations increase in proportion.

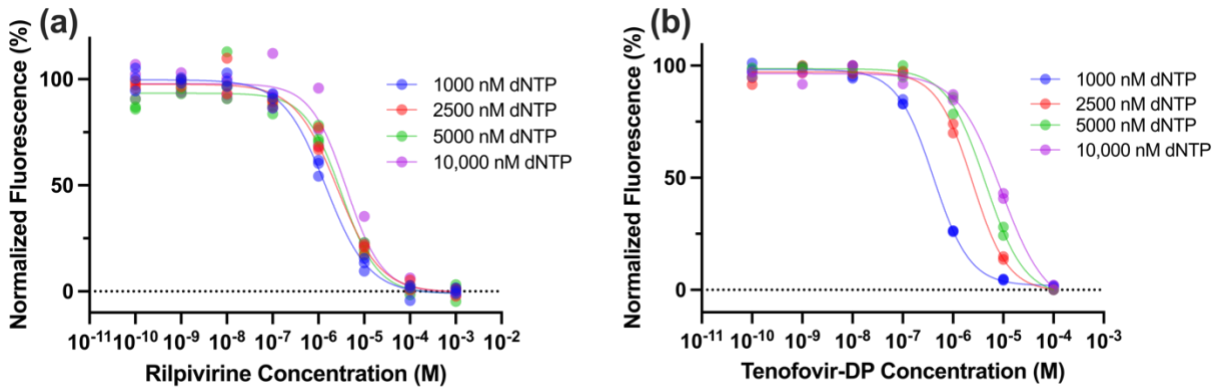


**Figure 8. Effect of dNTP concentration of NRTI vs NNRTI measurement**. **(a)** Normalized RPV fluorescence readout demonstrates a no shift in quantitative region with increasing dNTP

concentration. **(b)** Normalized Tenofovir-DP fluorescence readout demonstrates a shift toward higher NRTI concentrations with increasing dNTPs.

An increase in DNA synthesis reactants allowed for more dsDNA to be synthesized uninhibited. An increase in DNA production increases fluorescence signal output. For NRTIs like TFV-DP, increasing dNTP concentration shifts the resulting enzymatic inhibition curve horizontally along the x-axis. This effect is due to the mechanism of action NRTIs employ for inhibition. Since NRTIs are nucleotide analogs, NRTIs are competing with endogenous nucleotides for RT incorporation. As dNTP concentration increases in comparison to NRTIs, RT is more likely to incorporate the dNTP than the NRTI. This means that a higher concentration of NRTI is needed in the sample for detection, which is what the curve shifting towards higher drug concentrations indicates.

NNRTIs do not show this same curve shifting effect as dNTP concentration increases, reflected in Figure 8. Since NNRTIs interact directly with the RT enzyme and not dNTPs competitive binding is not an issue. This indicates that REACT will have the same quantitative range regardless of dNTP concentration, as long as they are in excess. Because dNTPs don't affect the quantitative range we have the freedom and flexibility to increase concentration. We know from previous work that RESTRICT struggles to operate in complex biological samples (86). We hypothesized that we could move forward with the highest concentration to provide free boost in signal. As we are optimizing REACT for NNRTIs, we want to make the signal very large and are able to do so without shifting drug concentrations measurable.

*7.1.3.2 Detection of Long-Acting Injectable Rilpivirine Alert Threshold*

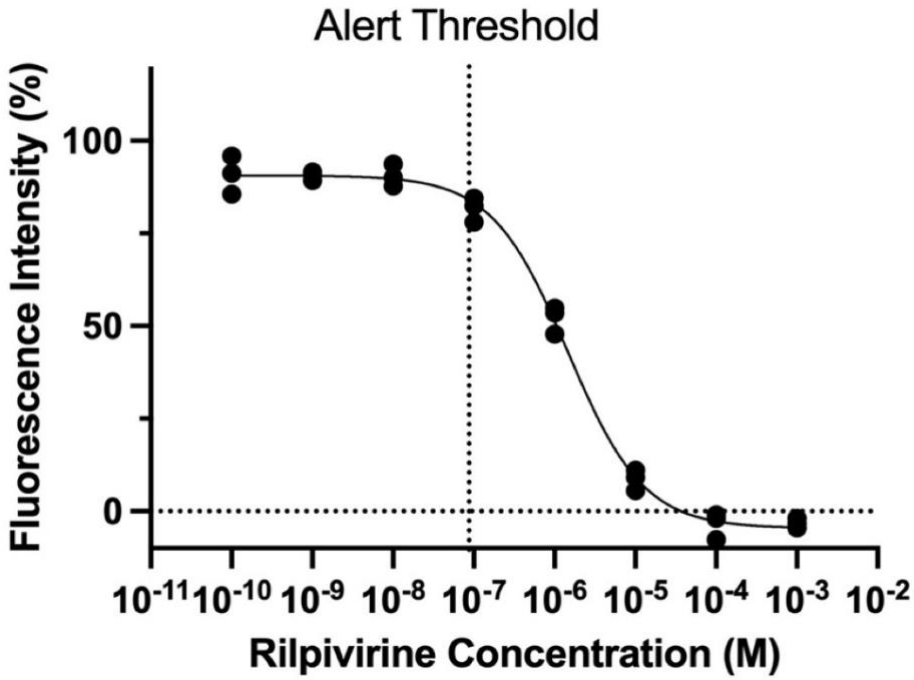


**Figure 9. REACT can detect RPV levels near the proposed Alert Threshold for LAI RPV**. N=3. Vertical line indicates the French National Agency alert threshold of 32ng/mL ($8.7x10^{-8}$ M).

The French National Agency for Research on AIDS and Viral Hepatitis Emerging Infectious Diseases alert threshold for long-acting injectable RPV is 32 ng/mL ($8.7x10^{-8}$ M). Below this threshold, PLWH are at risk of treatment failure and development of drug resistance (17). Figure 9 shows a REACT curve measuring RPV concentrations with the vertical line indicating the alert threshold. This threshold of interest falls at the edge of the quantitative range, enabling REACT to detect if someone taking LAI is above or below the threshold. The introduction of possible inhibitors in biological matrices could exert some inhibition on the assay, shifting the curve right, and REACT would still be able to detect if RPV levels are above or below the alert threshold.

*7.1.3.3 Measurement of multiple NNRTIs used in HIV treatment and prevention*

We perform a proof-of-concept demonstration of REACT using five NNRTIs that each offer distinct advantages in HIV prevention and treatment, listed in Table 1. We employed $C_{max}$ values as an overarching benchmark for NNRTI concentration detection, which is defined as the peak concentration of drug in a specific sample type (99).

**Table 1. Summary of NNRTI use cases and $C_{max}$ plasma values.** The NNRTI class provides a variety of tailored options to meet the needs of different populations that can benefit from DLF. $C_{max}$ plasma values derived from clinical data.

| **NNRTI** | **Use Case for Drug** | **$C_{max}$ (nM)** | **Ref** |
|---|---|---|---|
| Rilpivirine | Bi-monthly injections for HIV treatment | 376 | (40) |
| Dapivirine | Monthly vaginal rings for HIV prevention | 1.40 | (191) |
| Doravirine | Used in alternative regimens for adults living with HIV to minimize metabolic effects associated with weight gain and cholesterol | 2,260 | (192) |
| Nevirapine | Cost-effective NNRTI available as a generic formulation with a strong record of tolerability and efficacy. Widely used, especially in low-resource areas, for preventing perinatal transmission. | 56,300 | (193) |
| Etravirine | Third-line regimen with a high genetic barrier to resistance and fewer neuropsychiatric adverse effects. | 903 | (194) |

REACT readout exhibited the anticipated sigmoidal curve, indicative of enzymatic inhibition reaction, across all five NNRTIs examined (Figure 10). Figure 10a is a replicate of the RPV data from Figure 9 with a different threshold. Notably, there was minimal variability between replicates, with an average coefficient of variation (CV) of <5%. While $C_{max}$ values can vary

significantly due to inter- and intra-pharmacokinetic variability, sample type, and specific NNRTI, $C_{max}$ values are a useful metric for evaluating whether an assay provides clinically relevant results (14,104,105). The linear region of REACT sigmoidal curves overlapped with the $C_{max}$ for four of the five NNRTIs tested, illustrating the potential utility of the platform for distinguishing clinically relevant values across populations of interest. REACT demonstrates the capacity to measure a broad spectrum of NNRTI drug levels, illustrating the versatility of the platform.

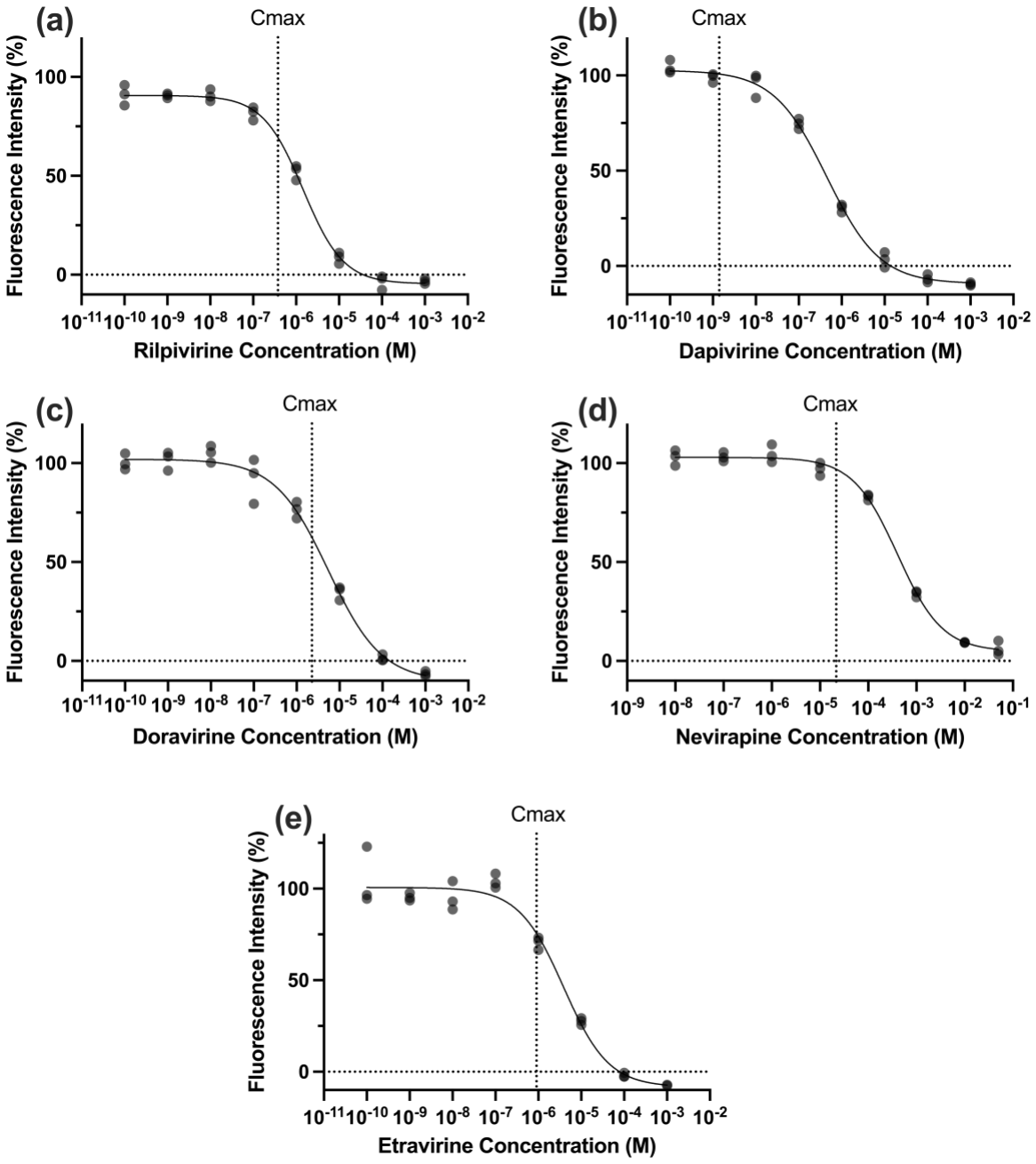


**Figure 10. Measurement of multiple NNRTIs using REACT**. Normalized fluorescence is plotted against drug concentrations of **(a)** Rilpivirine **(b)** Dapivirine **(c)** Doravirine **(d)** Nevirapine and **(e)** Etravirine. N=3. Vertical lines indicate $C_{max}$ values in Table 1.

*7.1.3.4 NRTI vs NNRTI Detection*

As has been previously demonstrated, the REACT system can detect RT inhibitors in either the NRTI or NNRTI class. Since the mechanism of action for RT inhibition is different for NRTIs and NNRTIs, REACT can distinguish between NRTIs and NNRTIs depending on the template used in the reaction.

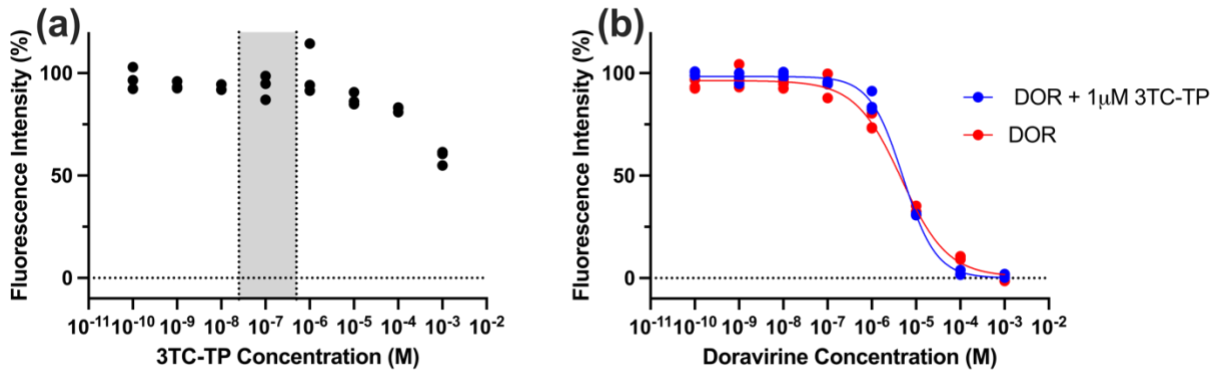


**Figure 11. REACT is able to detect NNRTI inhibition in the presence of an NRTI. (a)** Normalized fluorescence from REACT detection of the cytosine analog 3TC-TP does not demonstrate inhibition when used with a template lacking the G nucleotides. Clinical range shown in shaded region. **(b)** Normalized fluorescence values of DOR detection are not significantly impacted by the presence of 3TC-TP.

The original REACT platform (RESTRICT) measured NRTIs, nucleotide analogs, that was specific to the template used. Since NRTIs are nucleotide analogs, the template design used determines the amount of complementary binding sites available for the nucleotide analog to be incorporated into the growing DNA chain. For example, Olanrewaju et al. demonstrated that the optimal template for detection of TFV-DP, a deoxyadenosine analog, includes a sequence rich in thymidine (T) bases(14,176,177). Each T base provides an opportunity for TFV-DP incorporation,

which initiates chain termination. Since detection is dependent on template design, RESTRICT can selectively detect different NRTIs depending on their analog structure(177). The TTCA template found optimal for TFV-DP detection, does not contain G nucleotides. For any NRTIs that are C analogs, such as 3TC-TP, there are no opportunities for the NRTI to induce early chain termination since there are no places on the template strand that are complementary to the analog demonstrated in Figure 11a. Since no chain termination was possible, reverse transcription is not inhibited. Interestingly, fluorescence appears to decrease significantly at the highest concentration tested (1e-3M), however this concentration of 3TC-TP is significantly higher than any clinically relevant concentrations that could be found in clinical samples(177). REACT can detect NNRTIs regardless of the template design, since NNRTIs interact directly with the reverse transcriptase enzyme for inhibition. This allows for REACT to detect NNRTI inhibition in the presence of NRTIs if the template used does not contain complementary nucleotides for the NRTI analog present. Figure 11b demonstrates how the detection of NNRTI DOR by REACT is not affected by the presence of 1 μM 3TC-TP.

While it is possible for REACT to detect NNRTI inhibition in the presence of an NRTI, this version of REACT cannot distinguish between the inhibition of an NRTI in the presence of an NNRTI. This is again because NNRTIs work directly on the RT enzyme, which is necessary for incorporation of nucleotide analogs. The inhibition seen by REACT would show the combined inhibitory effect of both NRTIs and NNRTIs.

However the utility of a one-pot reaction assay to distinguish between NRTI and NNRTI inhibition may have limited utility as the field is currently moving away from NRTI and NNRTI-based regimens in favor of INSTIs due to their increased barrier to genetic resistance(195). Current guidelines for recommended first-line regimens involve an INSTI (like bictegravir and

dolutegravir) and 2 NRTIs (like TAF or TDF and FTC or 3TC)(113). In some circumstances, NNRTI-based regimens that are recommended include DOR/TDF/3TC, DOR plus TAF/FTC, or RPV/TAF/FTC (113).

As demonstrated in Figure 11 REACT can distinguish between the inhibition of DOR and 3TC using the TTCA template. The third ARV in the recommended first-line NNRTI-based ART in addition to DOR and 3TC, is TDF. TDF is metabolized into TFV-DP, which is an adenosine analog which we have previously demonstrated is measurable using this enzymatic assay platform(14,176,177). It is theoretically possible for REACT to determine the inhibition of DOR in a clinical sample of someone on this combined ART regimen if the template used did not contain guanosine or thymidine, depending on thermodynamics of template design and RT enzyme sequence preference.

Additionally, NRTIs and NNRTIs are primarily found in different biological matrices, which suggests that optimized sample prep steps could allow the use of REACT to detect NNRTIs and NRTIs in a complex clinical sample from someone on a multi-drug regimen. NRTIs and NNRTIs are found in different biological compartments based on their necessary mechanism of action and distribution properties. NRTIs are prodrugs that require intracellular phosphorylation to become active, and due to their hydrophilic nature, the activated form accumulates intracellularly, predominantly in PBMCs(1,155,196). NNRTIs do not require any transformation to become active, and are lipophilic so they distribute well in plasma(197).

There are several methods to separate whole blood components plasma and PBMCs/RBCs. Standard laboratory practices of active separation of whole blood can be performed using benchtop centrifugation(198). Other methods more amenable to low resource settings include hand-operated centrifuge devices such as a “paperfuge”(199), a modified eggbeater(200), and the use of a fidget-

spinner(201) to attain similar sedimentation-based methods of separation. Passive filtration methods have also been explored using plasma separation membranes that separate plasma from larger components in whole blood through size exclusion dictated by the pore size and material of the membrane(198). If sample separation techniques are employed to separate the biological components NRTIs and NNRTIs are found and primarily measured in, REACT could then be used to determine NRTI and NNRTI levels with NRTI-dependent template sequences.

### *7.1.4 Conclusion*

REACT is able to measure NNRTI concentrations across 5 different drugs tested. Increasing the Master Mix concentration by a factor of 10 provided an additional boost in fluorescence signal without shifting the quantitative range. This will be especially important when testing spiked plasma samples or clinical samples, since Olanrewaju et al., determined that biological matrices can inhibit DNA synthesis within the assay(86). Additionally, REACT is able to determine the inhibition of NNRTIs in a sample containing an NRTI, based on specific template design.

## 7.2 Aim 2. Validate REACT Performance in Plasma

### *7.2.1 Introduction*

NNRTIs can be highly protein-bound and have a high affinity for plasma proteins, particularly albumin and alpha-1 acid glycoprotein. As a result, NNRTIs are primarily found in the plasma compartment, with minimal distribution into other bodily fluids or tissues (106). Plasma is a complex matrix that contains various components, including salts, proteins, lipids, and other

endogenous molecules (107). Previous RESTRICT experiments have demonstrated that biological matrices can inhibit DNA synthesis (86). Olanrewaju et al., 2020, determined a 1:4 dilution with water was an easy sample preparation method to dilute components in the biological matrix that may inhibit RT assay performance. We evaluated REACT performance in 1:4 diluted plasma samples spiked with DOR to evaluate its performance in a more complex sample matrix and simple sample preparation.

### *7.2.2 Material and Methods*

REACT was performed with DOR spiked in diluted plasma with readout on the plate reader and the portable reader to simulate clinical samples. Gender-pooled, unfiltered human plasma from healthy participants collected into K2 EDTA anticoagulant was acquired from BIOIVT (HUMANPLK2-0000283) and diluted 1:4 in nuclease-free water (Invitrogen, AM9937). Plasma was spiked with DOR dilutions in DMSO for final DOR concentrations spanning eight orders of magnitude matching the spiked buffer testing conditions ($10^{-3}$ M to $10^{-10}$ M).

REACT was performed by substituting 10 µL of DOR-spiked plasma for the 10 µL of DOR in DMSO in the reaction. After readout on the plate reader, each sample was transferred to PCR tubes for measurement on the portable reader according to the above methods. “No enzyme” controls consisted of 10 µL of Master Mix, 20 µL of RT buffer, 2.5 µL DMSO, and 7.5 µL of diluted plasma. “No drug” controls consisted of 10 µL of Master Mix, 10 µL of RT buffer, 2.5 µL DMSO, 7.5 µL of diluted plasma, and 10 µL of RT. Fluorescence readouts were normalized to “no enzyme” and “no drug” controls and fit to a four-parameter logistic regression curve. To account for the 76% protein binding in plasma of DOR in plasma, the spiked sample concentration was multiplied by 0.24 (108).

### *7.2.3 Results and Discussion*

As mentioned previously, NNRTIs can be highly protein bound. This can be a challenge when measuring in vitro spiked plasma samples as the introduction of the NNRTI to the plasma sample induces protein binding that sequesters free drug levels. For spiked plasma sample optimization and evaluation we used DOR, which has a moderately low protein binding of 76% (108). Using a lower protein binding NNRTI allows us to test higher concentrations of free drug and evaluate a full enzymatic sigmoidal curve to optimize REACT.

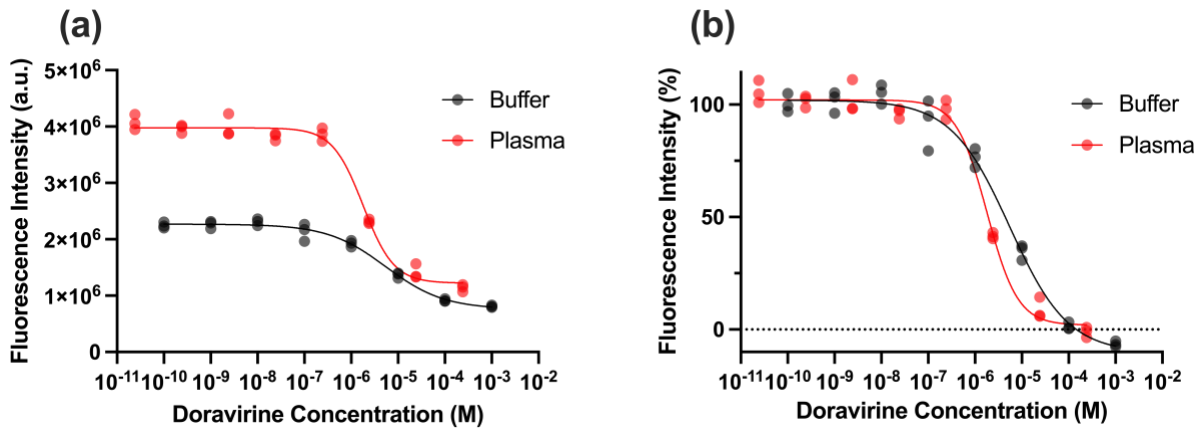


**Figure 12. Detection of spiked DOR plasma samples with REACT in a plate reader.** Spiked plasma concentrations are protein-adjusted. **(a)** Plate reader raw fluorescence data from buffer and spiked plasma samples indicate increased background fluorescence in plasma samples. **(b)** Normalized plate reader buffer and diluted plasma values indicate minimal inhibition in complex biological matrices.

To evaluate the performance of REACT in a more complex sample matrix, we spiked doravirine (DOR) into human plasma obtained from healthy volunteers not receiving any ARVs.

As in our past work, we diluted samples in water (25% v/v) as a simple sample preparation method to reduce non-specific HIV RT inhibition. Figure 12a compares REACT with DOR spiked into aqueous buffer or human plasma. The x-axis of the plasma curve was adjusted to account for the 76% protein binding of DOR in plasma (108). Raw fluorescence values for plasma samples were higher than buffer values of equivalent drug concentration, likely due to endogenous proteins and other components found in plasma that are known to exhibit autofluorescence (109). This resulted in a vertical shift of the curve but not a shift along the x-axis, indicating similar levels of DOR were detected in buffer and plasma samples suggesting minimal inhibition of assay performance in diluted plasma samples. As seen in Figure 12b, normalized fluorescence values to account for baseline fluorescence of plasma samples show similar dose-response curves sigmoidal positioning across DOR levels in plasma and in buffer, suggesting minimal inhibition of assay performance in diluted plasma samples.

#### *7.2.4 Conclusion*

REACT is capable of detecting a wide range of DOR concentrations in 1:4 diluted plasma. The normalized buffer curve and protein-adjusted spiked plasma curve overlap with alignment, indicating that inhibitory factors that may be present in plasma do not significantly inhibit REACT.

## 7.3 Aim 3. Characterize REACT Compatibility on Portable Reader

#### *7.3.1 Introduction*

To demonstrate the potential of REACT as a portable POC DLF test, we next translated fluorescent measurements from a traditional plate reader to the Harmony, a portable heater and

fluorescence reader that was previously developed and validated for point-of-care COVID-19 diagnostics (19). The Harmony portable reader offers several practical advantages over traditional plate readers, particularly in terms of reduced size, weight, and cost. For example, the iD3 Spectramax plate reader used in this work weighs 40 kg and occupies 3.18 $m^2$ of bench space, necessitating a dedicated, stationary workstation. In contrast, the Harmony portable reader weighs only 0.1 kg, representing 0.25% of the plate reader weight, and occupies just 1% of the area on a benchtop in comparison (0.035 $m^2$) (Figure 13). Notably, Harmony costs only ~$300 USD to assemble whereas the iD3 SpectraMax costs ~$30,000 USD, which is a 100-fold reduction in price (19). The reported $300 USD price for the Harmony does not consider the reduction in per unit cost in a scale-up in manufacturing, which would decrease the per unit price. While the benchtop plate reader requires a computer for data readout and analysis, Harmony results can be obtained on a cell phone. Integration into Harmony increases access to REACT especially in low-resource settings where HIV is endemic.

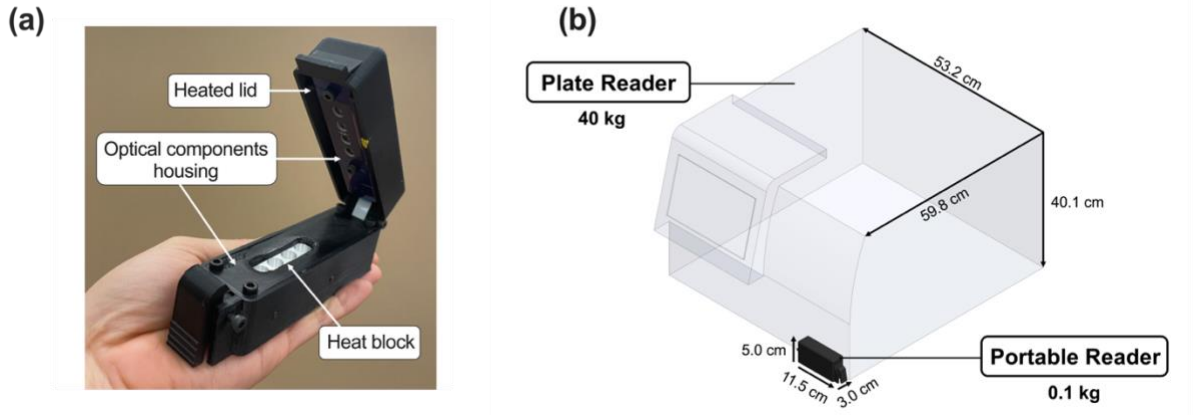


**Figure 13. The portable reader offers a significant reduction in size and weight compared to a traditional plate reader. (a)** The Harmony device consists of fluorescence detectors and a heat block with four reaction tube wells. **(b)** The Harmony portable reader offers a significant reduction in size and weight compared to a traditional plate reader.

### *7.3.2 Materials and Methods*

To validate the compatibility of the portable reader with REACT, the readout of identical samples was performed in the plate reader and a portable reader that was previously developed for isothermal incubation and fluorescence readout for a point-of-care COVID-19 diagnostic (19). REACT was performed according to the methods described in Aim 1 with DPV in DMSO spanning eight orders of magnitude in concentration ($10^{-3}$ M to $10^{-10}$ M). Mechanical, optical, and electrical components of the Harmony reader were assembled as previously described. Samples were incubated in the plate reader under light agitation for 30 minutes at 37 °C. 40 µL of PG was then added and readout was performed on the plate reader.

After fluorescence readings on the plate reader were completed, 60 µL of each sample tested was transferred from the 96-well plate to individual PCR tubes (Millipore Sigma, BR781320) and placed in the portable reader. Each sample was measured in each of the four available wells for 25 seconds, with a fluorescence measurement taken every 1.2 seconds. The average of these values during the 25-second measurement was taken as the representative fluorescence value to reduce the impact of background noise on the reading. Fluorescence readout was normalized to "no enzyme" and "no drug" controls and fit to a four-parameter logistic regression curve. The resultant fluorescence values from the portable reader were compared to measurements from the plate reader by performing a simple linear regression and calculating the Pearson correlation coefficient (GraphPad Prism).

### *7.3.3 Results and Discussion*

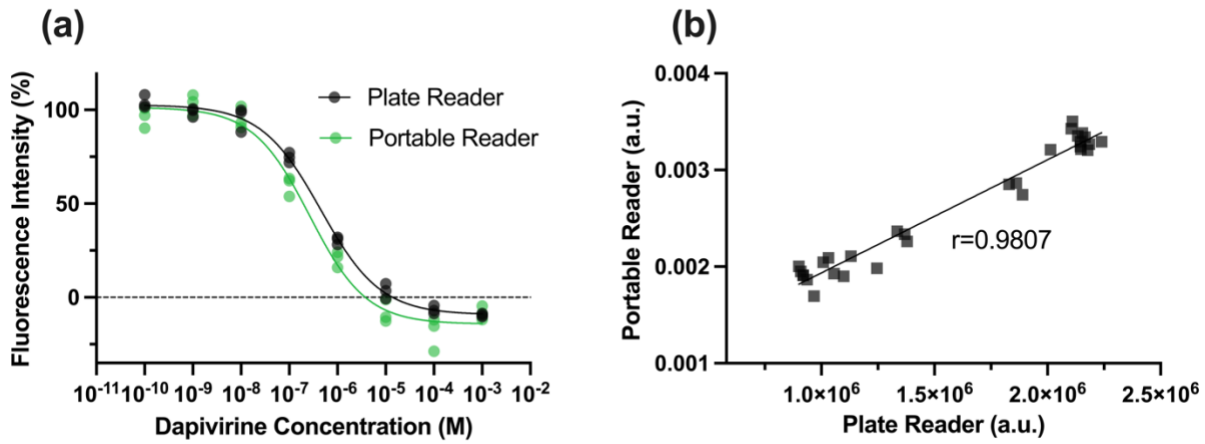


**Figure 14. Detection of DPV in buffer with REACT in a plate reader and a portable reader**. **(a)** Normalized fluorescence values indicate similar performance of REACT measurements of DPV in both readers. N=3. **(b)** Simple linear regression shows a correlation (Pearson's r = 0.9807, P<0.0001) between REACT measurements in the plate reader and measurements from a representative well in the portable reader. N=30.

There was high correlation between measurements from Harmony and the plate reader (Pearson's r=0.9807, P<0.0001) **(**Figure 14a & b**)**. Because Harmony only has 4 wells, we incubated REACT assays at 37˚C in the plate reader during high-throughput characterization experiments before measuring fluorescence output in either Harmony or the plate reader. However, we verified that the complete incubation and fluorescence readout process on Harmony did not result in appreciable differences in fluorescence intensities (Figure S1). Fluorescence output indicated in Figure 14a was derived from a representative well in Harmony (see Figure S2 for fluorescence from all Harmony wells). Harmony uses low-cost components for detection (LED, photodiode) and temperature control and does not include any calibration between wells or runs, so it might be expected to be more variable than the lab plate reader. However, normalization of

the signals to controls (no-enzyme, no-drug) likely reduced variations, and the data shows that Harmony results compare well to the plate reader (Figure 14a) and across the four wells of a Harmony device (Figures S1 & S2). We provide detailed comparisons between assay variation in the Harmony and plate reader in Tables S1 and S2. Taken together, Figure 14 shows that REACT can be performed in a portable reader that offers substantial reductions in size, weight, and cost relative to a traditional plate reader.

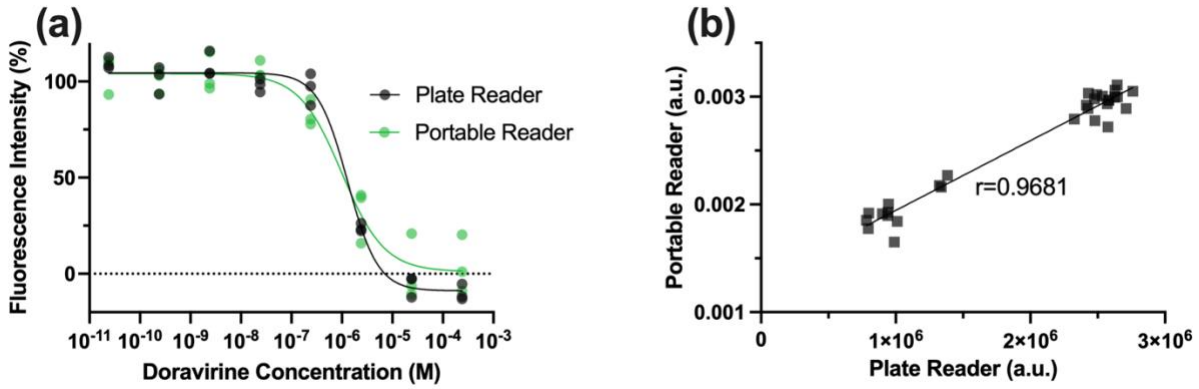


**Figure 15. Detection of spiked DOR plasma samples with REACT in a plate reader and a portable reader. (a)** REACT results in plasma were comparable between the plate reader and the Harmony portable reader. **(b)** Simple linear regression shows high correlation (Pearson's r=0.9681, P<0.0001) between REACT measurements in the plate reader and measurements from a representative well in the portable reader with plasma samples. N=30. Drug concentrations in REACT assays in plasma samples were adjusted for the 76% protein binding of DOR.

The portable reader was able to maintain excellent agreement with the plate reader when measuring plasma samples with a exhibits the normalized fluorescence values of identical samples measured on the plate reader and portable reader (Figure 15). This indicates high correlation

between the two instruments plate reader and portable reader values as demonstrated in Figure 14b (Pearson r=0.9681, P<0.0001).

### *7.3.4 Conclusion*

The correlation of fluorescence readout of REACT was very high between the Harmony portable reader and plate reader in both buffer (Pearson r=0.9807) and diluted plasma samples (r=0.9681). This indicates the comparable fluorescence detection ability of the plate reader and the portable reader for a 100-fold reduction in price. This supports the feasibility of REACT to be integrated into a portable platform to expand access to DLF.

## 7.4 Summary of Completed Work

The REACT assay offers a versatile solution for measuring NNRTI drug levels, addressing limitations of the gold standard LC-MS/MS such as cost and centralized laboratory requirements. We have demonstrated the detection of five NNRTIs, including RPV, DPV, DOR, NVP, and ETV, including drugs used in long-acting injectable and extended-release HIV treatment and prevention regimens. Moreover, we verified the ability to rapidly measure REACT fluorescence output in a portable reader with comparable performance to that of a traditional plate reader. This supports the feasibility of replacing the large, expensive benchtop equipment required for REACT with an inexpensive portable reader to increase accessibility to rapid DLF, particularly in resource-limited settings. Finally, we validated the ability of the REACT platform to detect an NNRTI in a complex biological matrix using both the plate reader and the portable reader. This work is innovative because it expands our prior work on enzymatic assays for HIV DLF to a new class of RT inhibitor. This activity-based assay strategy could also be useful for measuring HIV drug classes beyond RT

inhibitors. Future work will validate REACT with clinical samples and compare its performance against gold standard LC-MS/MS testing.

## 8. Perspective on Work

Since the approval of the very first HIV antiretroviral in 1987, the field of HIV treatment and prevention has seen incredible advances (202). From the introduction of combination therapy in 1995 (202), to the development of once-daily single-tablet regimens in 2006 (203), to the introduction of PrEP in 2012 (204), and now the emergence of long-acting treatments such as injectables, vaginal rings, and implants, the HIV landscape is constantly evolving (47). In this dynamic and ever-evolving HIV landscape, the technology and tools available also evolve. In the context of long-acting treatments and with the potential for a vaccine or even a cure on the horizon, the role of DLF is poised to shift.

The preliminary success of LAI CAB as PrEP and CAB and RPV as ART signals a transformative shift toward long-acting regimens that significantly reduce dosing frequency, potentially alleviating the compliance burden for patients (11). In this context of LAI, the role of DLF in adherence monitoring may diminish, as these long-acting dosages are more forgiving and minimize adherence issues (111). However, multiple factors may still impact virological outcomes based on levels of drug exposure, which still asserts DLF as an essential tool in supporting successful treatment and prevention strategies (11,111). The potential for subtherapeutic drug levels in long-acting regimens poses significant risks, which could result in severe personal and public health consequences, including treatment failure and the emergence of drug resistance.

Arguably, the most significant concerns about long-acting regimens is the risk of drug resistance, due to the risk of prolonged subtherapeutic drug concentrations (111,146,147). If these ARV-resistant strains are transmitted globally, the consequences could be severe, particularly if resistance extends to other drugs within the same class (4). This risk, and its relation to drug levels, will remain a pertinent scenario in which monitoring drug levels may be important. The inherent pharmacokinetic variability between individuals, along with factors such as injection site and technique, BMI, and other risk factors, underscores that a one-size-fits-all approach to dosing is not feasible.

Evidence from the use of other long-acting medications, such as antipsychotics and contraceptives, also provides valuable insights into managing suboptimal drug exposure identified through DLF (111). This can be addressed by adjusting the frequency of LAI administration. As treatment options expand, individuals living with HIV or those at risk will have an increased range of choices for their regimens. This evolving landscape may lead to a dynamic decision-making process, as patients are more likely to explore various options when more are available (143). In this context, DLF can be utilized to assess residual drug concentrations and guide the initiation or adjustment of treatment regimens. The long-acting formulations are particularly appealing for managing individuals with inconsistent or limited access to healthcare, a group that is at greater risk of experiencing subtherapeutic drug levels. Consequently, this population may benefit from DLF testing to accurately evaluate their medication coverage, assess risk, and ensure adherence.

Additionally, as the HIV treatment and prevention landscaping is evolving, so is the population of PLWH. Some projections suggest that by 2025, nearly 25% of PLWH will be over 65 years of age (205). A growing elderly population will likely increase cases of polypharmacy, suggesting that the role DDIs have on HIV treatment and prevention is likely to increase (111).

Currently, suspected DDI is one of the most commonly reported reasons for requesting DLF, and this influencing variable remains a concern for long-acting treatments (111). This in combination in the context of an aging person who develops comorbidities that may potentially alter the pharmacokinetics of ARVs (such as severe renal or hepatic insufficiency) creates increasingly complex cases that may need DLF to support clinical decisions. For example, it may be challenging to determine the clinical relevance of a DDI between RPV and a proton pump inhibitor in someone undergoing gastric surgery or the inducing effect of rifampicin on DOR metabolism in someone with severe liver failure. In such cases, the utility of widely used conventional drug interaction checkers may be limited, providing a strong justification for DLF (111). DLF may not only inform drug regimen clinical decisions but also inform a larger understanding of DDI and ARV pharmacokinetics as well as increase our knowledge on new drugs.

DLF also maintains a role for as a tool for use in the development and validation of new long-acting therapies both in clinical trials and monitoring in real world rollout. For example, the investigation of long-acting ISL regimens saw unexpected declines in CD4 cell counts, leading to several clinical trials being placed on hold (48). While the potency and long half-life of ISL indicated possible use as a long-acting formulation, the doses required to maintain required drug levels for sustained periods resulted in dose-dependent adverse effects (48). This has led to ongoing efforts to optimize dosing schedules that balance efficacy and safety and highlights how monitoring drug levels remains crucial to inform risk levels of adverse effects, especially in the case of novel ARV formulations with limited pharmacokinetic information available (48).

Additionally, the emergence of long-acting strategies will not preclude the use of oral medications, especially during the transition period of development of longer acting strategies that can be used as complete regimens. Several long-acting strategies like every 6 months lenacapavir

(LEN) and ibalizumab's every 2-week infusion, currently require coadministration with an oral backbone regimen (OBR) to be effective (206). For example, the only other FDA-approved LAI for HIV prevention is LEN, which is a first-in-class capsid inhibitor approved by the FDA in 2022 for use in combination with other ARVs for heavily treatment-experienced adults with multidrug-resistant HIV-1 who are failing their current ART due to resistance, intolerance, or safety considerations. Since these therapies are currently only approved for use in heavily-treatment experienced adults with multi-drug resistance (MDR), many patients taking LEN may have underlying adherence issues at baseline to oral medications that led to the development of their MDR status (206). Individuals using LEN as a monotherapy due to nonadherence or high levels of resistance to the OBR have been shown to develop resistance to LEN, and while those LEN-specific mutations did not confer cross-resistance to other ARV classes, there is a concern of potential loss of activity of future agents that may target capsid proteins (206). DLF of the OBR used in conjunction with LEN may be used to support the use and roll-out of long-acting LEN regimens to preserve efficacy especially for those most at-risk.

Ultimately an effective vaccine or cure will be needed to end the epidemic. A cure in the classic sense would be very difficult to achieve because it would require the elimination of the reservoir of all virus carrying cells (207). This is difficult because the virus can survive in a resting state for years, or even life, while remaining invisible to the immune system and ARV drugs (207). Alternatively, some approaches aim to achieve sustained ART-free remission, which would not involve eradicating the HIV reservoir, but would allow PLWH to keep latent virus suppressed without daily medication. Most approaches to achieve sustained ART-free remission involve altering or stimulating the immune system to induce long-term control of HIV (207).

Unfortunately, an effective vaccine remains elusive, as evidence by the unsuccessful results of the HVTN 702/Uhambo, HVTN 705/Imbokodo, and HVTN706/Mosaico large-scale efficacy trials (208). This has prompted the field to consider alternative approaches, especially those that can elicit cross-reactive and potent neutralizing antibodies and cellular responses (208). Broadly neutralizing antibodies (bNAbs) have the unique ability to target and neutralize a wide range of HIV variants by targeting conserved regions of the HIV virus (207,209). While earlier studies have shown that infusion of bNAbs directly into individuals confers temporary detection, currently bNAbs do not maintain their ability to suppress the virus because viral mutations occur. Strategies that include administering a combination of bNAbs are currently being developed, but even then, development of mutations conferring resistance remains a concern (209).

The design of future vaccine efficacy trials faces challenges due to the rapidly evolving landscape of HIV prevention. One significant challenge is balancing the ethical obligation to provide participants with the best standard of HIV prevention while also enabling the assessment of vaccine efficacy through adequately powered trials (207,210–212). With the incorporation of highly effective non-vaccine prevention modalities like PrEP, vaccine efficacy trials must now navigate challenges related to varying levels of adherence and prevention choices among participants. It is increasingly becoming best practice to provide PrEP options to all participants and to randomize those who decline PrEP and those found to be non-adherent through DLF into either placebo or vaccine groups (210,212). DLF is crucial for understanding whether observed differences in efficacy are due to suboptimal adherence to PrEP regimens or inherent effectiveness of the vaccine itself and allows for tailored secondary analyses. Other investigational products, such as novel ARVs, vaginal rings, or implants, will face similar challenges for efficacy trial design (212).

This role becomes even more relevant with the advancements of long-acting agents, since they cannot be easily removed from the body once administered. If use of a long-acting drug is interrupted in a vaccine or cure study, the terminal decay period would need to be to be supplemented with more conventional short-acting agents to protect against selection of drug resistance. DLF may be required to assure the washout of long-acting or sustained-release agents to demonstrate that any sustained remission after the intervention is not due to ongoing ARV exposure (207). As treatment options become more complex and available, innovative trial design, including DLF, will be necessary for balancing ethical mandates, scientific goals, and the practical realities of diverse participant behaviors.

In the case of a successful cure or ART-free remission, other monitoring than DLF will likely take priority, such as viral load monitoring to confirm continued remission. However, in cases where sustained ART-free remission is not achieved and ART must be restarted, careful monitoring of viral load and drug levels may be needed to evaluate virological response. If a cure strategy does not result in complete protection from reinfection of multiple variants of the virus, PrEP may be used for additional protection. Indeed, the Berlin Patient, Timothy Brown, is one of at least four other people who were cured with an allogenic stem cell transplant from a donor whose immune system did not express a key receptor used by HIV to enter cells (CCR5). While uncommon, infection can occur with viruses that use the CXCR4 receptor for cell entry. Due to concern of reinfection, Brown has opted to continue with a two-drug PrEP post-cure (207). As advances in ART-free remission and cure research progress, many complex issues in testing and validation will need to be addressed.

In conclusion, while the specific role may change, DLF remains a critical tool in the era of long-acting treatments, vaccine development, and potential HIV cures. While advancements in HIV prevention and treatment have ushered in a new era of long-acting therapies that promise greater convenience and adherence, they also bring complexities such as pharmacokinetic variability, risks of drug resistance, and challenges in ensuring optimal drug levels. DLF not only serves as a vital tool for tailoring individual treatments and managing risks in clinical practice but also plays an indispensable role in the design and execution of clinical trials. From assessing the pharmacokinetics of investigational products to addressing adherence challenges in vaccine efficacy studies and monitoring real-world rollout of novel interventions, DLF provides insights that are pivotal for advancing HIV research and safeguarding public health. As the HIV landscape continues to evolve, the integration of DLF into research and practice will be instrumental in optimizing outcomes, addressing diverse patient needs, and ultimately working toward the eradication of HIV.

# 9. Publications and Presentations

## 9.1 Manuscripts

1. Brainerd C, Singh MA, Tatka J, Craig C, Gilligan-Steinberg S, Panpradist N, et al. REverse transcriptase ACTivity (REACT) assay for point-of-care measurement of established and emerging antiretrovirals for HIV treatment and prevention Anal Bioanal Chem. 2024 Dec 1;416(29):6809–18. https://doi.org/10.1007/s00216-024-05602-4

## 9.2 Conference Posters

1. Brainerd C, Craig C, Tukei L, Vuong M, Olanrewaju A. Therapeutic monitoring of long-acting injectable rilpivirine using a rapid enzymatic assay. In: The 12th IAS Conference on HIV Science International Aids Society (IAS). 2023.
2. Qiu A, Vuong M, Walters S, Brainerd C, Olanrewaju A. Therapeutic Monitoring of Dapivirine Using a Rapid Enzymatic Assay. In: University of Washington Undergraduate Research Symposium. 2024.

## 9.3 Conference Presentations

1. Brainerd C, Tatka J, Craig C, Chang M, Vuong M, Panpradist N, et al. Towards Rapid Dapivirine Measurement with a Portable, Low-Cost Platform. In: Continuum 2024. *Selected for Oral Abstract Presentation*

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1. Spinelli MA, Haberer JE, Chai PR, Castillo-Mancilla J, Anderson PL, Gandhi M. Approaches to Objectively Measure Antiretroviral Medication Adherence and Drive Adherence Interventions. Curr HIV/AIDS Rep. 2020 Aug;17(4):301–14.

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# 11. Supplemental Information

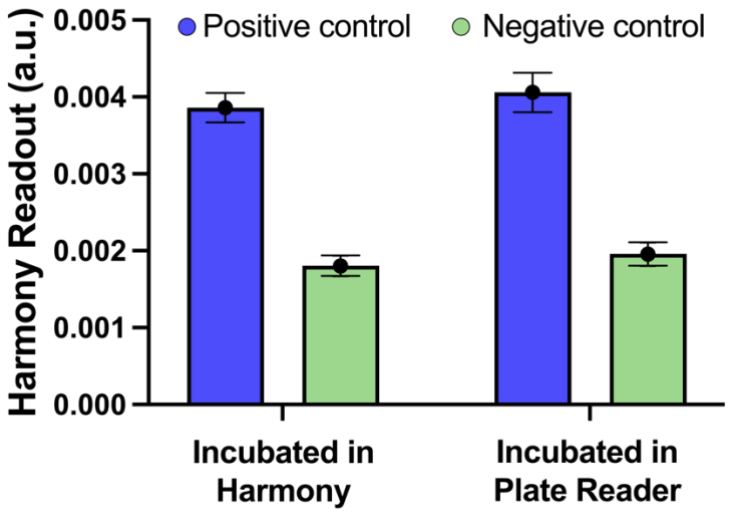


**Figure S1. Fluorescence measurements from Harmony are not significantly affected by incubation in either the Harmony or plate reader.** Positive and Negative controls were either incubated in the Harmony or plate reader before readout of each replicate on the Harmony in all 4 wells. N=2 for Harmony incubation reactions. N=3 for plate reader incubation reactions. Error bars indicate mean and standard deviation.

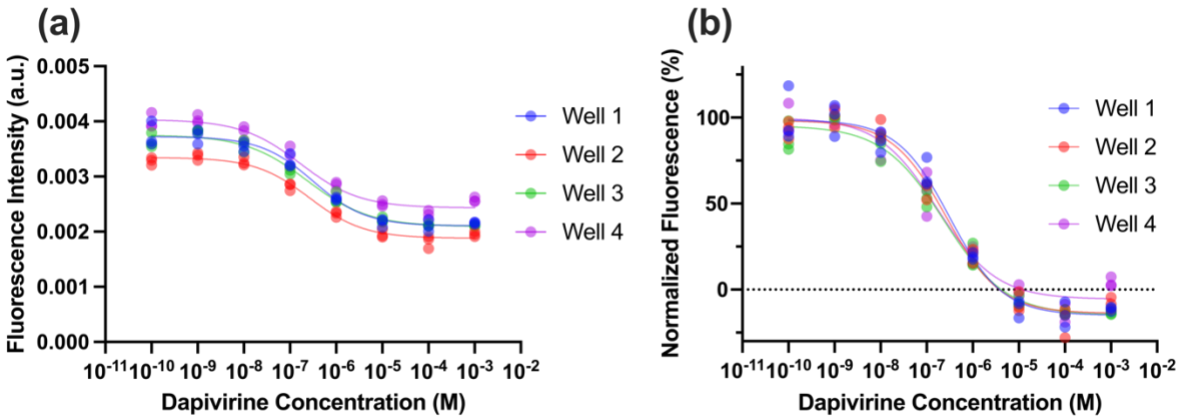


**Figure S2. REACT detection of dapivirine in all Harmony wells**. **(a)** Raw fluorescence output of REACT from shows slight variation in intensities across the four wells. **(b)** When normalized to positive and negative controls in each well, fluorescence output of REACT is not appreciably different between wells in the Harmony. Eight drug concentrations were tested in triplicate in all four Harmony wells.

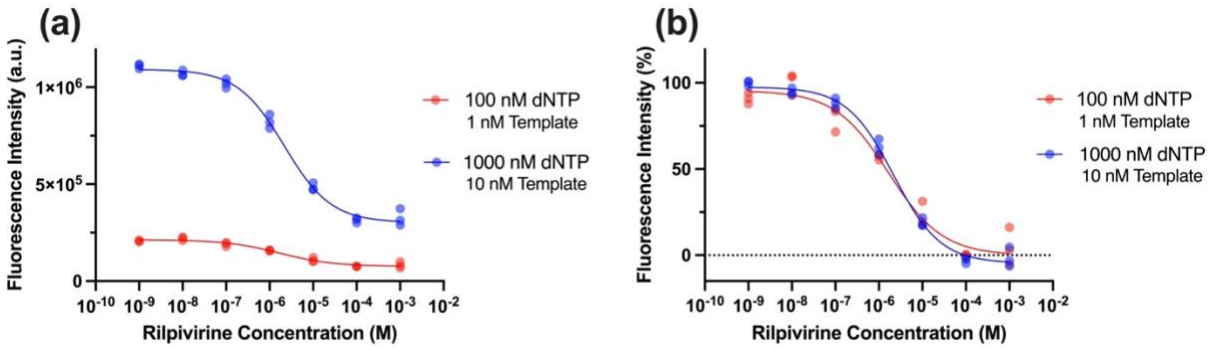


**Figure S3. Increasing the concentration of dNTPs does not adversely impact the ability of REACT to detect lower concentrations of NNRTI. (a)** Raw REACT fluorescence values increase with increased dNTP, template, and primer concentrations. **(b)** Normalized fluorescence

values demonstrate no curve shift when with increased dNTP concentration. Increasing dNTPs, with corresponding increases in template and primer concentrations, provides a fluorescence signal boost without shifting the assay's quantitative region and makes the assay more amenable to readout using an inexpensive reader and diluted plasma samples.

**Table S1. REACT assay variation in buffer (from Fig. S2) was slightly higher in Harmony compared to the plate reader.** We calculated the coefficient of variation (%CV) in triplicate for eight dapivirine drug concentrations in buffer tested across each of the four Harmony wells.

| Drug Condition (M) | Plate Reader (%CV) | Well 1 (%CV) | Well 2 (%CV) | Well 3 (%CV) | Well 4 (%CV) |
|---|---|---|---|---|---|
| Negative Control | 0.272 | 4.924 | 3.262 | 3.345 | 3.295 |
| $10^{-3}$ | 1.024 | 0.862 | 2.333 | 0.762 | 1.677 |
| $10^{-4}$ | 2.578 | 4.962 | 6.196 | 1.073 | 3.422 |
| $10^{-5}$ | 4.269 | 3.408 | 3.948 | 2.377 | 2.000 |
| $10^{-6}$ | 1.690 | 1.077 | 2.306 | 4.158 | 2.799 |
| $10^{-7}$ | 1.603 | 3.767 | 2.347 | 2.234 | 5.798 |
| $10^{-8}$ | 3.467 | 2.433 | 2.296 | 3.178 | 3.308 |
| $10^{-9}$ | 1.250 | 3.535 | 2.073 | 0.426 | 1.952 |
| $10^{-10}$ | 1.800 | 6.114 | 2.070 | 3.631 | 3.572 |
| Positive Control | 1.725 | 8.934 | 4.622 | 1.771 | 5.234 |
| **Average %CV** | **1.968** | **4.002** | **3.145** | **2.296** | **3.306** |

**Table S2. REACT assay variation in diluted plasma was slightly lower in Harmony compared to the plate reader.** We calculated the coefficient of variation (%CV) in triplicate for eight doravirine drug concentrations in (25% volume/volume) diluted plasma tested across each of the four Harmony wells.

| Drug Condition (M) | Plate Reader (%CV) | Well 1 (%CV) | Well 2 (%CV) | Well 3 (%CV) | Well 4 (%CV) |
|---|---|---|---|---|---|
| Negative Control | 3.367 | 9.626 | 8.828 | 6.374 | 6.234 |
| $2.4x10^{-4}$ | 7.732 | 1.940 | 1.133 | 1.614 | 1.844 |
| $2.4x10^{-5}$ | 9.505 | 4.298 | 2.691 | 4.785 | 0.760 |
| $2.4x10^{-6}$ | 2.330 | 2.672 | 3.678 | 1.233 | 5.470 |
| $2.4x10^{-7}$ | 5.149 | 1.386 | 4.076 | 0.864 | 3.424 |
| $2.4x10^{-8}$ | 2.068 | 0.437 | 1.711 | 3.050 | 0.678 |
| $2.4x10^{-9}$ | 3.897 | 1.640 | 1.298 | 2.496 | 1.547 |
| $2.4x10^{-10}$ | 4.303 | 2.616 | 1.368 | 3.344 | 2.908 |
| $2.4x10^{-11}$ | 1.682 | 3.643 | 3.960 | 4.166 | 2.689 |
| Positive Control | 4.556 | 1.974 | 2.994 | 2.415 | 2.139 |
| **Average %CV** | **4.459** | **3.023** | **3.174** | **3.034** | **2.769** |