The Complexities of Adapting and Validating a Novel Assay for Adherence Monitoring of Oral HIV Pre-Exposure Prophylactics

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Abstract

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Oral pre-exposure prophylaxis (PrEP) is effective in preventing HIV, but its efficacy depends on adherence. The gold standard for adherence monitoring, liquid chromatography-mass spectrometry, is accurate but costly and complex. Therefore, a simpler method is needed. The REverSe TRanscrIptase Chain Termination (RESTRICT) assay measures oral PrEP drug levels in blood by detecting tenofovir-diphosphate, an active PrEP metabolite. RESTRICT has a turnaround time of under 24 hours, is easy to prepare, and uses standard clinical lab equipment. The dose-dependent normalized fluorescence result can be plotted as a standard curve for semi-quantitative adherence measurement. The assay must be validated as a Laboratory Developed Test, assessing sensitivity, specificity, accuracy, precision, range, linearity, and interference. The validation showed RESTRICT as a promising alternative to mass spectrometry, potentially improving PrEP uptake and adherence.

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i. Abbreviations

ART	Antiretroviral Therapy
ASC	Average Standard Curve
BM	BaseMatrix by SeraCare
cDNA	Cell DNA
CLIA	Clinical Laboratory Improvements Amendment
HIV	Human Immunodeficiency Virus
LC-MS	Liquid Chromatography-Mass Spectrometry
LDT	Laboratory-Developed Test
MM	Master Mix
MSM	Men who have sex with Men
PoCT	Point of Care Test
PrEP	Pre-exposure Prophylaxis
RBC	Red Blood Cell
RT	HIV Reverse Transcriptase
RTB	HIV RT Buffer
TAF	Tenofovir alefenamide
TAT	Turnaround Time
TDF	Tenofovir disproxil fumarate
TFV-DP	Tenofovir Diphosphate
UNAIDS	United Nations Program on HIV/AIDS
WB	Whole Blood

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I. INTRODUCTION

HIV is still an incredibly prevalent and challenging disease ravaging certain regions, primarily the Global South. In sub-Saharan Africa, HIV prevalence was estimated at 2.6 cases per 1000 people in 2019, with approximately 690,000 incident cases [22]. Ending the HIV epidemic with an eye to eventual eradication is the core goal of the United Nations AID (UNAID), which aims to bring incidence down to fewer than 200,000 cases annually worldwide– an ambitious goal. The organization released its goals for 2030 in accordance with the UN's Sustainable Development Goals (SDGs). SDG 3 aims for good health and well-being for all people worldwide, and one target is to address the prevalence of HIV and AIDs. UNAID enumerates its 95-95-95 Fast Track goals to reduce HIV prevalence: by 2023, 95% of people living with HIV are aware of their status, 95% of those are taking antiretroviral medication, and 95% of those are virally suppressed. The goal for medication distribution also extends to individuals who are HIV-negative but may be at increased risk of transmission and would therefore be afforded significant protection by taking a Pre-Exposure Prophylactic, referred to as PrEP. Unfortunately, these goals have not been met, and the deadline has been extended to 2025.

PrEP has been recommended by the World Health Organization (WHO) for HIV prevention for key at-risk populations since 2012. It has been shown to be safe and effective across all studied populations and strata. The landmark paper published by Baeten *et. al.* in 2012 studied the efficacy of Tenofovir Disproxil Fumarate (TDF), a class of tenofovir PrEP, in serodiscordant couples in Kenya and Uganda. They rigorously demonstrated that PrEP caused a 67% reduction in HIV acquisition between partners,

and up to 71% efficacy compared to placebos [5]. The addition of PrEP into Public Health guidelines and endeavors has since allowed up to 41% reduction in incidence among at-risk individuals [27]. Groups defined as “high risk” for HIV include men who have sex with men (MSM), IV drug users, sex workers, and people in status-discordant relationships [13]. Other at-risk groups often include members of the LGBTQ+ community– in particular transgender individuals.

There are many reasons countries in sub-Saharan Africa have higher incidence, prevalence, and lower therapeutic uptake than anywhere else in the world. Much of this inequity results from prolonged resource deprivation resulting from colonial projects across the continent which have limited financial capacity, infrastructure development, and sociopolitical flexibility when it comes to introducing public health interventions [16]. However, the populations themselves also come with unique challenges. In more remote areas of sub-Saharan Africa, cultural and social pressures can lead to mistrust of vaccines and medications, particularly those provided by Western public health organizations. This is particularly prominent in the context of HIV, as the virus has a significant stigma attached, so many patients are afraid that prophylactics may lead to social ostracization or violence [15].

A negative consequence of this is that many individuals who may be at elevated risk of HIV transmission are unable or unwilling to take PrEP. Some innovations, such as the preferential use of oral PrEP instead of clinic-based injections, have improved uptake. Unfortunately, current oral PrEP therapies must be taken daily to achieve adequate protection– something that many patients struggle to do. The number of weekly doses taken by a patient compared with the prescribed regimen is called

adherence. The simplest method for monitoring adherence is through direct patient surveys. However, patients are often uncertain or dishonest about their adherence, raising the need for clinical testing to measure PrEP drug levels.

Several methods exist to measure adherence to oral PrEP regimens, all based on the detection of a metabolite, tenofovir diphosphate (TFV-DP). Oral PrEP therapies are based on a variety of tenofovir analogs such as tenofovir disoproxil fumarate or tenofovir alafenamide. These are rapidly metabolized and converted to TFV-DP which functions as a direct HIV reverse transcriptase inhibitor. TFV-DP has a half-life of up to 17 hours making it an ideal analyte for adherence [20]. Currently, the gold standard for TFV-DP detection is liquid-chromatography-mass spectrometry (LC-MS), which can discriminate with high specificity between 2, 4, and 7 doses per week. Due to the clearance rate of TFV-DP, 4 doses per week constitutes the approximate minimum concentration for protective function. Therefore, patient PrEP adherence is split into three categories: 7 doses/perfect adherence, 4 doses/moderate or minimum adherence, and 2 doses/poor adherence. The precise cutoff concentrations for each dosage level have been validated by Paul Drain's lab across a variety of clinical experiments [12].

While LC-MS is an excellent diagnostic tool for PrEP adherence monitoring, it is complex, expensive, and requires equipment inaccessible to many labs. These features make it infeasible for remote or low-resource settings that may lack the facilities, staff, or training to conduct LC-MS testing. Consequently, there is a need for PrEP adherence testing that is more rapid and accessible.

To fulfill this need, Ayokunle Olanrewaju *et. al.* developed the REverSe TRanscrIptase Chain Termination or RESTRICT assay [24]. RESTRICT works similarly

to existing PrEP detection assays in that it senses the metabolite of oral PrEP, TFV-DP. Unlike current tests, RESTRICT leverages the inherent activity of HIV Reverse Transcriptase (HIV RT) to accurately detect the concentration of TFV-DP in whole blood using a fluorescent reporter. Because the assay requires only a small quantity of venous whole blood and a fluorescent plate reader, it has a rapid TAT (including diagnostic analysis) of less than 24 hrs. The assay itself can be completed in approximately 1 hour and can separate adherent PrEP users (≥ 4 doses/wk) from nonadherent users (≤ 2 doses/wk). RESTRICT is designed to be potentially used as a point-of-care-like assay that can be completed in a basic clinical lab while a patient is at the clinic. In time, it will help address an important need for specific and rapid adherence monitoring in cases where patients struggle to remain adherent.

Point of care tests (PoCTs) are clinical diagnostic tests that can be performed in a short amount of time, with minimal training or specialized/complex instrumentation, and return actionable results to patients rapidly. The downside of PoCTs is they often must sacrifice some sensitivity and specificity in the name of speed. As such, they must be rigorously validated for diagnostic efficacy to ensure they can serve as a suitable alternative to gold-standard testing.

CLIA Validation

The validation of clinical care tests is dictated by the Clinical Laboratory Improvements Amendment (CLIA) which specifies standards and regulations for any clinical activities involving human specimens collected for non-forensic purposes. CLIA is enforced through collaboration between the Food and Drug Administration, Health

and Human Services, and the Center for Medicare and Medicaid Services. CLIA separates clinical testing and devices by complexity, awarding specific certification levels to each type. Each aspect of a clinical test from setup and sample collection to implementation to analysis and diagnostic application, is awarded a complexity level 1-3. Level one, or low complexity tests are typically simple, pre-prepared, and require minimal scientific or clinical knowledge and training to implement and interpret. Low-complexity tests include dipstick urinalysis, home glucometers, basic rapid tests, etc. Level three, or high complexity tests may involve labile reagents/materials, special collection and handling of samples, clinical and scientific understanding to implement and interpret, or complex instrumentation. Examples include non-automated PCR, antigen testing, Liquid Chromatography-Mass Spectrometry (LC-MS), etc [1].

There is also a category of high-complexity CLIA tests called Laboratory Developed Tests (LDTs), which are also sometimes called “home-brew” tests. These are *in vitro* clinical tests designed, manufactured, and used by a single CLIA-approved laboratory. LDTs must still be validated to certain CLIA standards before any of their results can be released [6]. These standards essentially verify that the tests detect the correct analyte to a reasonable degree of sensitivity and specificity. In this context, sensitivity refers to a test’s ability to detect the presence of its target analyte while specificity describes the ability to discriminate between the target analyte and peripheral substances. These can also be understood as the false negative rate and the false positive rate, respectively.

Care Gap

The introduction of PrEP alongside other HIV transmission preventatives such as condoms has been widely successful in reducing overall HIV incidence globally. Since the 1990s, HIV incidence has decreased by 59% globally [13]. While this number can be attributed to many factors including improved healthcare access, standards of care, and diagnostic and surveillance methods, proper use of PrEP has been shown to reduce sexual transmission risk by more than 95%. However, since oral PrEP is administered as a daily pressed pill, maintaining adequate adherence can be challenging.

There are a multitude of obstacles to PrEP distribution and adherence. A recent review by Haberer *et. al.* identified barriers at the healthcare system level, the community/family level, and the individual level (Fig. 1). They evaluated these as challenges to uptake (how many people receive prescriptions), executed adherence (if patients follow their prescriptions), or persistence (whether patients can maintain adherence over time). Uptake is one of the primary issues surrounding PrEP use.
In many populations, individuals are often unaware that they would benefit from PrEP because of inadequate public health guidance and social stigma. Unfortunately, this is particularly true within higher-risk populations including unhoused people, drug users, and LGBTQ+ communities because access to overall healthcare is frequently limited and trust in providers can be compromised. Haberer describes how social associations between PrEP and HIV status or promiscuity feed harmful stigma and limit those who might otherwise seek HIV preventatives.

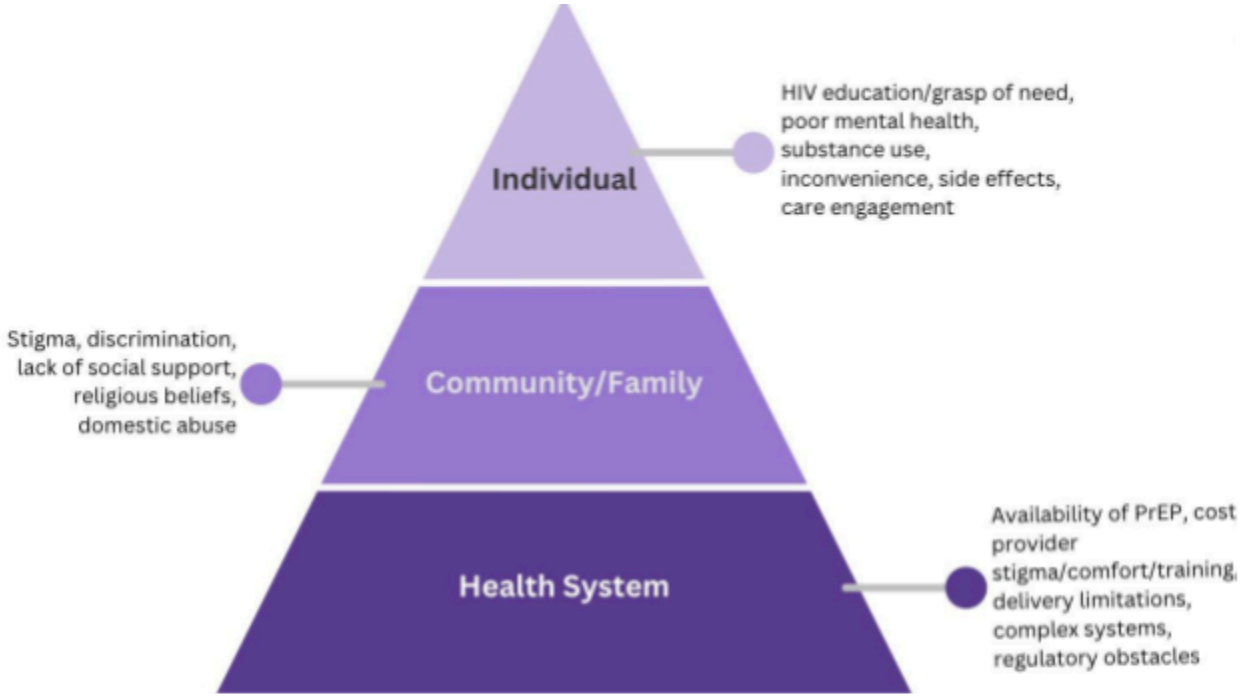


Figure 1: *According to Haberer et. al., the plethora of obstacles to adequate HIV prevention and treatment access can be broken into three levels. The Health System includes the pharmaceutical seller, the physical healthcare institution, the providers, clinicians, lab staff, etc. and impact availability of PrEP. Community and family can cause obstacles in initiating and consistently taking PrEP through social, cultural, or religious pressures regarding healthcare or HIV specifically. At the individual level, a person's internal biases or concerns, other physical and mental health conditions, and education on HIV risk and prevention impact their odds of taking PrEP.*

It is important that providers can properly educate patients on the need for and targets of PrEP and that public health institutions endeavor to meet different communities where they are. This can be done by adjusting for a patient's housing status, sexual and gender orientation, and mental or other health comorbidities.

The next category of barriers is executed adherence. This is where adherence monitoring becomes crucial. Currently, most adherence monitoring for PrEP is done by self-reported survey data. However, as Haberer points out, both survey data and things like pharmacy pill counts are subject to "social desirability and recall bias" meaning patients have the potential to alter their responses due to things like shame,

forgetfulness, or poor mental health. It can be very difficult to adhere to a routine medication dose, especially for those who don't have a consistent living situation or a safe, private place to store and use their medications. The review points out that there are several opportunities to improve executed adherence including different dosing regimens, electronic monitoring systems (ex: digital pill counting bottles), and expanded molecular monitoring. While urinalysis detection of tenofovir is relatively cheap, is it not a widespread monitoring method in clinical practice. There is a need for molecular adherence monitoring methods that are rapid and relatively easy to implement with basic clinical instrumentation. This will also assist in promoting persistent adherence, which may look different for different patients. For example, some may only need PrEP for a limited period due to non-monogamous exploration, serodiscordant relationships, etc. However, others could benefit from PrEP in the long term, such as sex workers. Molecular adherence monitoring could help providers modulate their dosing regimens based on the patient's intended use for PrEP [17].

Molecular adherence monitoring assays have the potential to increase PrEP uptake and persistence across the board; providers will be better able to educate and advise patients on PrEP usage and patients will have more ownership over their medication and a more direct method to understand HIV risk and prevention.

Assay Design

RESTRICT is designed to use the existing activity of HIV RT as a tool for deductive drug detection. Broadly, RT generates double-stranded cDNA as part of HIV's reproductive process. Since PrEP drugs inhibit RT activity, there is a direct correlation between the volume of cDNA created and the quantity of TFV-DP in a system. Therefore, we can deduce the concentration of PrEP metabolites based on how much or how little the RT activity is inhibited.

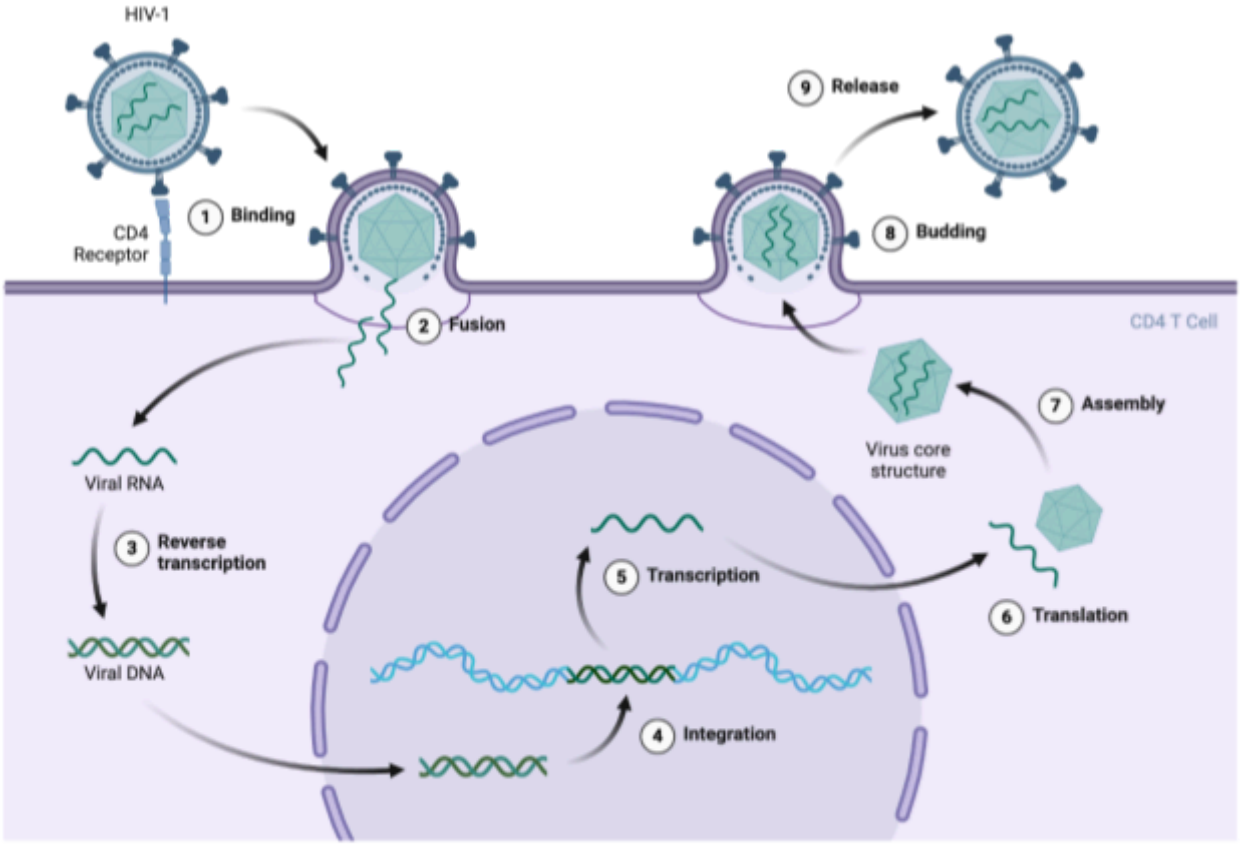


Figure 2: *An HIV virion binds to the CD4 receptor on a CD4 T cell (1) and initiates cellular invasion by membrane fusion (2). In the cytoplasm, the viral RNA undergoes reverse transcription into dsDNA (3) with its own HIV RT and free nucleotides. The viral DNA enters the nucleus where it integrates into the cellular genome (4) via a viral-encoded integrase. Now treated by the cell as regular DNA, the viral genome is transcribed (5) and translated (6) into viral proteins via the normal cellular DNA replication process. The viral proteins spontaneously assemble into the viral capsid (7) containing viral RNA and exits the cell by budding (8) off in a CD4-receptor-bearing membranous envelope where it once again becomes infectious (9). Created with Biorender.*

HIV-1 reproduces in a nine-step cycle (Fig. 2). When a virus encounters a host, it works its way into the bloodstream and binds to CD4 T cells, or “helper” T cells. Once bound, it fuses with the cell membrane, dumping the contents of the viral capsid into the host cell. These contents include HIV RNA, the natural genetic material of the virus, and HIV RT whose role it is to convert the RNA into DNA that can enter the nucleus and be incorporated into the host genome. Once integrated, the virus can take advantage of the host replication machinery to translate and assemble viral proteins. These are packaged into a viral capsid and exit the host cell through a process called budding. Only once the new virus has left the cell does it become infectious. Because HIV targets CD4 T cells– one of the most critical cell types in adaptive immunity– these cells' replication process is compromised, reducing T cell count and overall immunity. If left untreated, HIV infections can progress to Acquired Immunodeficiency Syndrome or AIDS which can be fatal [20].

Oral PrEP medications target the activity of HIV RT which requires free nucleotides to synthesize dsDNA from the viral ssRNA. TFV-DP mimics the structure of dATP which, when incorporated by the RT, causes a critical error leading to chain termination. Thus, the viral DNA is never incorporated into that of the host, preventing viral replication. It is possible to use fluorescent interpolating dyes such as PicoGreen (PG) to detect the presence of double-stranded (ds) cDNA which, in a controlled setting, can only be generated by functional RT. Thus, the degree of inhibition of RT can be determined based on the relative fluorescence of PicoGreen.

The RESTRICT assay extracts TFV-DP stored in patient red blood cells (RBCs) for use in a simulated HIV RT reaction. Instead of viral RNA, the enzyme is supplied

with a single-stranded DNA template, specifically designed to facilitate the incorporation of TFV-DP. This template was designed and validated by Olanrewaju *et. al.* as a 200-nucleotide repeating TTCA sequence. The thymidine-rich sequence requires primarily dATP to synthesize a complementary strand, creating ample opportunity for the drug to induce chain termination. This template was also designed with an ideal binding site for RT to improve the efficiency of the reaction; 16S forward and reverse RNA primers are included to initiate transcription.

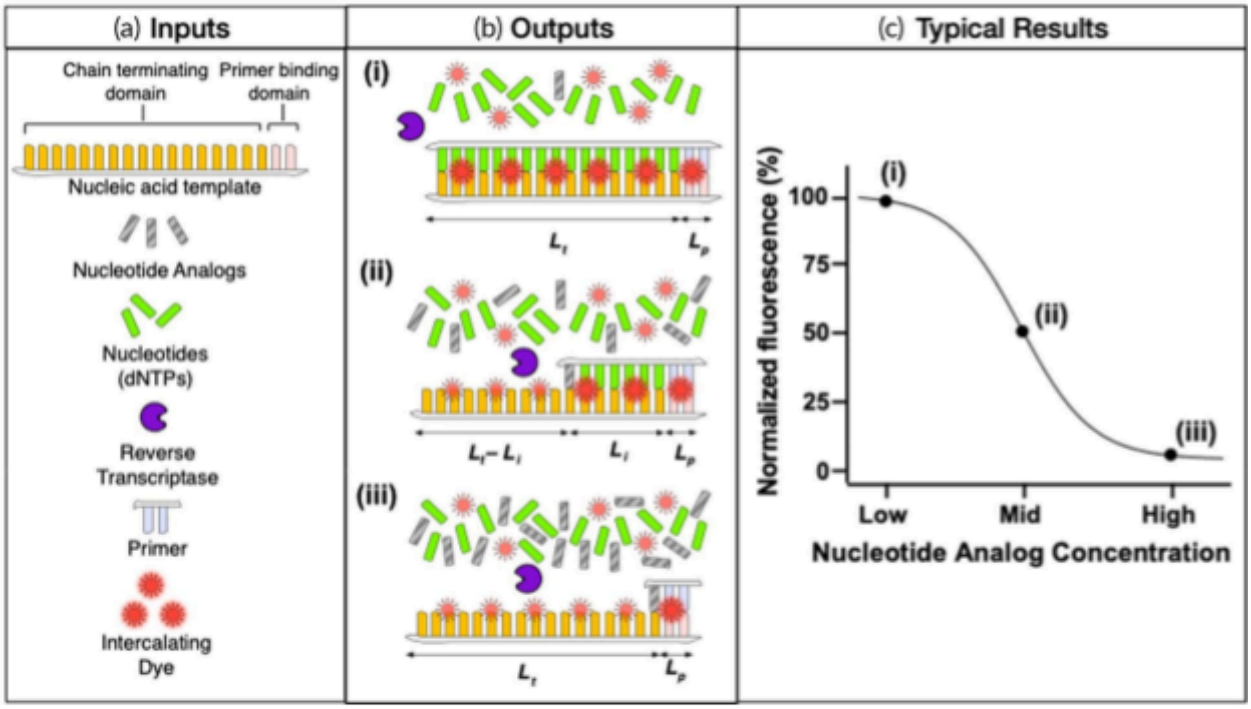


Figure 3: *Overview of RESTRICT. (a) RESTRICT requires a single-stranded DNA template with repeating sequence TTCA combined with a 16S rRNA primer and free nucleotides. The nucleotide analog refers to the reverse transcription-terminating drug, in this case TFV-DP. HIV RT is added to this Master Mix with an intercalating dye that responds to dsDNA to measure how quickly chain termination occurs. (b) The HIV RT uses the primer and free nucleotides or analogs to synthesize a cDNA strand complementary to the TTCA template. The dye binds to dsDNA and fluoresces (i). In the presence of less drug, chain termination occurs less often meaning more dsDNA and brighter fluorescent signal (ii). More drug increases the rate of chain termination, leaving less dsDNA and producing a lower signal. (c) PrEP drug concentration can be plotted against fluorescent intensity to generate an inverse curve where less drug/low adherence corresponds to high fluorescence (i) and more drug/high adherence produces low fluorescence (iii). These results follow a sinusoidal path similar to a dose-response curve. Figure created by Dr. Ayo Olanrewaju [24].*

Once the template strand, primers, processed whole blood (WB) sample, and free nucleotides (dNTPs) are combined, the addition of HIV RT and 30-minute isothermal incubation facilitates the formation of ds-cDNA. Wherever TFV-DP is incorporated instead of dATP, the RT will be induced to chain termination, limiting the final amount of cDNA synthesized. Since the intensity of fluorescence can be directly correlated with the inhibition of RT by TFV-DP by using PicoGreen, a sinusoidal relationship can be plotted where maximum fluorescence corresponds to minimal drug concentration and vice versa (Fig. 3).

Using the gold standard method, LC-MS, it is possible to separate different oral PrEP adherence levels based on roughly graded ranges of TFV-DP concentration. In studies conducted in urine, Drain *et. al.* was able to define cutoffs for low, moderate, and high adherence [12]. RESTRICT aims to rapidly identify which adherence range a patient falls into. While the overall sensitivity of the assay may not rise to that of LC-MS, it is still possible to tell whether a patient is perfectly or moderately adherent or nonadherent. Part of the validation process is relating the fluorescent intensity readings from RESTRICT to results from LC-MS so that adherence ranges can be benchmarked on the RESTRICT standard curve. This can be achieved by creating Dried Blood Spots (DBS) of each patient sample assayed with RESTRICT so the same patient's adherence and the accuracy of RESTRICT can be verified against LC-MS. The assay can be applied directly to DBS but there is a possibility that the drug concentration may not remain stable on the card, whereas using fresh WB ensures the determined drug levels are as close to the patient's current ones as possible.

It has yet to be validated exactly how effectively RESTRICT can recapitulate LC-MS data nor how the patient's drug levels vary based on their dosing schedule. The CLIA validation process will confirm the sensitivity and specificity of RESTRICT relative to LC-MS but the time-dependent aspect of adherence will be further elucidated during the subsequent validation by a Randomized Control Trial (RCT).

II. METHODS

General Assay Preparation (standard or patient)

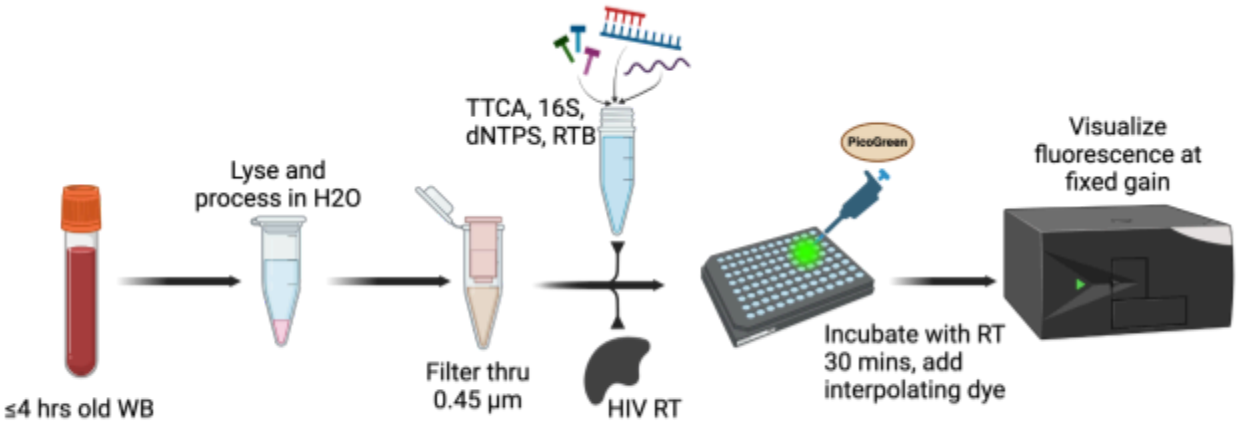


***Figure 4**: Fresh whole blood, processed within four hours of the draw, is diluted 50/50 with water and lysed through a process of repeated vortexing, heating, and centrifuging. The lysate is filtered on a 0.45 µm Eppendorf spin column and visually inspected for contaminants or remaining debris. Drug can be artificially spiked into the sample at this point to create contrived concentrations. The cleared lysate is combined with the Master Mix which contains the template, primer, free nucleotides, and buffer necessary for reverse transcription. This mixture is added to a 196-well black-bottom plate with HIV RT immediately before incubation. After 30 minutes at 37°C, the PicoGreen intercalating dye can be added and visualized on the microplate reader. Created with Biorender.*

A stock of RT buffer (RTB) was made with 60 mM Tris, 30 mM Hydrochloric acid, and 30 mM Potassium Chloride to improve the Potassium-facilitated activity of RT and 8 mM Magnesium Chloride. 100 mM DTT was added to daily batches to help with cell porosity and protein denaturation. The solution was buffered to a pH of 8.3 and vortexed

vigorously. This RTB formed the basis of all dilutions in the assay preparation and was batch-prepared into 50 mL aliquots.

For each assay run, summarized briefly in Figure 4, the Master Mix (MM) had to be prepared and combined with each patient sample. All assay preparation was undertaken on ice unless otherwise specified. First, the number of patient samples to be run was entered into a purpose-built Excel template to calculate the respective volume of each reagent needed to achieve the concentrations in Table 1. An aliquot of RTB was thawed, to which the according volume of Dithiothreitol (DTT) was added to reach 100 mM. This would be the working stock for the day. In a screw-top tube, an appropriate volume of RTB was aliquoted. To this TTCA template, 16S primer, and dNTPs were added. Each patient sample was mixed 1:1 with MM in 1.5 mL Eppendorf tubes as well as a control replacing patient sample with molecular-grade water (MGW). Diluting the sample prior to plate addition improves the pipetting accuracy since the patient sample is often slightly viscous and can be difficult to pipette on its own. It was critical that the HIV RT was not added to this mix until the assay was ready to be run on the plate reader to ensure consistent activity and conditions.

***Table 1**: RESTRICT master mix reagent concentrations and point of addition*

Reagent	Preparation concentration	Addition route
TTCA template	4 nM	Master Mix
16S rRNA primer (fwd & rvs)	40 nM	Master Mix
dNTPs	400 nM	Master Mix
RTB	N/A	Diluent
HIV RT	0.212 nM	Direct to reaction
PicoGreen	1X (stock at 400X)	Direct to reaction

Next, the HIV RT was thawed on ice (just until pipetable to avoid activation) and the calculated quantity was added to chilled RTB. The time the enzyme spent out of the -20°C freezer or off the ice was kept to a minimum. The MM-patient sample mixtures were added to black, opaque-bottom plates (Fig. 4). Each well ultimately consisted of four equal volumes of 10 µL: MM, sample or MGW, RTB, and RT dilution. It was important to calculate all additions based on final assay concentration (the concentration of the reagent in the well after adding all the respective components), ensuring nothing is diluted more than it should be. 20 µL of the sample-MM mixture was added to each well along with 10 µL RTB. The plate was placed on the Tecan Infinite™ M-Plex microplate reader. 10 µL of the RT dilution was then added to all relevant wells using a multichannel pipette so addition times were as close to simultaneous as possible. From Tecan Magellan, the plate was inserted and incubated for 30 minutes at 37ºC. At the end of 30 minutes, each well was quenched with 40 µL (1:1) of PicoGreen interpolating dye via multichannel. After approximately 1 minute, the fluorescent intensity scan (Table 2) was initiated and the results were saved from Tecan Magellan™.

***Table 2**: Tecan Magellan Infinite™ MPlex microplate reader protocol program settings*

Parameter	Setting
Temperature	37ºC
Excitation wavelength	485 nm
Emission wavelength	535 nm
Shaking	20 sec, orbital
Gain	142
Z-Position	16490 µm

Sample Prep

RESTRICT uses fresh, whole blood (WB) collected in EDTA tubes to ensure the most accurate picture of a patient's PrEP use. Venous WB was processed within 4 hours or less of the draw. First, 450 µL MGW was aliquoted into a 1.5 mL screw-top tube. The WB sample was inverted 10-20 times to ensure homogeneity. Next, 150 µL WB was diluted into the MGW. The samples were vortexed for one minute, then spun down for 5 minutes at 10,000xg.

The tubes were then heated for 5 minutes at 95°C, with agitation. The heated samples were spun down for another 5 minutes at 10,000xg. The supernatant was carefully extracted and spun through a 0.45µm-pore centrifugal filter for 5 minutes at 10,000xg. The supernatant was then transferred to a clean 1.5 mL screw-top tube.

Standard Curve Generation

To generate a standard curve for benchmarking RESTRICT measurements, known quantities of blood were spiked into PrEP-negative, HIV-negative WB.

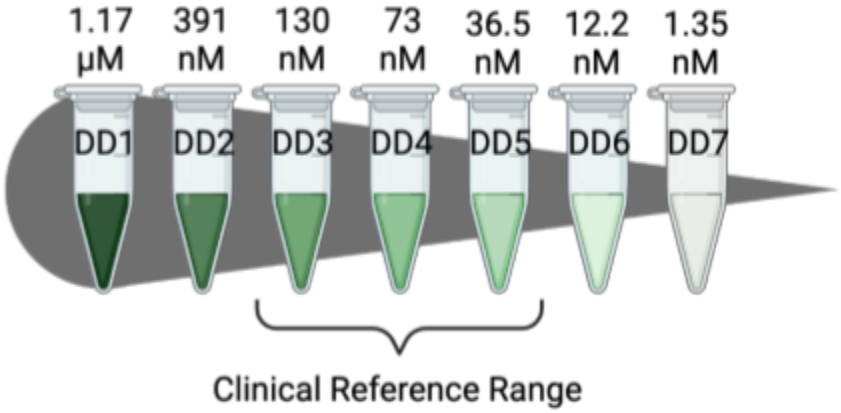


***Figure 5**: The TFV-DP dilution series can be prepared by spiking volumes of the higher concentration into additional buffer to create subsequent concentrations. The seven-point series spans the clinically relevant range with two levels on either side to account for patient variation and outliers. Created with Biorender.*

To prepare the 7-point TFV-DP dilution series (Fig. 5), the Entry Template was used to calculate the necessary volumes relative to the number of reactions planned. TFV-DP was spiked into RTB to generate Drug Dilution 1 (DD1) and mixed thoroughly by pipetting. The necessary volume of DD1 was diluted into RTB to create DD2. This process of diluting and spiking into subsequent RTB aliquots was continued until DD7 was reached. It was important to ensure that there was enough remaining volume of each drug dilution to complete the plate preparation (the Entry Template has built-in overage volumes for this reason). As specified in the general preparation section, RT was added well-wise to the assay immediately before the 30-minute incubation.

To create the contrived samples, each drug dilution was added to respective aliquots of the processed patient sample. The final plate layout is shown in Fig. 6.

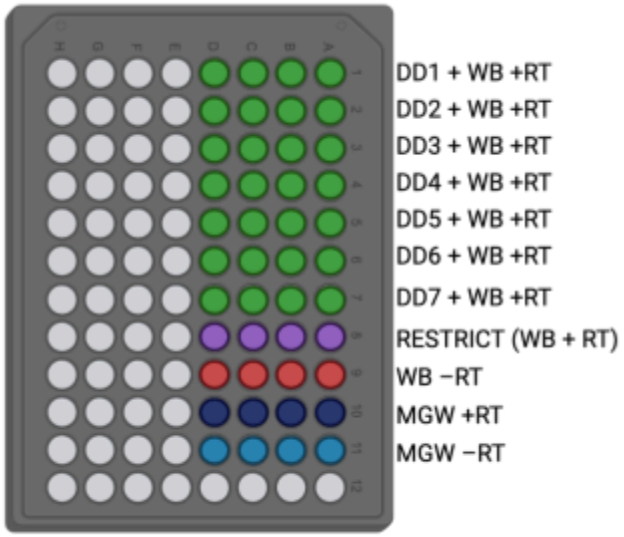


***Figure 6**: The assay reactions are performed in quadruplicate to maximize precision. The seven-point TFV-DP dilution series (green) is added in order with buffer and HIV RT. Patient sample +/- controls (purple, red) and enzyme +/- controls (navy, blue) with water instead of sample are run on every plate for normalization and comparison. Water controls demonstrate that the RT is working at all while sample controls show the patient sample doesn't hinder assay functionality. "DD" = drug dilution (Fig. 3). "WB" = whole blood. "MGW" = molecular-grade water. RESTRICT measurement means patient sample with RT, no drug added. Grey wells in this example are unused, but two patient samples can be run on a single plate. Created with Biorender; plate rotated 90° right for readability.*

The water controls define the overall range of measurement while the blood controls determine the functionality of the assay and the expected range of fluorescence. To minimize variability and improve sensitivity and specificity, standard curves used for validation were generated based on the averaged results of 54 negative patient samples. Each sample was processed across the full 7-point drug series and the results were averaged to determine the most accurate dynamic range for each TFV-DP concentration.

III. VALIDATION PLAN

CLIA guidance requires that LDTs be validated in the following ways: analytical sensitivity, analytical specificity, reportable range, accuracy, precision including inter-assay (between run) and intra-assay (between reactions), linearity (for quantitative results), and interference. In general, accuracy is tested by comparison to the gold standard– in this case, LC-MS. Precision can be validated through repeated runs across different patients and different days and evaluated by calculating percent cumulative variance (%CV). Sensitivity refers to the assay's ability to detect the analyte of interest and can be evaluated by calculating the false negative rate, ie. how often there is insufficient detection in the presence of the analyte. Specificity, conversely, is the assay's ability to distinguish the target analyte from other substances. This can be reported as the false positive rate, ie. how often the assay shows off-target detection. Reportable range can be distinguished from reference range as the total dynamic range of the assay versus the clinically relevant range; both are important for validation. In the case of RESTRICT, the normalized reportable range follows a sinusoidal trend but the reference range can be assessed for linearity. Linearity can be thought of as how

predictably results follow a linear relationship. Finally, CLIA requires a basic profile of any substances/sample conditions that may interfere with the aforementioned attributes of the assay. This can include hemolyzed or lipemic samples, common medications that may interfere, or patient conditions that could skew results.

A validation plan was drafted with experiments designed to meet all these criteria. This plan is dynamic and has been adjusted or repeated as needed to ensure the most optimized version of the assay is validated.

Analytical Specificity

Analytical specificity can be evaluated via the false positive rate of the assay. To validate specificity, 60 samples of PrEP-/ART-/HIV- whole blood (WB) were assayed. Drug concentrations were contrived by spiking known concentrations of TFV-DP into the processed WB samples. By generating a standard curve for each patient, the overall Limit of Blank (LoB) could be established for the assay. This is important for specificity because it provides a lower limit to detection, allowing the elimination of out-of-range results. The LoB could be calculated by the equation $LoB = Mean_{Blank} + 1.645\sigma$ where σ = standard deviation. The 1.645 is based on a normal distribution and incorporates one standard deviation of the distribution. The consistency in results for the negative controls indicated that the assay has a high degree of specificity and, in a properly controlled reaction, does not detect non-target cDNA. This indicates that the sample preparation is effective at isolating the TFV-DP from other nucleic acids that may be present in patient WB.

Analytical Sensitivity

Sensitivity can be evaluated by the false negative rate. In this case, sensitivity was validated using the same Average Standard Curve (ASC). From this, it was possible to calculate the Limit of Detection (LoD) which describes the minimum drug concentration to achieve chain termination and produce a fluorescent signal. Since the drug-fluorescence relationship is inversely proportional, the minimum drug concentration achieves the highest possible fluorescence. However, since the assay detects drug concentration indirectly by way of cDNA concentration, it is possible that any other dsDNA present in the reaction could confound readings. Based on the LoB, the LoD can be described by the following equation: $LoD = LoB + 1.645\sigma$ [3].

Reportable Range

The reportable range can be distinguished from the reference range. The former refers to the total range of values the assay might return; generally the span between the positive and negative water controls (ie. 100% inhibition (no RT) vs 0% inhibition (RT, no drug)). The reference range falls within the reportable range and describes the clinically relevant values of the assay. In the case of RESTRICT, this refers to the 36.5-130 nM range which corresponds to 2, 4, or 7 doses of PrEP per week [11]. The validation plan was to determine both the reportable and reference range from the 51-sample ASC. However, both the ASC and subsequent runs infrequently yielded a maximum normalized fluorescence (nFU) of 0.8, or about 80% of the desired maximum. This meant that the reportable range of the assay was 20% less than hoped. Thus, it was necessary to perform optimization testing to determine the limiting factor on the

reportable range. While it was observed that increasing the DNA template concentration drove up the maximum nFU values, doing so also increased variability and off-target detection. It was expected that the assay would require additional optimization and re-validation.

Accuracy

To demonstrate the accuracy of RESTRICT, the assay results had to be compared to the gold standard method: LC-MS. LC-MS can be run on Dried Blood Spots (DBS) and detect quantities of TFV-DP in fmol/punch with at least 85% specificity and sensitivity [11]. Based on previous data collected by the Drain Lab, PrEP adherence could be separated into nonadherent, moderate, and optimal based on the 2, 4, and 7 doses/week cutoffs. These corresponded to 350 fmol/punch, 700 fmol/punch, and 1250 fmol/punch, respectively. TFV-DP ≥700 fmol/punch is considered adequate adherence [11].

To test accuracy, ten DBS cards were prepared from PrEP-/ART-/HIV- samples and sent for LC-MS processing in the Anderson Lab at the University of Colorado Anschutz. An additional two samples were contrived at DD5 or 36.5 nM TFV-DP spiked into the processed samples and blotted on DBS cards. The purpose of starting with negative samples is to verify the LoD and ensure the assay does not detect any off-target substances in the absence of the drug. If the results of the LC-MS adequately match those of RESTRICT, an additional ten negative samples were prepared with contrived drug levels spanning the clinical range as well as ten PrEP-patient samples. All will be assayed with RESTRICT and sent for LC-MS analysis. If there is greater than

90% identity between the results, the assay will be validated as having acceptable accuracy.

Precision

Precision can be described by the reproducibility of results. For CLIA, it must be evaluated from two perspectives: inter- or between-assay variability and intra- or within-assay variability. To assess inter-assay precision, two patient samples were compared at clinically relevant drug concentrations on the same plate over multiple runs. This was repeated with a total of 10 patients. If the nFUs were at least 80% similar between assay runs, the inter-assay precision was deemed acceptable. During the process of these validation experiments, there was consistently nonideal variability both between samples and runs. The sample-to-sample discrepancy was suggested to be a result of patient variability and was assessed by studying trends in patient hematocrit and sample UV-vis absorbance. Between-run variability was largely minimized by streamlining the assay workflow. This was achieved by batch preparing and freeze-storing the RTB, pre-mixing the patient samples with MM for easier addition to the plates, and use of multichannel pipettes to minimize the delay between wells for RT and PG additions. Reagents susceptible to eroded functionality due to freeze-thaw cycling such as RT and DNA template were aliquoted into single-use units to avoid unnecessary thaws. Cumulative variance (%CV) was generally well below the acceptability threshold of 20%.

Intra-assay variability could be assessed through a comparison of reaction replicates. All RESTRICT reactions including positive and negative blood and water

controls and contrived drug-dilution series samples are run in a minimum of three replicates– most frequently in four. Much of the variability between replicates could be controlled through careful workflow and pipetting techniques. Cumulative variance (%CV) was generally well below the acceptability threshold of 20%. For runs with >20%CV, UV-vis absorbance was collected to potentially elucidate whether the sample itself yielded the variability.

Interfering Substances

Per CLIA recommendations, any potential substances or sample formulations that interfere with the functionality of RESTRICT had to be assessed. A standard inhibition testing panel includes three sample conditions that frequently interfere with blood-based assays: hemolysis, icterus, and lipemia (HIL).

Hemolysis: an excess of hemoglobin in the blood due to ruptured RBCs, such as in the event of excessive shaking of the sample. It can be identified by redness in the plasma of an EDTA sample and can interfere with analyte detection due to the release of intracellular material into the sample.

Icterus: an excess of bilirubin, sometimes seen in patients with compromised liver function. Icteric samples can present with yellow plasma in EDTA samples. It interferes with the sample's spectral properties and may react with assay reagents.

Lipemia: an excess of lipids, sometimes caused by insufficient fasting before sample collection or certain diseases. Lipemic samples often appear

cloudy and turbid or may even have a fatty layer on top. This can interfere with sample processing such as filtering and can cause light scattering.

HIL interference can be assessed with a simple pre-made panel containing hemolytic, icteric, and lipemic plasma.

Other potential sources of interference assessed were EDTA and heparin. These are anticoagulants commonly added to blood samples to improve benchtop stability. However, they can sometimes interfere with the function of assays. These can also be tested using pre-made plasma samples.

The consideration of potential interference of other drugs or disease conditions is also an important one in evaluating RESTRICT, as many PrEP users may be receiving other medications or therapies. Of particular concern are any other nucleotide analog inhibitors like TFV-DP which may confound the assay's specificity. However, this testing requires multiple draws from a patient and as such is relegated to the clinical validation phase of development rather than the CLIA validation. The upcoming RCT will be run by the Drain Lab.

IV. RESULTS AND ANALYSIS

Initial Average Standard Curve

A total of 62 samples were provided by the Harborview Hematology Laboratory, 60 of which were from individuals not taking PrEP. All samples were assayed with a full drug dilution series to generate standard curves for each. A total of 51 of these runs were kept for use in the master ASC. The 9 PrEP- samples excluded from the data set were removed due to errant data resulting from assay preparation errors and/or "Overflow" results from the plate reader. "Overflow" means the fluorescence intensity is too high for the reader's reportable range and can be solved by either lowering the gain or altering the reagent composition to increase the final concentration of dsDNA. The results were entered into the data entry template per the specifications of the plate map (see *Methods*). From here, they were automatically compiled into a single sheet for each assay run called "Combined Data" where they could be exported into R for analysis and visualization.

An R script was constructed which compiled data from the "Combined Data" sheets of each run file into a single data frame. All of the raw fluorescent intensity values (RFUs) results were processed through the normalization equation

$$Normalized = \frac{RFU(sample+RT) - RFU(sample-RT)}{RFU(water+RT) - RFU(water-RT)}$$

to reduce background and more clearly see trends. From here, the data was sorted by Date > Run number > Patient > Well type and plotted on a graph of [TFV-DP] (nM) x nFU. Each plot consisted of nine total points: 7 drug dilutions, a positive blood control, and a negative blood control.

A standard curve for a single patient/run/date was created using a four-parameter logistic regression (4PL) model, sometimes used in plotting dose-response relationships for pharmaceuticals. The Dittmer Lab created an R package, *dr4pl,* which accepts four parameters to create a logic regression curve. The parameters are the maximum and minimum values of the dynamic range (in this case, nFU), the Hill Slope coefficient, and the 50% inhibitory capacity (IC50) [10]. The Hill Slope is derived from the Hill Equation which describes receptor-ligand binding interactions. For use in the R model, the Hill coefficient for RESTRICT is assumed to be 1. The IC50 or the log(EC50) refers to the point on the curve halfway between the maximum and minimum points. This describes the degree of inhibition of RT by TFV-DP. For RESTRICT, the IC50 should fall around the 4 doses/week level of adequate adherence, in the approximate middle of the clinically relevant range. These four parameters are used to construct the regression curve.

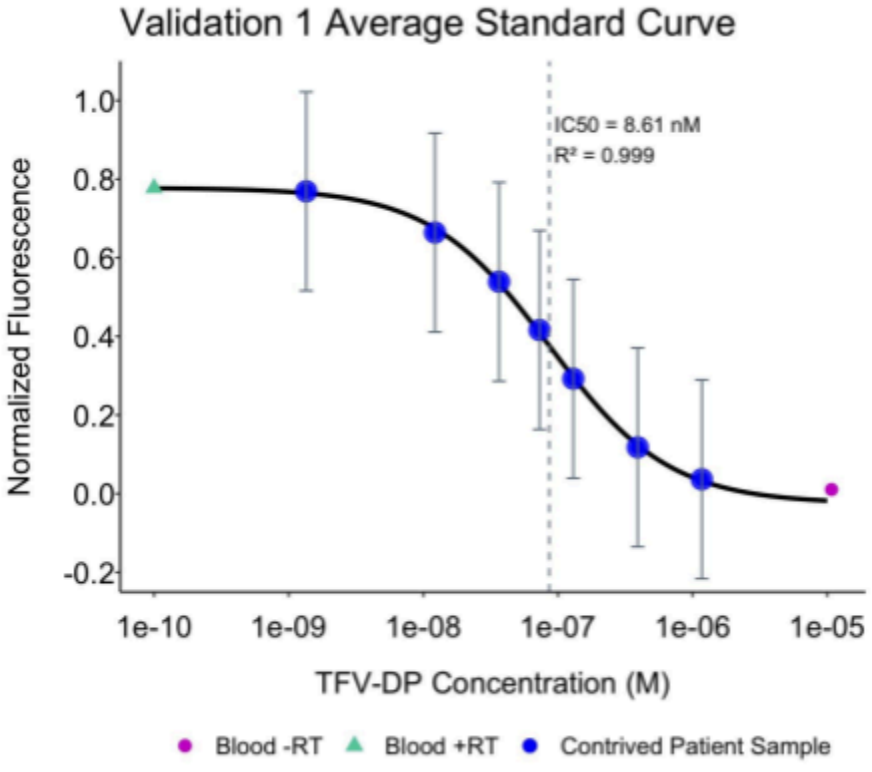


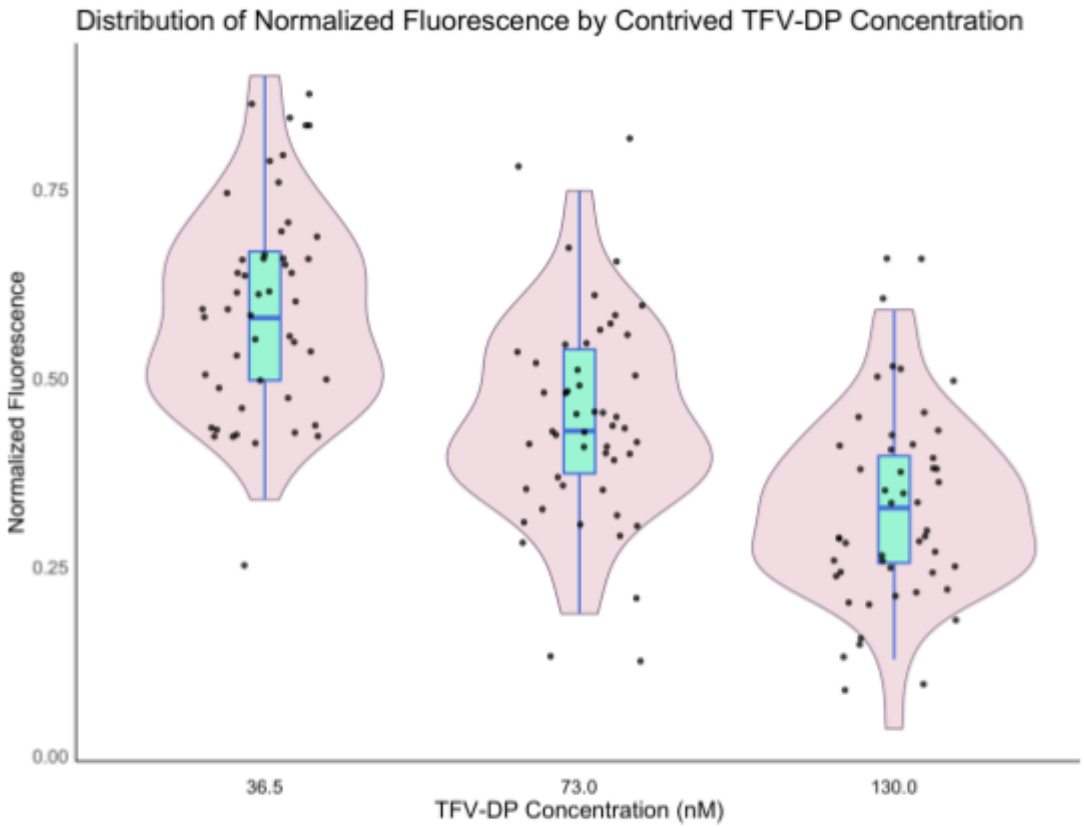
***Figure 7**: A 4-point logistic regression applied to the average normalized fluorescence across 51 contrived patient samples to create an average standard curve which can be used for reference for PrEP-patient sample testing. Error bars represent the 95% confidence interval (CI) for each average.*

To create the ASC, the same model was applied to a single data frame of the compiled data from the “Combined Data” sheet of each of the 51 standard curve runs. Instead of the four replicate points for each drug concentration, the results were averaged across replicates and across patient samples to create a single ASC (Fig. 7).

The IC50 of this ASC was 8.61 nM which is well within the expected range of TFV-DP concentrations, which corresponds to the clinical range. From 36.5 nM TFV-DP spiked into the processed sample, it undergoes a 1:4 dilution on the plate, meaning approximately 9 nM corresponds to 4 doses/week. The most troubling aspect of this ASC was the dynamic range. None of the standard runs had a maximum nFU ≥1.0 as had been observed in the development data. The average maximum nFU value was approximately 0.8. For water controls where the RT acts on the assay components in complete isolation from unintended substances, the +RT control effectively models 0% inhibition while the –RT control models 100% inhibition. While it is expected for blood to have some impact on the span of this dynamic range, since the RFUs are normalized based on the water controls, perfect recapitulation of those controls should return an nFU of 1.0. As such, this low maximum indicates the assay is only achieving 80% of its potential reportable range. This meant that it would be more difficult to discern adequate from perfect adherence, rendering the assay a qualitative “drug or no drug” determination. To follow the original design of a quantitative assay capable of distinguishing three levels of adherence, it will be necessary to optimize the assay’s composition and workflow in the future.

Precision and Variability

One of the greatest challenges in optimizing RESTRICT was controlling for sample variability. While it is expected that WB will always introduce some variability, it is important for the interpretation of the assay that normalized fluorescence values are



[TFV-DP] (nM)	Minimum	Lower Quartile	Median	Upper Quartile	Maximum
36.5	0.3390	0.497	0.580	0.668	0.901
73.0	0.1880	0.374	0.430	0.538	0.749
130.0	0.0359	0.256	0.328	0.397	0.591

***Figure 8/Table 3**: Violin boxplot showing the distribution of normalized fluorescence (nFU) values across patients and runs at the three clinically relevant drug concentrations. In order, the plots correspond to low medium, and high adherence. The table below summarizes the distribution of nFU values for each concentration.*

distinguishable at each TFV-DP concentration. This allows the establishment of hard cutoffs between levels of adherence. The average normalized fluorescence across patients and runs (Fig. 8, Table 3) shows the overlap between results at the three clinically relevant drug concentrations. The median results are sufficiently distinct, implying it is possible to adequately distinguish between different adherence levels. However, the upper quartiles and maxima show that enough of the patient samples at different concentrations overlap enough to make the cutoffs uncertain. For example, the upper quartile of the high adherence group (130 nM) with a value of 0.397 overlaps with the moderate adherence (73 nM) lower quartile at 0.374 and almost with the median at 0.43. The closeness of these values means it is more difficult to determine whether patients with normalized fluorescence values between 0.35-0.45 fall into the moderate or high adherence category.

The %CV was computed across patients, replicates, and concentrations. The average inter-replicate %CV across patients was 2.4%, which demonstrates excellent workflow consistency. Across all drug dilutions, the %CV was 2.7%, solidifying the integrity of the serial dilution process and the assay's reproducibility across TFV-DP concentrations. Per the parameters of the Validation Plan, the %CVs were well within tolerance. However, this did not address the adherence-level separation issue. Further optimization would be necessary.

Assay Optimization

Due to the unsatisfactory results of the initial validation, several tweaks were made to the assay protocol to improve dynamic range and variability. To determine optimization targets, the assay was run both in the validation and the development labs, swapping reagents used in the development scheme for existing reagents one at a time to understand which might be causing increased variability and limited range. The current and development protocols were also examined closely to reveal any discrepancies.

An initial observation was the presence of agitation during the sample preparation procedure. The initial validation entailed agitating the sample during the heating cycle just prior to filtering while the development protocol did not include this step. Several experiments were able to show that allowing the sample to sit still on the heating block slightly improved inter-sample variability. This was likely because agitation during heating introduced additional debris from the lysis which may have been difficult to filter out with a 0.45 μm filter.

Additionally, the protocol from the development stage included continuous agitation throughout the plate incubation as opposed to the 20-second interval in our existing method. Extending the shaking period in the Tecan programmed protocol to the full 30 minutes improved both inter-sample and inter-replicate variability. It is thought that this change enabled more even mixing within the reaction well, increasing the efficiency of the HIV RT reaction, though no further experiments were done to elucidate this point.

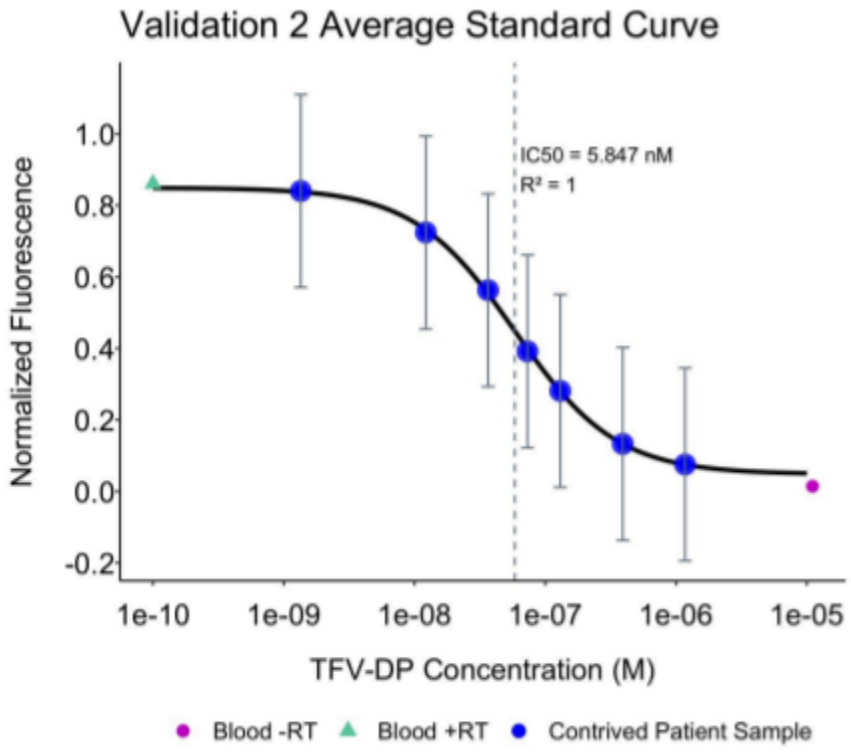


***Figure 9**: The 4-point logistic regression curve applied to the average normalized fluorescence across 30 contrived patient samples. Negative control includes some jitter to be visible on the plot. The increased dynamic range of this standard curve ensures more spread between the three clinically relevant drug concentrations (three points nearest IC50). Error bars indicate 95% confidence interval.*

A final adjustment to the protocol was ordering the TTCA template PAGE-purified to ensure maximum efficiency. With these changes, the validation was re-started, thus far yielding promising results. At the time of writing, 30 samples have been tested and plotted on a new Average Standard Curve (Fig. 9). The first notable improvement is the dynamic range. The changes to agitation not only improved the variability but increased the upper limit of the assay, bringing the total range to the desired ≥0.9. Additionally, the R^2 value increased to 1.0 which implies the 4-PL model fit perfectly. The IC50 decreasing from 8.61 to 5.88 also corresponds to the shift in the dynamic range of the assay. This means that less drug was required to see 50% inhibition, suggesting greater efficiency of the assay.

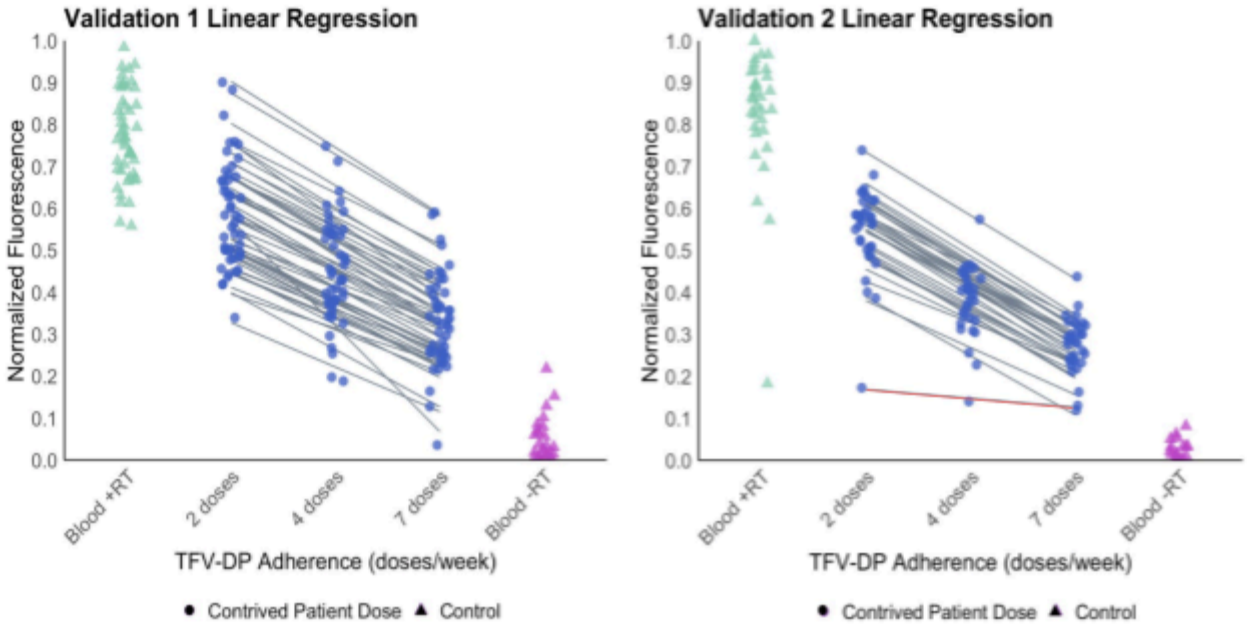


***Figure 10**: A comparison of the linear regression across the clinically relevant drug concentration range including all contrived patient samples used to generate the Average Standard Curves. Validation 1 (left) includes 51 samples while Validation 2 (right) shows 30 samples. The outlying regression in Validation 2 (red) with an abnormally low normalized fluorescence across the span of the clinical range was excluded from the Standard Curve but is shown here for comparison.*

One of the most stark comparisons between the first and second validation is the linear regression across the clinical concentration range (Fig. 10). As discussed previously, a primary difficulty with the initial validation data was the ability to distinguish cleanly between different adherence levels. These linear regressions demonstrate already how much less overlap there is between nFUs at clinical concentrations in Validation 2, despite only 30 samples being shown versus 51 samples in Validation 1. There also appears to be less variability between patients and within the controls, thus solidifying the dynamic range across clinical concentrations. The lowest curve (red) from Validation 2 represents a patient already taking PrEP– TAF rather than the TDF upon which RESTRICT is based. This patient slipped through our screening and, while excluded from the ASCs and ROC curves, was included in the linear regression. The

minor error offers an opportunity to to see the impact of existing TFV-DP on the assay which is to lower, almost flatten, the dose-response curve. However, since TAF is more potent than TDF, the final serum concentration of TFV-DP is lower meaning the +RT control does not go all the way to zero, as would be expected from a TDF patient.

An additional method for analyzing the performance of RESTRICT is Receiver Operating Characteristic (ROC) curves that compare sensitivity and specificity. Based on the data, a threshold is calculated between two drug levels of interest.

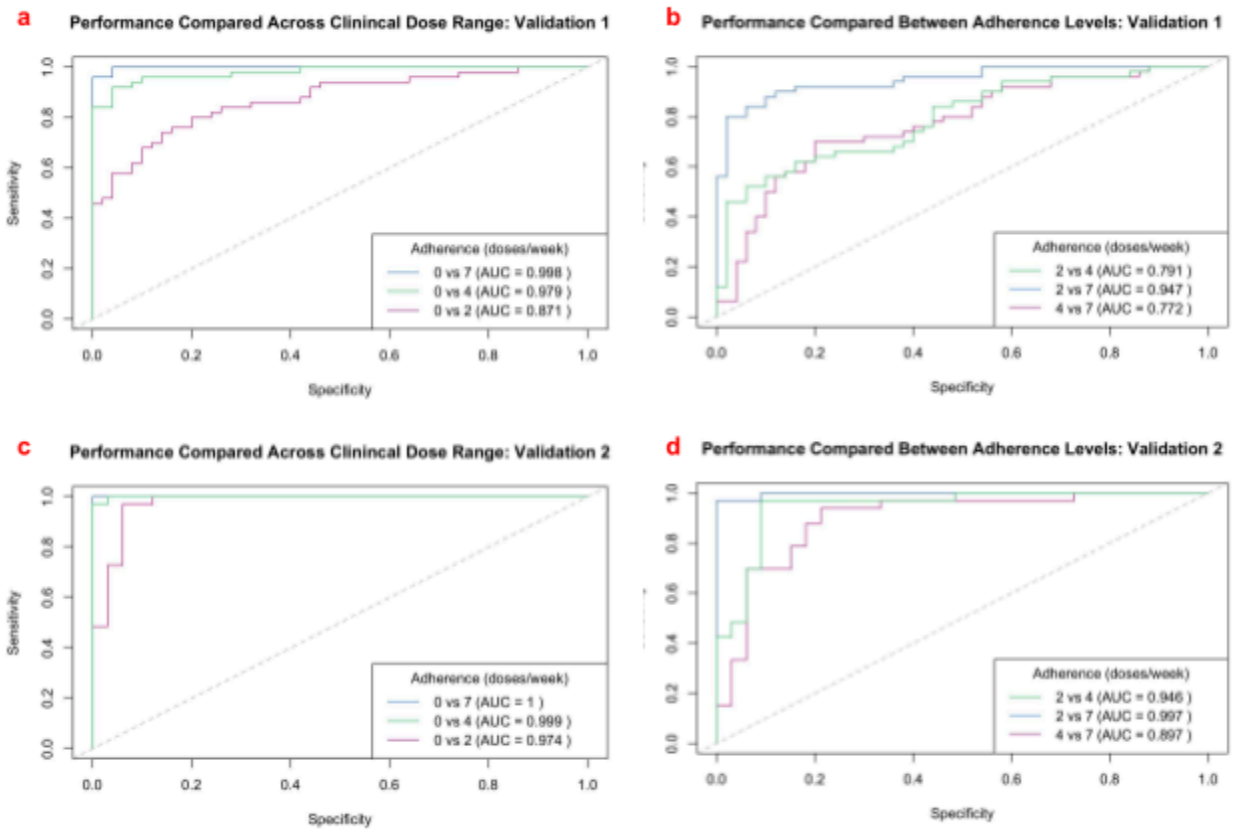


Figure 11*: The initial validation results (a, b) demonstrated that RESTRICT was excellent at detecting the presence of TFV-DP but lacked the sensitivity to adequately distinguish between drug levels. In particular, the AUC separating moderate (4) from high (7) adherence was not acceptable (b). With the changes made in Validation 2 (c, d), the assay improved both its sensitivity and specificity allowing for a more semi-quantitative result. The separation between 4 and 7 doses (d) is improved and these curves confidently show that RESTRICT can be used to identify individuals out of adherence to their PrEP regimen.*

The distributions of nFU values at each drug level are plotted either above or below this threshold. The Area Under the Curve (AUC) measures the degree of overlap of the two distributions. Perfectly overlapping sets will align on the diagonal reference line meaning AUC values closest to 1 indicate high performance, and values closer to 0.5 indicate low performance.

To create the ROC curves (Fig. 11), the data from the clinical range of each Standard Curve dataset was separated by TFV-DP concentration into the three adherence levels: 2 doses/week (low/non-adherent), 4 doses/week (moderate) and 7 doses/week (high/perfect). For each level, the data was compared to the positive controls (Blood +RT) or to one another to create a binary predictor. Using the ROCR package, the performance of the assay in distinguishing each adherence level was calculated and plotted. The ROC curves generated from Validation 1 suggested that the assay was sufficiently sensitive to distinguish between patients taking or not taking PrEP and between nonadherence and moderate to high adherence (Fig. 11a). However, it could not adequately separate total adherence from minimum to moderate adherence (Fig. 11b). This confirmed that the current assay design would serve only as a quantitative assay with a binary output: adherent or nonadherent.

With the changes implemented for Validation 2 and the expanded dynamic range, the assay performance notably improved. The AUC values for all comparisons increased in Validation 2 demonstrating a greater ability of the assay to separate between adherence levels. A particularly notable improvement was for moderate versus high adherence (Fig. 11d), which were essentially indistinguishable in Validation 1. These are very promising results.

V. DISCUSSION

The validation process for RESTRICT revealed some inefficiencies and areas for improvement within the assay preparation and workflow. Initially, it was necessary to adapt the assay from the plate reader on which it was developed to the Tecan Infinite® M-Plex microplate reader for validation. Before starting the formal validation, the assay was optimized from the ground up. This involved running the assay with negative and positive water controls without patient blood multiple times to ensure assay functionality and determine the approximate range of RFUs. Once the non-sample controls adequately reflected those from the development data, PrEP-negative patient WB was introduced and compared to the water controls. It was clear from the start that the addition of WB reduced the span of the reportable range and, despite several tweaks to workflow such as pipetting techniques and sample agitation, the average maximum RFU was consistently lower than the development data.

To better understand and benchmark the trend between RFU and PrEP drug concentration, the RFU values are normalized based on the water and sample controls (nFUs). The development data boasted a reportable range of 0.0-1.2 nFU while the optimization runs on the new microplate reader typically showed a range of 0.05-0.8 nFU, or less than 80% of the target range. To elucidate this discrepancy, it was necessary to run through the assay step by step and understand the role of each component. In the hands of a trained Medical Laboratory Scientist (MLS), the assay was run under a broad variety of different conditions. Initially, a pre-made buffer that approximately matched the composition of the original was used. However, it was determined that to properly compare the results, it was necessary to mix a custom

buffer based on the development protocol. The DNA template and RT concentrations were varied across runs and the SOP was adjusted with small workflow changes. It was clear from these early runs that the complexity of the assay preparation required an easy-to-use template with which to calculate required volumes, final assay concentrations, etc.

To fulfill these needs, an Excel template was designed which enabled the technician to map the plate by sample type and automatically calculate the reagent volumes based on the number of reactions and sample volume (Appendix A). This template reduced human error and standardized the reagent calculations and additions. It also enabled the raw fluorescence data to be formatted based on the plate map, allowing for greatly simplified data export and processing. The use of this template significantly reduced inter-replicate variability and the frequency of outliers.

The consistency provided by the template also enabled the standardization of the microplate reader protocol. In early runs, it was necessary to leave the settings on automatic to compensate for variable fluorescence. After standardizing the reagent and assay preparation protocols, it was possible to pinpoint exact settings to ensure comparability across runs. The most variable setting was the fluorescent gain, or the voltage at which the fluorescence is amplified. It was important to find a fixed gain value that maximized fluorescent intensity values without causing “Overflow” (results above the reader’s upper LOD). Single plates were run multiple times at different gains to determine the highest possible value that would consistently produce interpretable results; in this case, 142. Once an optimal gain was found, the other settings could be fixed: read height (distance of detector above plate), emission wavelength, and

excitation wavelength. For PicoGreen, the documented range for excitation and emission is 480 nm-520 nm but through repeat testing, the range for RESTRICT was set to 485 nm-535 nm as the lower drug dilutions and therefore increased cDNA concentration slightly raised the range.

One challenge was being forced to adjust expectations on the overall timeline of the project. While research and experimentation are always more complex than they seem on the surface, we had not accounted for the amount of optimization necessary to prepare RESTRICT for CLIA validation. Between training, reagent ordering, and open experimentation, it took nearly four months to get the assay ready for the initial validation. It then took an additional four months to complete every section of the validation and analyze the data for optimization targets. In the beginning, the goal was to complete the validation and hand it off to the Drain Lab for randomized clinical testing within six months. Having to reevaluate our expectations and timeline was frustrating but necessary for the integrity of the assay. It is now a much more effective semi-quantitative PrEP assay for a clinical setting than it was at the beginning as a research-only assay.

This optimization process will likely continue past the second validation as experimentation has revealed several possibilities for improving the assay sensitivity and specificity significantly. One avenue of continued exploration is changing the diluent from RTB. At issue was how to make the controls with and without patient samples (blood and water controls) more closely resemble one another. Simply, we needed the blood to behave more like water or the water to behave more like blood. Since further diluting or otherwise significantly altering the sample preparation would likely have

consequences for the overall sensitivity of the assay, we considered diluent options that made the water controls more blood-like. BaseMatrix Diluent by SeraCare is a negative plasma developed for use in LDTs for HIV, HSV, HPV, and similar. It is prepared from defibrinated, dialyzed human plasma meaning it makes an excellent blood-like alternative for negative controls [28].

Initial testing of RESTRICT substituting BaseMatrix (BM) for all instances of RTB suggests that BM increases the dynamic range of the assay while retaining the other performance characteristics. The individual standard curves (by plate) seem much more likely to reach an upper limit of 1.0 nFU in both the blood and water controls, expanding the range of the assay even beyond the desired ≥0.9. Raising the upper limit will allow a broader distribution of nFUs within the clinical range, improving the ability of RESTRICT to distinguish between adherence ranges. However, further experimentation is needed before RESTRICT can be re-validated using BM.

While the assay will continue to be optimized and re-validated as seems necessary, RESTRICT as it stands in the second round of CLIA validation is ready to be validated clinically through RCT. In conjunction with CDC researchers also pursuing a similar means of PrEP adherence tracking, the Drain Lab will soon commence a trial in the Seattle, WA area with 100 participants taking some form of tenofovir-based oral PrEP or tenofovir-based antiretroviral therapy. RESTRICT results will be compared to LC-MS readings on DBS. While most participants will be taking TDF (commercially Truvada) which was the only medication tested during the CLIA validation phase, the study aims to recruit some taking tenofovir alefenamide (commercially Descovy). TAF generally functions at much lower serum concentrations than TDF meaning it is much

more difficult to monitor by way of chain termination interpolation. One aim of the study will be to examine the efficacy of RESTRICT on monitoring TAF adherence. Another area of focus will be assessing the impact of existing HIV RT in the blood of virally unsuppressed patients on the functionality of the assay.

Additional questions posed in the upcoming trial include the stability of TFV-DP in WB samples after draw and after freezing/thawing. According to the development data, the window for sample processing was limited to ≤4 hours after collection. However, this is a relatively limited timeframe, particularly in areas where phlebotomy may be a significant distance from the clinical lab. If RESTRICT results prove durable on WB more than 4 hours post-collection, it would be another step toward improving the accessibility of adherence monitoring in low-resource settings. More than this, it would be ideal to be able to run RESTRICT on thawed samples. Exploratory testing performed on samples frozen post-processing and thawed 24 hours later for assy seem to suggest that RESTRICT sensitivity drops off precipitously. However, this has neither been confirmed with additional testing nor does it necessarily imply that samples cannot be frozen immediately after collection and processed post-thaw so there is hope this can be elucidated during trial. The CDC and Drain Lab will be running separate trials but collaborating on results to generally improve study power and demographics.

VI. CONCLUSION

The CLIA validation requirements are relatively straightforward but, as demonstrated, the process can be fraught with complexities and unforeseen considerations. Although the validation experiments themselves may not take long, hindsight shows that it is necessary to budget a broader timeframe to adapt and optimize an assay from the development stage, especially if development and validation are performed in different laboratories.

The challenges faced during this process highlighted the difference between research science and clinical work. One such difference is scalability. To ensure consistency across every run of RESTRICT, it was necessary to batch-prepare reagents sufficient for up to 100 plates. Diluting and storing such batches added new variables to the assay performance and meant that the workflow for generating the Master Mix was altered. This was ultimately beneficial, however, because it meant that reagents could be stored in single-use aliquots, limiting the exposure of stock reagents to freeze-thaw cycles and prolonging stability and usability.

Another crucial consideration in the change of labs was the use of a different microplate reader. This meant that the first step in preparing for validation was to recapitulate the results and assay performance in the development stage. To compare results between the different readers, the raw fluorescence data had to be normalized. Initially, there were many challenges in recapitulating old data– particularly the dynamic range of normalized values.

Adjustments to the protocol yielded beneficial effects, improving the dynamic range, precision, specificity, and signal-to-noise ratio. Small changes to workflow limited

variability across a plate but to address the dynamic range, it was necessary to do more extensive experimentation. Ultimately, the goal was to improve the comparability of the patient sample (blood controls) and non-sample controls (water controls) or to “make the water look more like blood.” This was accomplished by completely changing the diluent for the assay from RTB to BaseMatrix DNAse-treated plasma. This helped to control for patient-sample variability and provided more optimal conditions for the RT, meaning the upper limit of the dynamic range increased and the blood and water controls matched more closely.

Overall, the assay has undergone significant changes and improvements from the development stage to better meet the CLIA validation criteria for LDTs. However, further changes will likely be necessary during the clinical testing phase once results are based on patients’ serum PrEP levels instead of manually spiked-in drug. In addition to validating RESTRICT in an RCT setting, a goal of the trials is to further elucidate the time-dependent side of PrEP adherence. While the assay may be able to discern between approximate adherence levels, it is yet unknown how the dosing consistency and potential lapse intervals impact perceived adherence.

Finally, a longer-term goal for RESTRICT is to adapt it into a point-of-care assay that can be implemented in low-resource settings. The assay is simple enough that it has strong potential for such status, but it will need to undergo significant adjustments to the reagent storage and specialized material needs, such as the custom TTCA oligomer. The validation process has demonstrated that RESTRICT can be an extremely beneficial alternative to LC-MS and may improve the uptake and efficacy of PrEP around the world.

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While I started the earliest optimization of the assay, all of the CLIA validation work and Standard Curve data were produced by Ally Studer and Trever Kuralt, our wonderful Medical Laboratory Scientists. Additional optimization and troubleshooting work was performed by Cole Martin from the Posner Group. LC-MS testing was run on RESTRICT DBS samples by Dr. Peter Anderson's lab at the University of Colorado Anschutz Medical Campus.

Background and troubleshooting for the project were provided by Dr. Andrew Bender and Dr. Ayokunle Olanrewaju. Dr. Olanrewaju created Figure 3. I would like to thank him for allowing me to use his work in this paper.

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Figures were created with BioRender or generated in R Studio using *tidyverse*. ChatGPT was used for R code learning support (not used to create original content).

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v. Appendix A: Data Entry Table Design

RESTRICT ASSAY

Run Date:	
Run Tech:	
Plate Number:	

Patient Samples		
ID#		
Subject ID		
Graph Title		

KEY:
Patient A, B, etc: PTC, PTD, PTV'
+vs- RT: Identifies if RT added to sample (-RT = MGW added instead)
DD#: Drug Dilution Series Identifier
H2O/MGW: Molecular Grade Water
MM: MASTER MIX
WB: Whole Blood
RT: Reverse Transcription
PB: Processed Blood
RTB: Reverse Transcription Buffer

PLATE LAYOUT

Drug Concentrations

	2.93E-07	9.78E-08	3.25E-08	1.83E-08	9.13E-09	3.05E-09	3.38E-10	Blood +RT	Blood -RT	Water +RT	Water -RT	
	1	2	3	4	5	6	7	8	9	10	11	12
ex: A	PTC1 +RT DD1	PTC1 +RT DD2	PTC1 +RT DD3	PTC1 +RT DD4	PTC1 +RT DD5	PTC1 +RT DD6	PTC1 +RT DD7	PTC1 +RT	PTC1 -RT	H2OA +RT	H2OA -RT	
PTC1 +RT B	PTC1 +RT DD1	PTC1 +RT DD2	PTC1 +RT DD3	PTC1 +RT DD4	PTC1 +RT DD5	PTC1 +RT DD6	PTC1 +RT DD7	PTC1 +RT	PTC1 -RT	H2OA +RT	H2OA -RT	
C	PTC1 +RT DD1	PTC1 +RT DD2	PTC1 +RT DD3	PTC1 +RT DD4	PTC1 +RT DD5	PTC1 +RT DD6	PTC1 +RT DD7	PTC1 +RT	PTC1 -RT	H2OA +RT	H2OA -RT	
D	PTC1 +RT DD1	PTC1 +RT DD2	PTC1 +RT DD3	PTC1 +RT DD4	PTC1 +RT DD5	PTC1 +RT DD6	PTC1 +RT DD7	PTC1 +RT	PTC1 -RT	H2OA +RT	H2OA -RT	
E	PTD1 +RT DD1	PTD1 +RT DD2	PTD1 +RT DD3	PTD1 +RT DD4	PTD1 +RT DD5	PTD1 +RT DD6	PTD1 +RT DD7	PTD1 +RT	PTD1 -RT	H2OB +RT	H2OB -RT	
F	PTD1 +RT DD1	PTD1 +RT DD2	PTD1 +RT DD3	PTD1 +RT DD4	PTD1 +RT DD5	PTD1 +RT DD6	PTD1 +RT DD7	PTD1 +RT	PTD1 -RT	H2OB +RT	H2OB -RT	
G	PTD1 +RT DD1	PTD1 +RT DD2	PTD1 +RT DD3	PTD1 +RT DD4	PTD1 +RT DD5	PTD1 +RT DD6	PTD1 +RT DD7	PTD1 +RT	PTD1 -RT	H2OB +RT	H2OB -RT	
H	PTD1 +RT DD1	PTD1 +RT DD2	PTD1 +RT DD3	PTD1 +RT DD4	PTD1 +RT DD5	PTD1 +RT DD6	PTD1 +RT DD7	PTD1 +RT	PTD1 -RT	H2OB +RT	H2OB -RT	

+RT/-RT		DD Wells		RESTRICT SAMPLE	
Well Product	Diluent Used	Well Product	Diluent Used	Well Product	Diluent Used
10µL MM	RTB	10µL MM	RTB	10µL MM	RTB
10µL MGW/Sample	MGW/None	10µL MGW/Sample	MGW/None	10µL Sample	None
10µL RT/MGW	RTB	10µL RT	RTB	10µL RT/MGW	RTB
10µL MGW	MGW	10µL DD#	MGW	10µL MGW	MGW

Reagent	LOT	Expiration
MGW		
PG		
1X TE Buffer		
TFV-DP		

Reagent	LOT	Expiration
RTB Stock		
Working RTB (w/ DTT)		
DTT		
RT		

Reagent	LOT	Expiration
TTCA		
16s		
dNTPs		

Date:	26-Feb-24	Plate #:	1

WORKING RTB + DTT:
Used for All Plates

Buffer Volume (mL):	5			
Manufacturer Stock DTT [ ] (mM)	Target DTT [ ] in Working RTB (mM)	Stock DTT Volume (µL)	Stock RTB Volume (µL)	[ ] DTT in MM (mM)
100	10	500	4500	1.16

Master Mix Calculation — 10µL per well
MM Made Per Plate

Total Sample Wells:*	100	Rounded Volume Used for Calculations (20% over) 120

*Round as needed for precise reagent volumes

Total volume MM needed:	1200 µL		
Working Reagent	Working Reagent Concentration (nM)	Final Concentration in MM (nM)	Working Reagent Volume (µL)
TTCA	80	4	60
16s	800	40	60
dNTPs	10000	400	48
Working RTB	-	-	1032

Plate Notes: Standard curve

RT Calculation — 10µL per well
RT Made Per Plate

Total Sample Wells with RT:*	72	Rounded Volume Used for Calculations (25% over) 90

*Round as needed for precise reagent volumes

Volume Needed (µL)	Target RT Concentration	RT Concentration for LOT:4105648	VOLUME RT (µL)	VOLUME RTB (µL)
900	0.212	20.2	9.4	890.6

NO RT Calculation — 10µL per well
"NO RT" made for All Plates

Total Sample Wells w/o RT:*	72	Rounded Volume Used for Calculations (25% over) 90

*Round as needed for precise volumes

Volume Needed (µL)	Target RT Concentration	RT Concentration for LOT:4105648	VOLUME MGW (µL)	VOLUME RTB (µL)
900	0.212	20.2	9.4	890.6

PG Calculation — 40µL per well
PG Made Per Plate

Total Sample Wells:	88	Rounded Volume Used for Calculations (25% over) 110

Volume Needed (µL)	PG Conc Needed	PG Original Conc	VOLUME PG (µL)	VOLUME 1xTE (µL)
4400	1.0	200.0	22.0	4378.0

vi. Appendix B: Data Processing and Visualization Code

```
# Load libraries
library(tidyverse)
library(kableExtra)
library(knitr)
library(pROC)
library(ROCR)
library(readxl)
library(writexl)
library(scales)
library(dr4pl)

# Define the list of Excel files
file_paths <- list.files(path =
"Z:\\Divisions\\Virology\\Retrovirus\\Retrovirus_ABI\\RESTRICT\\RESTRICT RUN DATA\\RUN
DATA\\Validation 2\\Compiled Runs\\Standard Curves",
                         full.names = TRUE)

# Function to read and preprocess a specific sheet from an Excel file
read_excel_sheet <- function(file) {
  read_excel(file, sheet = 'Combined Data') %>%
    filter(!is.na(Result)) %>%
    select(-c(Patient, `Final Concentration`, Gain, `Gain Type`, `Run Notes`, Title, TECH)) %>%
    mutate(
      Result = as.numeric(Result),
      Date = format(Date, format = "%m%d%Y"),
      Run_id = paste(Date,"_", `Run number`),
      rowgroup = substr(`Row Group`, 1, 4),
    ) %>%
    filter(Dilution != 'Water +RT DD4')
}

# Read and combine data from all files and remove extraneous columns
all_data <- file_paths %>%
  map_dfr(read_excel_sheet)

all_data <- all_data %>%
  select(-c(HCT, `Group 1`, `Group 2`)) %>%
  filter(!is.na(Result))

###################################PROCESSING#################################

# Subset relevant columns and preprocess data
pat_graphs <- all_data %>%
  filter(!is.na(Result)) %>%
  select(Run_id, Subject_ID, Dilution, `Prep Concentration`, Result, `Row Group`, Well) %>%
  mutate(
    concentration_M = case_when(
      Dilution == 'Blood -RT' ~ 1e-5,
```

```
      Dilution == 'Blood +RT' ~ 1e-10,
      Dilution %in% c('H2O -RT', 'H2O +RT') ~ NA_real_,
      Dilution == 'Water +RT DD4' ~ 1.83e-8,
      TRUE ~ `Prep Concentration`
    ),
    concentration_M = as.numeric(concentration_M)
  ) %>%
  filter(!is.na(Well), Dilution != 'Water +RT DD4')

# Create average control columns and normalize results within each Run_id
norm_data <- pat_graphs %>%
  group_by(Run_id) %>%
  filter(!is.na(Result) & !Subject_ID == "R0212") %>% # Remove TAF patient from Validation 2
  mutate(
    conc_nM = concentration_M,
    avg_negb = mean(Result[Dilution == "Blood -RT"]),
    avg_posb = mean(Result[Dilution == "Blood +RT"]),
    avg_negw = mean(Result[Dilution == "Water -RT"]),
    avg_posw = mean(Result[Dilution == "Water +RT"]),
    norm_fluor = (Result - avg_negb) / (avg_posw - avg_negw)
  )


avg_result_conc <- norm_data %>%
  group_by(Dilution, conc_nM) %>%
  summarize(avg_normResult = mean(norm_fluor, na.rm = TRUE)) %>%
  ungroup()

##################################CALCULATIONS################################

# 4PL calculations
obs1 <- avg_result_conc %>%
  filter(!Dilution %in% c('Blood -RT', 'Blood +RT', 'Water +RT', 'Water -RT')) %>%
  filter(!is.na(conc_nM)) %>%
  group_by(conc_nM) %>%
  summarize(avg_normResult = mean(avg_normResult, na.rm = TRUE))

cntrl1 <- avg_result_conc %>%
  filter(Dilution %in% c('Blood -RT', 'Blood +RT', 'Water +RT', 'Water -RT'))

drpl1 <- dr4pl(
  avg_normResult ~ conc_nM,
  method.init = "logistic",
  init.parm = dr4pl_theta(
    theta_1 = 1, # Upper
    theta_2 = NA, # IC50
    theta_3 = 1, # Slope
    theta_4 = 0 # Lower
  ),
  data = obs1
)
```

```
# Calculate IC50
ic_table1 <- IC(drpl1, inhib.percent = c(20, 50, 80))
tabledf1 <- as.data.frame(t(ic_table1))
colnames(tabledf1) <- c("IC20", "IC50", "IC80")
tabledf1[] <- lapply(tabledf1, function(x) round(x * 1e8, digits = 3))
norm_data$IC50 <- tabledf1$IC50
ic50 <- tabledf1$IC50

# Calculate R squared
ob1 <- obs1$avg_normResult
res1 <- residuals(drpl1)
mean_ob1 <- mean(ob1)
SSres1 <- sum(res1^2)
SStot1 <- sum((ob1 - mean_ob1)^2)
r2_1 <- 1 - (SSres1 / SStot1)
tabledf1$R_squared <- round(r2_1, digits = 3)

# Clinical Dilution Range calculations
avg_clin <- norm_data %>%
  filter(conc_nM %in% c(3.65e-08, 7.3e-08, 1.3e-07)) %>%
  group_by(Subject_ID, conc_nM) %>%
  summarize(avg_normResult = mean(norm_fluor, na.rm = TRUE)) %>%
  mutate(conc_nM = case_when(
    conc_nM == 3.65e-08 ~ 36.5,
    conc_nM == 7.3e-08 ~ 73.0,
    conc_nM == 1.3e-07 ~ 130.0
  ))

lrc1x <- lm(avg_normResult ~ conc_nM, data = avg_clin)
slopec1 <- coef(lrc1x)[2]
c1rsq <- summary(lrc1x)$r.squared
clintab1 <- data.frame(Slope = slopec1, R_Sqr = round(c1rsq, digits = 3))
clintab1$line <- paste0("y=", round(lrc1x$coefficients[2], digits = 4), "x+",
round(lrc1x$coefficients[1], digits = 4))

# Create tibble of stats
all_stats <- all_data %>%
  select(c(Subject_ID, Dilution, `Prep Concentration`, Run_id, rowgroup,
           `Mean 1`, `Standard Deviation 1`, `CV Percentage 1`,
           `Mean 2`, `Standard Deviation 2`, `CV Percentage 2`))
pat_wise_stats <- all_stats %>%
  mutate(rowgroup = case_when(
    rowgroup == "PTA_" ~ "PTA",
    rowgroup == "PTB_" ~ "PTB",
    rowgroup == "PTC_" ~ "PTC",
    rowgroup == "PTD_" ~ "PTD",
    rowgroup == "PTD1" ~ "PTD"))

# Pair stats with patient
```

```
patAC_stats <- pat_wise_stats %>%
  filter(rowgroup %in% c("PTA", "PTC")) %>%
  select(-c("CV Percentage 2", "Standard Deviation 2", "Mean 2"))
patBD_stats <- pat_wise_stats %>%
  filter(rowgroup %in% c("PTB", "PTD")) %>%
  select(-c("CV Percentage 1", "Standard Deviation 1", "Mean 1"))

# Find the average %CV and Standard Deviation across runs by drug dilution for even #
subjects
avg_by_sub_A <- function(patAC_stats) {
  patAC_stats %>%
    filter(!is.na(Subject_ID)) %>%
    group_by(Subject_ID) %>%
    summarize(
      `%CV` = mean(`CV Percentage 1`, na.rm = TRUE),
      SD = mean(`Standard Deviation 1`, na.rm = TRUE)
    ) %>%
    ungroup()
}
stats_by_sub_A <- avg_by_sub_A(patAC_stats)

avg_by_type_A <- function(patAC_stats) {
  patAC_stats %>%
    filter(!is.na(Dilution)) %>%
    group_by(Dilution) %>%
    summarize(
      `%CV` = mean(`CV Percentage 1`, na.rm = TRUE),
      SD = mean(`Standard Deviation 1`, na.rm = TRUE),
      Mean = mean(`Mean 1`, na.rm = TRUE)
    ) %>%
    ungroup()
}
stats_by_type_A <- avg_by_type_A(patAC_stats)

# Find the average %CV and Standard Deviation across runs by drug dilution for odd # subjects
avg_by_sub_B <- function(patBD_stats) {
  patBD_stats %>%
    filter(!is.na(Subject_ID)) %>%
    group_by(Subject_ID) %>%
    summarize(
      `%CV` = mean(`CV Percentage 2`, na.rm = TRUE),
      SD = mean(`Standard Deviation 2`, na.rm = TRUE)
    ) %>%
    ungroup()
}
stats_by_sub_B <- avg_by_sub_B(patBD_stats)

avg_by_type_B <- function(patBD_stats) {
  patBD_stats %>%
    filter(!is.na(Dilution)) %>%
    group_by(Dilution) %>%
```

```
    summarize(
      `%CV` = mean(`CV Percentage 2`, na.rm = TRUE),
      SD = mean(`Standard Deviation 2`, na.rm = TRUE),
      Mean = mean(`Mean 2`, na.rm = TRUE)
    ) %>%
    ungroup()
}
stats_by_type_B <- avg_by_type_B(patBD_stats)

# Summarize SD and %CV in formatted tables
stats_by_sub <- bind_rows(stats_by_sub_A, stats_by_sub_B)
stats_by_type <- bind_rows(stats_by_type_A, stats_by_type_B)

# Average CV and SD across unique Dilutions
stats_by_type <- stats_by_type %>%
  group_by(Dilution) %>%
  summarize(
    `%CV` = mean(`%CV`, na.rm = TRUE),
    SD = mean(SD, na.rm = TRUE),
    Mean = mean(Mean, na.rm = TRUE)
  ) %>%
  ungroup()

# Calculate mean across patients
mean_CV_pat <- mean(stats_by_sub$`%CV`)
mean_SD_pat <- mean(stats_by_sub$SD)

##################################VISUALIZATION#################################

# Plot 4PL standard curve of average fluorescence values
plot1 <- plot(drpl1) + geom_point(color = 'royalblue3', size = 1)
graphic1 <- plot1 +
  geom_point(data = cntrl1 %>% filter(Dilution != "Blood -RT"),
             aes(x = conc_nM, y = avg_normResult,
               color = Dilution, shape = Dilution),
             size = 3) +
  geom_point(data = obs1,
             aes(x = conc_nM, y = avg_normResult,
               color = "Contrived Patient Sample", shape = "Contrived Patient Sample"),
             size = 3) +
  geom_jitter(data = cntrl1 %>% filter(Dilution == "Blood -RT"),
              aes(x = conc_nM, y = avg_normResult,
                  color = Dilution, shape = Dilution),
              size = 3, width = 0.05, height = 0.05) +
  scale_x_log10(
    breaks = scales::trans_breaks("log10", function(x) 10^x, n = 6),
  ) +
  scale_y_continuous(
    expand = c(0, 0),
    limits = c(0, 1),
    breaks = scales::breaks_extended(8)
```

```
  ) +
  geom_vline(
    xintercept = IC(drpl1, 50),
    linetype = "dashed",
    color = "slategray"
  ) +
  annotate(
    "text", x = IC(drpl1, 50) + 0.1 * IC(drpl1, 50), y = 0.9, label = paste0("IC50 = ", round(ic50, 3), "
nM\nR² = ", round(tabledf1$R_squared, 3)),
    color = "black", size = 4, hjust = 0
  ) +
  xlab("TFV-DP Concentration (M)") +
  ylab("Normalized Fluorescence") +
  ggtitle("Validation 1 Average Standard Curve") +
  theme(
    axis.text.x = element_text(margin = margin(10, 0, 0, 0, "pt"), color = "black"),
    axis.text.y = element_text(margin = margin(10, 0, 0, 0, "pt"), color = "black"),
    text = element_text(size = 16),
    panel.border = element_blank(),
    panel.grid = element_blank(),
    panel.background = element_blank(),
    axis.line = element_line(color = "black")
  ) +
  labs(color = "", shape = "") +
  scale_color_manual(values = c("Blood +RT" = "aquamarine3", "Blood -RT" = "magenta3",
"Contrived Patient Sample" = "blue")) +
  scale_shape_manual(values = c("Blood +RT" = 17, "Blood -RT" = 16, "Contrived Patient
Sample" = 19)) +
  theme(legend.position = "bottom")


# Create violin plot of clinical range
violin_plot <- ggplot(avg_clin, aes(x = as.factor(conc_nM), y = avg_normResult)) +
  geom_violin(fill = "pink2", color = "plum4", alpha = 0.4) +
  geom_boxplot(width = 0.1, fill = "aquamarine", color = "royalblue", linewidth = 0.8, outlier.shape
= NA) +
  geom_point(color = "black", alpha = 0.8 , position=position_jitter(width=0.2, height=0.1)) +
  theme_minimal() +
  theme(
    axis.text.x = element_text(margin = margin(10, 0, 0, 0, "pt"), color = "black"),
    text = element_text(size = 16),
    panel.border = element_blank(),
    panel.grid = element_blank(),
    panel.background = element_blank(),
    axis.line = element_line(color = "black")) +
  scale_x_discrete(labels = c("36.5", "73.0", "130.0")) +
  xlab("TFV-DP Concentration (nM)") +
  ylab("Normalized Fluorescence") +
  ggtitle("Distribution of Normalized Fluorescence by Contrived TFV-DP Concentration")

# Calculate the stats of the violin plot and put in a table
```

```
boxplot_stats <- avg_clin %>%
  group_by(conc_nM) %>%
  summarize(
    min = min(avg_normResult),
    Q1 = quantile(avg_normResult, 0.25),
    median = median(avg_normResult),
    Q3 = quantile(avg_normResult, 0.75),
    max = max(avg_normResult)
  )
boxplot_stats <- boxplot_stats %>%
  rename(
    `[TFV-DP] (nM)` = conc_nM,
    `Minimum` = min,
    `Lower Quartile` = Q1,
    `Median` = median,
    `Upper Quartile` = Q3,
    `Maximum` = max
  ) %>%
  mutate(
    across(where(is.numeric), ~ signif(., 3))
  )
boxplot_table <- boxplot_stats %>%
  kbl() %>%
  kable_material(c("striped", "hover"))

# Linear regression of average clinical range
set.seed(1)

avg_clin <- avg_clin %>%
  mutate(category = case_when(
    conc_nM == 36.5 ~ "2 doses",
    conc_nM == 73.0 ~ "4 doses",
    conc_nM == 130.0 ~ "7 doses"
  ))

cntrl_pat <- norm_data %>%
  filter(Dilution %in% c("Blood +RT", "Blood -RT")) %>%
  group_by(Subject_ID, Dilution, conc_nM) %>%
  summarize(avg_normResult = mean(norm_fluor, na.rm = TRUE)) %>%
  filter(avg_normResult >= 0)

lr_model <- lm(avg_normResult ~ as.numeric(factor(category, levels = c("2 doses", "4 doses", "7
doses"))),
               data = avg_clin)

# Combine clinical and control data sets
combined_data <- bind_rows(
  avg_clin %>% mutate(source = "Contrived Patient Dose"),
  cntrl_pat %>% mutate(source = "Control")
)
avg_clin$category <- factor(avg_clin$category, levels = c("2 doses", "4 doses", "7 doses"))
```

```
cntrl_pat$Dilution <- factor(cntrl_pat$Dilution, levels = c("Blood +RT", "Blood -RT"))

#Plot linear regression curves
lin_reg_clin <- ggplot() +
  geom_smooth(data = avg_clin %>% filter(category %in% c("2 doses", "4 doses", "7 doses")),
              aes(x = factor(category, levels = c("2 doses", "4 doses", "7 doses")), y =
avg_normResult, group = Subject_ID),
              method = "lm", se = FALSE, color = "slategray", linewidth = 0.5, alpha = 0.5) +
  geom_point(data = avg_clin %>% filter(category %in% c("2 doses", "4 doses", "7 doses")),
             aes(x = factor(category, levels = c("2 doses", "4 doses", "7 doses")), y =
avg_normResult, shape = "Contrived Patient Dose"),
             size = 3, color = "royalblue3", alpha = 0.8,
             position = position_jitter(width = 0.1)) +
  geom_point(data = cntrl_pat %>% filter(Dilution == "Blood +RT"),
             aes(x = factor(Dilution, levels = c("Blood +RT", "Blood -RT")), y = avg_normResult,
shape = "Control"),
             size = 3, color = "aquamarine3", alpha = 0.7,
             position = position_jitter(width = 0.1)) +
  geom_point(data = cntrl_pat %>% filter(Dilution == "Blood -RT"),
             aes(x = factor(Dilution, levels = c("Blood +RT", "Blood -RT")), y = avg_normResult,
shape = "Control"),
             size = 3, color = "magenta3", alpha = 0.7,
             position = position_jitter(width = 0.1)) +
  scale_x_discrete(
    limits = c("Blood +RT", "2 doses", "4 doses", "7 doses", "Blood -RT")
  ) +
  scale_y_continuous(
    expand = c(0, 0),
    limits = c(0, 1),
    breaks = scales::breaks_extended(8)
  ) +
  scale_shape_manual(values = c("Contrived Patient Dose" = 16, "Control" = 17)) +
  labs(
    title = "Validation 1 Linear Regression",
    x = "TFV-DP Adherence (doses/week)",
    y = "Normalized Fluorescence",
    shape = ""
  ) +
  theme_minimal() +
  theme(
    plot.title = element_text(size = 18, face = "bold"),
    axis.text.x = element_text(size = 14, angle = 45, hjust = 1),
    axis.text.y = element_text(size = 14),
    axis.title = element_text(size = 16),
    panel.grid = element_blank(),
    axis.line = element_line(color = "black", linewidth = 0.5),
    legend.position = "bottom",
    legend.text = element_text(size = 14)
  )

# ROC curve creation: subset clinical doses and positive control for benchmark
```

```
roc_data <- norm_data %>%
  filter(conc_nM %in% c(3.65e-08, 7.3e-08, 1.3e-07, 1e-10)) %>%
  group_by(Subject_ID, conc_nM) %>%
  summarize(avg_normResult = mean(norm_fluor, na.rm = TRUE)) %>%
  mutate(conc_nM = case_when(
    conc_nM == 1e-10 ~ 0.0,
    conc_nM == 3.65e-08 ~ 36.5,
    conc_nM == 7.3e-08 ~ 73.0,
    conc_nM == 1.3e-07 ~ 130.0
  ))

# Assign binary identifier to dose concentrations
# Comparing to the positive control
roc_data_base <- roc_data %>%
  mutate(
    dose7 = ifelse(conc_nM == 130.0, 1, ifelse(conc_nM == 0.0, 0,  NA)),
    dose4 = ifelse(conc_nM == 73.0, 1, ifelse(conc_nM == 0.0, 0, NA)),
    dose2 = ifelse(conc_nM == 36.5, 1, ifelse(conc_nM == 0.0, 0, NA))
  )
# Comparing adherence levels
roc_data_mid <- roc_data %>%
  mutate(
    dose27 = ifelse(conc_nM == 130.0, 1, ifelse(conc_nM == 36.5, 0,  NA)),
    dose47 = ifelse(conc_nM == 130.0, 1, ifelse(conc_nM == 73.0, 0, NA)),
    dose24 = ifelse(conc_nM == 73.0, 1, ifelse(conc_nM == 36.5, 0, NA))
  )

# Prepare data for ROC comparison
roc_07 <- roc_data_base %>%
  filter(!is.na(dose7)) %>%
  select(-c(dose4, dose2)) %>%
  mutate(dose7 = factor(dose7, levels = c(0, 1)))
roc_02 <- roc_data_base %>%
  filter(!is.na(dose2)) %>%
  select(-c(dose4, dose7)) %>%
  mutate(dose2 = factor(dose2, levels = c(0, 1)))
roc_04 <- roc_data_base %>%
  filter(!is.na(dose4)) %>%
  select(-c(dose2, dose7)) %>%
  mutate(dose4 = factor(dose4, levels = c(0, 1)))

roc_27 <- roc_data_mid %>%
  filter(!is.na(dose27)) %>%
  select(-c(dose47, dose24)) %>%
  mutate(dose27 = factor(dose27, levels = c(0, 1)))
roc_47 <- roc_data_mid %>%
  filter(!is.na(dose47)) %>%
  select(-c(dose27, dose24)) %>%
  mutate(dose47 = factor(dose47, levels = c(0, 1)))
roc_24 <- roc_data_mid %>%
  filter(!is.na(dose24)) %>%
```

```
  select(-c(dose27, dose47)) %>%
  mutate(dose24 = factor(dose24, levels = c(0, 1)))

# Create predictions
pred_07 <- prediction(predictions = 1 - roc_07$avg_normResult,
                      labels = roc_07$dose7)
pred_02 <- prediction(predictions = 1 - roc_02$avg_normResult,
                      labels = roc_02$dose2)
pred_04 <- prediction(predictions = 1 - roc_04$avg_normResult,
                      labels = roc_04$dose4)

pred_27 <- prediction(predictions = 1 - roc_27$avg_normResult,
                      labels = roc_27$dose27)
pred_47 <- prediction(predictions = 1 - roc_47$avg_normResult,
                      labels = roc_47$dose47)
pred_24 <- prediction(predictions = 1 - roc_24$avg_normResult,
                      labels = roc_24$dose24)

# Calculate ROC performance
perf_07 <- performance(pred_07, "tpr", "fpr")
perf_02 <- performance(pred_02, "tpr", "fpr")
perf_04 <- performance(pred_04, "tpr", "fpr")

perf_27 <- performance(pred_27, "tpr", "fpr")
perf_47 <- performance(pred_47, "tpr", "fpr")
perf_24 <- performance(pred_24, "tpr", "fpr")

# Calculate Area Under Curve
auc_07 <- performance(pred_07, "auc")@y.values[[1]]
auc_02 <- performance(pred_02, "auc")@y.values[[1]]
auc_04 <- performance(pred_04, "auc")@y.values[[1]]

auc_27 <- performance(pred_27, "auc")@y.values[[1]]
auc_47 <- performance(pred_47, "auc")@y.values[[1]]
auc_24 <- performance(pred_24, "auc")@y.values[[1]]

# Plot ROC curves for comparison to control
plot(perf_07, col = "dodgerblue3", main = "Performance Compared Across Clinical Dose Range:
Validation 2",
    xlab = "False Positive Rate", ylab = "True Positive Rate")
plot(perf_02, col = "violetred", add = TRUE)
plot(perf_04, col = "springgreen3", add = TRUE)
abline(a = 0, b = 1, lty = 2, col = "grey")
legend("bottomright",
       legend = c(
         paste("0 vs 7 (AUC =", round(auc_07, 3), ")"),
         paste("0 vs 4 (AUC =", round(auc_04, 3), ")"),
         paste("0 vs 2 (AUC =", round(auc_02, 3), ")")
       ),
       col = c("dodgerblue", "violetred", "springgreen3"),
       lty = 1,
```

```
      title = "Adherence (doses/week)")


# Plot ROC curves for comparison to each other
plot(perf_27, col = "dodgerblue3", main = "Performance Compared Between Adherence Levels:
Validation 2",
    xlab = "False Positive Rate", ylab = "True Positive Rate")
plot(perf_47, col = "violetred", add = TRUE)
plot(perf_24, col = "springgreen3", add = TRUE)
abline(a = 0, b = 1, lty = 2, col = "grey")
legend("bottomright",
       legend = c(
         paste("2 vs 4 (AUC =", round(auc_24, 3), ")"),
         paste("2 vs 7 (AUC =", round(auc_27, 3), ")"),
         paste("4 vs 7 (AUC =", round(auc_47, 3), ")")
       ),
       col = c("dodgerblue", "violetred", "springgreen3"),
       lty = 1,
       title = "Adherence (doses/week)")
```