

Measurement of Antiretrovirals in Blood, Urine and Oral Fluids for
Oral Preexposure Prophylaxis Adherence Monitoring

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Abstract

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The efficacy of daily oral pre-exposure prophylaxis (PrEP) significantly depends on maintaining sufficient drug concentrations from consistent and timely medication adherence. Therefore, oral PrEP adherence monitoring is essential to identify individuals with sub-optimal adherence who may benefit from enhanced support or intervention. Direct pharmacologic measurement of biological samples offers an accurate reflection of real-time drug concentrations, minimizing social desirability/recall bias associated with traditional adherence monitoring methods. Pharmacological measurements of PrEP adherence, like PrEP itself, are not one-size-fits-all. Different metrics measure various dimensions of PrEP adherence, such as dosing recency, dosing frequency, and cumulative dosing time. This dissertation, based on a randomized trial of emtricitabine/tenofovir disoproxil fumarate (FTC/TDF) with directly-observed therapy (DOT), aimed to evaluate the feasibility and performance of measuring tenofovir diphosphate (TFV-DP) in blood; tenofovir (TFV) / emtricitabine (FTC) in oral fluids; and TFV in urine for PrEP adherence monitoring.

We first assessed the associations between PrEP adherence (dosing recency, dosing frequency and cumulative dosing time) and drug concentrations of TFV-DP in dried blood spots (DBS), and estimated the steady-state adherence benchmarks for individuals with varying levels of adherence. Our findings indicated that the steady-state adherence benchmarks for TFV-DP concentrations in DBS were

466, 779, and 1375 fmol/3mm punch for participants taking 2, 4, and 7 doses per week, respectively. These benchmarks were slightly higher but comparable to previously established estimates. TFV-DP levels in DBS were associated cumulative dosing time, with each additional week of cumulative dosing leading to a 158 fmol/3mm punch increase ($p < 0.001$) before achieving steady state.

Next, we tested oral fluids for FTC/TFV to evaluate the relationships between drug concentrations and the three dimensions of PrEP adherence, and assessed the diagnostic performance of FTC/TFV in oral fluids for predicting PrEP non-adherence. Our findings suggested that FTC detectability in oral fluids is associated with dosing recency (odds ratio (OR): 0.21, 95% confidence interval (CI): 0.11-0.38). An FTC threshold of < 7.5 ng/mL accurately classified 90% of recent PrEP non-adherence with one missed dose or more than 24 hours since the last dose. However, TFV was poorly diffused in oral fluids and its detectability was not associated with dosing recency, frequency, or cumulative dosing time.

Lastly, we detected urine TFV concentrations using the next-gen urine TFV lateral flow assay (LFA) with quantified readouts. We assessed the effect of sex on the LFA readouts and calculated sex-specific sensitivities/specificities for recent non-adherence (defined as not having taken a dose within the prior 24 hours). Males exhibited a 33% lower peak color intensity ($p < 0.01$) and a 1.82 unit lower averaged visual score of the LFA test line ($p < 0.01$) compared to females. With the same optimal cutoffs for the LFA test/control line color intensity ratio (> 0.095), we found similar sensitivities (93% vs. 93%) and specificities (99% vs. 94%) among males and females.

Together, the results presented in this dissertation provide valuable insights into the potential applications of measuring antiretrovirals for daily oral PrEP adherence. Our work enhances understanding of the relationships between PrEP adherence and drug concentrations in different specimens. These new findings will help establish adherence benchmarks of drug concentrations and guide the development of future point-of-care (POC) tests for pharmacologic adherence monitoring and interventions.

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Dedication

To my parents and chosen family, whose steady support, love and encouragement have carried me through this journey. Your belief in me has been my greatest source of strength and inspiration:

欣, 蒋秀美, 牛勇, 花妹, June, Cindy, Jun-Yi, Coco.

Chapter 1. Introduction

Daily oral preexposure prophylaxis (PrEP) with tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) significantly reduces the risk of human immunodeficiency virus (HIV) acquisition ¹. Despite the increasing availability of more preexposure prophylaxis (PrEP) regimens such as Long-Acting Cabotegravir (CAB-LA) or Event-Driven (ED) PrEP, daily oral PrEP with TDF/FTC remains the first-line option recommended by the World Health Organization (WHO) for individuals at high risk of HIV ^{2,3}. The efficacy of daily oral PrEP depends heavily on maintaining adequate drug concentrations through consistent and timely medication adherence ^{4,5}. However, non-adherence remains a global challenge, characterized by high discontinuation rates and suboptimal adherence levels ^{4,6}. To achieve the UNAIDS goal of reducing annual HIV new infections to under 200,000 by 2030 ⁷, effective PrEP adherence monitoring and interventions are essential.

PrEP adherence monitoring is crucial for identifying individuals who struggles with adhering to the daily oral regimen. Recognizing non-adherence enables targeted support and interventions to improve adherence. Early detection of non-adherence may also suggest the need to explore alternative PrEP options, such as CAB-LA, which might better meet individual needs. Traditional methods such as diaries, clinic visit attendance, self-report, pill counts and electronic pill bottles/caps, do not measure real-time drug concentrations and often suffer from limited accuracy and reliability due to recall and social desirability bias ⁸⁻¹⁰. Direct pharmacological measurement of drug levels in biological samples offers a more accurate and objective reflection of real-time drug concentrations. However, interpreting these measurements can be challenging in terms of the relationship between PrEP adherence and drug levels.

Pharmacological measurements of PrEP adherence, like PrEP itself, are not one-size-fits-all. Different metrics measure various dimensions of PrEP adherence, such as dosing recency, dosing frequency, and cumulative dosing time. Metrics with a long half-life, such as Tenofovir-diphosphate (TFV-DP) in dried blood spots (DBS), provide gradients of benchmarks to differentiate varying levels of average dosing frequency over the proceeding months ^{11,12}. However, these benchmarks are only applicable to steady-state drug concentrations which require 4-5 half-lives to achieve with repeated dosing at relatively consistent intervals. In contrast, measurements with a short half-life, such as tenofovir (TFV) in

plasma/urine, do not accumulate significantly over time but offer valuable insights into when the last dose was taken (dosing recency) ^{13,14}. This can be particularly informative when steady state is not achieved or maintained due to inconsistent dosing intervals or insufficient cumulative dosing time. This dissertation, based on a randomized controlled trial of FTC/TDF with directly-observed therapy (DOT), aimed to evaluate the feasibility and performance of measuring tenofovir diphosphate (TFV-DP) in DBS; FTC/TFV in oral fluids; and TFV in urine for PrEP adherence monitoring.

Prior studies evaluating TFV-DP in DBS have primarily focused on measuring only one dimension of adherence that reflects the dosing frequency over the preceding 2-3 months. TFV-DP benchmarks in DBS have been established as follows: <350 fmol/punch for less than 2 doses per week, 350-699 fmol/punch for 2-3 doses per week, 700-1249 fmol/punch for 4-6 doses per week, and ≥ 1250 fmol/punch for daily dosing of oral FTC/TDF ¹¹. Nevertheless, these steady state benchmarks of TFV-DP in DBS for weekly PrEP dosing frequency were established based on statistical simulations using untimed samples conveniently collected from populations where Asians and/or females were underrepresented ^{11,15}. Data are also limited on whether TFV-DP concentrations in DBS are associated with dosing recency or cumulative dosing time. In **Chapter 2**, we first assessed the associations between PrEP adherence (dosing recency, dosing frequency and cumulative dosing time) and drug concentrations of TFV-DP in DBS, and estimated the steady-state adherence benchmarks among an Asian population with varying levels of adherence.

Oral fluids collection is noninvasive, easy-to-collect and requires minimal privacy. These characteristics make oral fluid a preferable biological sample for PrEP adherence monitoring, especially for high-risk populations where rapid HIV self-tests based on oral fluids are already highly accepted and recommended as an innovative alternative strategy to increase access to and effective use of PrEP ³. Yet, there is limited research on the detectability of FTC/TFV in oral fluids and whether FTC/TFV levels in oral fluids can accurately predict PrEP non-adherence. To address these gaps, we evaluated the relationships between drug concentrations in oral fluids and three dimensions of PrEP adherence (dosing recency, cumulative dosing time, and dosing frequency) in **Chapter 3**, and assessed the diagnostic performance of FTC/TFV in oral fluids for predicting PrEP non-adherence.

Point-of-care (POC) testing for urine TFV may allow for better monitoring and interventions to enhance PrEP adherence. One urine tenofovir lateral flow assay (LFA) was developed based on a TFV threshold at 1500 ng/mL to have high specificity for detecting PrEP non-adherence (accurately classified 98% of users who took a dose within 24 hours as adherent) ¹⁶. However, females may require higher adherence to achieve equivalent therapeutic drug levels compared to males, suggesting that sex-specific cutoffs of urine TFV LFAs may be necessary ^{11,12,17}. Further efforts are also needed to improve the sensitivity of the urine LFA in detecting non-adherence during the 24 to 96-hour window since the last dose. Utilizing the next-gen urine TFV LFA ¹³, which does not have a predetermined threshold or binary interpretation, the primary goal of our **Chapter 4** was to determine if the next-gen LFA readouts would differ by sex and to evaluate its diagnostic performance for detecting recent PrEP non-adherence.

To develop effective and accurate PrEP adherence monitoring tools for improved PrEP adherence and outcomes, it is crucial to understand the relationships between detected drug concentrations and the three dimensions of PrEP adherence. We explored those associations in-depth through analyses of the potential effects on PrEP drug concentrations from dosing recency, dosing frequency and cumulative dosing time. Our analyses also comprehensively evaluated the performance of different benchmarks of pharmacological measurements for identifying recent PrEP non-adherence. These findings provide valuable insights into the potential applications of measuring antiretrovirals for daily oral PrEP adherence and will help guide the development of future tests for better PrEP adherence monitoring.

Chapter 2: Tenofovir-Diphosphate in Dried Blood Spots versus Tenofovir in Urine/Plasma for Oral Preexposure Prophylaxis Adherence Monitoring

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Running title: TFV-DP vs TFV for PrEP Adherence Monitoring

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Key Points: Urine and plasma TFV levels were modestly correlated with TFV-DP levels in DBS. The latter metric provides valuable insight into both dosing recency and cumulative doses from variable

adherence patterns. Observed benchmark TFV-DP concentrations were slightly higher than prior estimates.

2.1 Abstract

Background: Tenofovir-diphosphate (TFV-DP) measured in dried blood spots (DBS) and tenofovir (TFV) measured in urine/plasma have been used to measure tenofovir based oral pre-exposure prophylaxis (PrEP) adherence. However, there is limited data comparing these three metrics and their appropriate use for PrEP adherence monitoring.

METHODS: We collected DBS, urine, and plasma samples from HIV-negative adults randomized to a low (two doses/week), moderate (four doses/week), or perfect (seven doses/week) adherence group (via directly-observed therapy) of tenofovir diphosphate/emtricitabine (TDF/FTC) for six weeks, followed by a four-week washout phase. Drug concentrations were measured using liquid chromatography tandem mass spectrometry. Linear mixed-effects modeling was used to examine associations between drug concentrations and dosing time.

RESULTS: Among 28 participants, the median age was 33 years and 12 (43%) were female. At steady state, 25th percentile TFV-DP concentrations were 466, 779 and 1375 fmol/3mm punch in the low, moderate, and perfect adherence group respectively. Correlation was stronger between quantifiable TFV-DP and plasma TFV ($r=0.65$, $p<0.01$) than between TFV-DP and urine TFV ($r=0.50$, $p<0.01$). Among all participants, each additional week of cumulative dosing on average led to a mean increase of 158 fmol/3mm punch ($p<0.001$) in TFV-DP during the dosing phase. Each additional day after the last dose was associated with 43 fmol/3mm punch lower TFV-DP ($p=0.07$).

CONCLUSION: TFV-DP levels in DBS provide valuable insight into both dosing recency and cumulative doses from variable adherence patterns. Our observed benchmark TFV-DP concentrations were slightly higher than prior predicted estimates based on convenience samples.

2.2 Background

Daily preexposure prophylaxis (PrEP) of oral tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) reduces the risk of human immunodeficiency virus (HIV) acquisition and is recommended by the World Health Organization (WHO) for high-risk individuals ^{1,18–20}. PrEP can also be taken as an event-driven (ED) regimen for men who have sex with men (MSM), offering more flexibility and convenience for people with infrequent or predictable risks ²¹. The 2016 United Nations' Declaration on Ending AIDS by 2030 included a commitment to ensure three million eligible people accessed PrEP. PrEP use is projected to rise at an increasing speed as more countries continue to adopt and implement PrEP policies ¹.

The efficacy of oral PrEP greatly depends on maintaining sufficient drug concentrations from consistent and timely medication adherence ^{22,23}. Therefore, oral PrEP adherence monitoring is critical and can help identify people with sub-optimal adherence who might benefit from enhanced support or intervention. Accurate measurement of oral PrEP adherence is challenging. Methods such as diaries, clinic visit attendance, self-report, pill counts and electronic pill bottles/caps do not measure real-time drug concentrations and have only demonstrated limited accuracy and reliability ^{24–26}. The recent approval of the long acting injectable cabotegravir (CAB) for HIV prevention offers a potentially easier alternative for people with demonstrated poor adherence of oral PrEP. Yet, tenofovir-based oral PrEP regimens remain the recommended HIV prevention approach for high risk populations, particularly in resource limited areas²⁷.

Direct pharmacological measurement of drug levels in biological samples is more objective but often difficult to interpret regarding the relationship between efficacy, adherence and drug concentrations. Tenofovir-diphosphate (TFV-DP) accumulates appreciably in red blood cells with repeated daily doses and has a long half-life of 17 days in this cellular compartment ²⁸. Measurement of TFV-DP in dried blood spots (DBS) is useful to assess cumulative patterns of adherence over longer time periods. High concentrations of TFV-DP measured in DBS (>700 fmol/punch) were found associated with substantial reduction in HIV infection risks ²⁹. These 'benchmarks' of TFV-DP levels in DBS were simulated using

statistical extrapolation based on samples conveniently collected without regard to time since last dose taken and were mostly from populations where Asians and/or females were underrepresented ^{11,28}.

Additional data defining adherence benchmarks for different cumulative dosing patterns across different populations, coupled with timed sample collection, would be reassuring. Urine and plasma tenofovir (TFV) concentrations are short-term adherence monitoring metrics due to their short half-life (~15 hours) ^{30–32}. Short-term metrics may provide valuable information about whether TDF was ingested recently, which could be potentially helpful to understand ED PrEP adherence when cumulative dosing patterns are less informative than the recency of dosing ³³.

Prior studies evaluating urine/plasma TFV or DBS TFV-DP have primarily focused on only one aspect of “dosing time” in PrEP adherence monitoring: either dosing recency or total cumulative time of dosing. Data are still limited on how TFV levels in urine, plasma and TFV-DP levels in DBS correlate with each other. Our objectives were to assess the associations between different dosing time (dosing recency and cumulative dosing time) and drug concentrations of TFV-DP in DBS and TFV in urine/plasma among an Asian population, and to estimate the correlations between the three metrics.

2.3 Materials & Methods

Study Design

The TARGET (Tenofovir Adherence to Rapidly Guide and Evaluate PrEP and HIV Therapy) study (ClinicalTrials.gov #NCT0301260) was a three-arm randomized, open-label pharmacokinetic clinical trial among healthy adults enrolled in Thailand with controlled levels of PrEP adherence. The study protocol has been published previously ³⁴. Eligible volunteers without HIV or Hepatitis B infection, aged from 18-49 years old, were randomized to receive 2 doses/week (Monday and Thursday), 4 doses/week (Monday, Wednesday, Friday and Saturday) or 7 doses/week (once daily) of TDF 300 mg/ emtricitabine (TFC) 200 mg (Truvada, Gilead Sciences) for 6 weeks, representing low, moderate and perfect PrEP adherence respectively. During the 6-week dosing phase, we performed directly observed therapy (DOT) for each

study drug intake. All participants ingested their last dose at the beginning of week 7 and were followed for another 4 weeks during the drug washout phase.

Collection of Urine, Plasma and DBS Samples

We collected pre-dose time-matched spot-urine, plasma and DBS specimens before the second dose in week 1/3/5 and before the last dose in week 7. During the washout phase, blood and urine samples were collected on the Monday morning of week 8/9/10/11. We also conducted weekly liver and renal function assessment for all participants at week 1/3/5/7/10.

Measurement of Drug Concentrations

Urine, plasma and DBS samples were stored at -70°C until analysis. Tenofovir concentrations in urine and plasma, as well as TFV-DP in DBS, were measured using validated liquid chromatography tandem mass spectrometry (LC-MS/MS) assays over the concentration ranges of 50-50,000 ng/ml in urine, 3-2,500 ng/ml in plasma. An indirect LC-MS/MS assay to quantify intracellular concentration of TFV-DP in red blood cells was validated in Thailand based on a previously published assay^{11,35}. Briefly, whole blood was spotted on Whatman Protein Saver 903 cards (50 µL/spot), air-dried overnight at room temperature and then stored at -70°C until analysis. For analysis, a 3 mm whole punch was taken from a single DBS, red blood cells were lysed from the paper, and then TFV-DP was separated from partially dephosphorylated TFV-analytes by solid-phase extraction (SPE). TFV-DP was subsequently dephosphorylated using alkaline phosphatase. Finally, tenofovir (TFV) was separated using SPE, the eluted sample dried, reconstituted in mobile phase and injected into the LC-MS/MS system. The range of the standard curve was linear over the range 200-3,000 fmol/3mm punch.

Statistical Analyses

We calculated Spearman correlation coefficients (γ) to estimate the correlations between time-matched pre-dose TFV-DP levels in DBS and TFV concentrations in urine/plasma, during the dosing phase. Log transformation (natural base) was used to account for the different scales, ranges and units between three measurements. Concentrations in DBS, plasma and urine below the lower limit of quantification

(LLOQ) were imputed with the half of the LLOQ values for each metric, namely 100 fmol/3mm punch for DBS, 1.5 ng/mL for plasma and 25 ng/mL for urine. Sensitivity analysis was also performed by restricting the analysis among samples above the LLOQ. We used Linear Mixed-Effects Modeling (LMMs) to evaluate the effect of cumulative dosing time and dosing recency on drug concentrations detected within each clinical metric. To assess the associations between different metrics and dosing time for participants who were still on PrEP, we restricted our LMMs among samples collected during the dosing phase only. Cumulative dosing time was included in models as the number of weeks into study participation after the baseline visit. Dosing recency was represented with the number of days since last dose for each participant. All statistical analyses were performed using R (R Core Team, 2014).

2.4 Results

Twenty-eight participants (43% female) with 224 DBS samples were included in the analyses. Half of the samples were collected from four visits during the dosing phase and the other half from four visits during the washout phase. Overall, 212 of the DBS samples were time-matched with pre-dose trough plasma and spot urine samples. The median age of participants was 33 years (interquartile range [IQR], 28-40 years) (**Table 1**) and the moderate adherence group had fewer female participants (2/10 [20.0%]) compared with the low (4/9 [44.4%]) or perfect (6/9 [66.7%]) adherence groups. Participants in the moderate adherence group also had a slightly higher median eGFR of 124 (IQR, 97-149) than the low and perfect adherence groups. Other clinical/hematologic/lab measurements were similar between three adherence groups.

The median trough TFV-DP levels in pre-dose DBS samples during the dosing phase were 329 (IQR, 176-555) fmol/3mm punch in the low adherence group, 622 (IQR, 332-875) fmol/3mm punch in the moderate adherence group, 914 (IQR, 317-1530) fmol/3mm punch in the perfect adherence group (**Table 2**). Female participants showed about 40 fmol/3mm punch higher mean, but similar median, TFV-DP concentrations than male participants during the dosing phase. (**Table 3**). When we restricted our analysis among TFV-DP concentrations above the LLOQ (30 samples excluded with 15 from each sex),

median TFV-DP was 640 (IQR, 395-1047) and 584 (IQR, 438-902) fmol/3mm punch among females and males respectively for the entire study phase (dosing + washout). **Figure 1** shows the observed TFV-DP concentrations accumulated during the dosing phase until week 7 when the last dose of each regimen was administered. The 25th quantile of TFV-DP levels in DBS samples before the last dose at the start of week 7 were 466, 779 and 1375 fmol/3mm punch for the low, moderate and perfect adherence group, respectively. Within the same adherence group, participants with higher TFV-DP concentrations appeared to maintain higher drug levels throughout the study course.

Compared to the low adherence group (2 doses/week), moderate (4 doses/week) adherence was on average associated with an estimated 250 fmol/3mm punch higher TFV-DP level during the dosing phase (Standard Error (SE)=108 p=0.02); and the corresponding estimate was 622 fmol/3mm punch higher for the perfect (7 doses/week) group (SE=144, p<0.01). Each additional cumulative week of TDF dosing was on average associated with 158 fmol/3mm punch (SE=12, p<0.001) higher TFV-DP concentration for participants with the same weekly PrEP regimen (in the same adherence group). In another mixed model examining the association between “days since last dose” (dosing regency) and TFV-DP levels in DBS during the dosing phase, we found each additional day since last dosing was on average associated with 43 fmol/3mm punch lower TFV-DP in DBS (SE=24, p=0.07) for participants with the same length of cumulative dosing.

Separate mixed models to evaluate the effect of dosing time (dosing recency & cumulative dosing time) on urine or plasma TFV concentrations showed that each additional day since last dosing on average contributed to 2435 (SE=456, p<0.001) and 9.01 (SE=0.98, p<0.001) ng/mL lower TFV levels in urine and plasma, respectively. Each additional week of cumulative dosing time was also associated with an increase of 192(SE=129, p=0.14) and 0.95 (SE=0.36, p=0.013) ng/mL in urine and plasms TFV during the dosing phase. Correlation was stronger between quantifiable TFV-DP and plasma TFV during the dosing phase (n=79, Spearman r=0.65, p<0.01, 95% CI (0.51-0.76)) than that between TFV-DP and urine TFV (n=85, Spearman r=0.50, p<0.01, 95%CI (0.30-0.64)). The difference between two correlation coefficients was significant (p<0.01) with a bootstrapped 95% CI at 0.08-0.32.

2.5 Discussion

In a study of directly-observed administration of TDF/FTC among HIV-negative participants in Thailand, we found the steady state adherence benchmark for TFV-DP concentrations in DBS were 466, 779, and 1375 fmol/3mm punch for participants taking 2, 4 and 7 doses per week, respectively. TFV-DP levels in DBS were associated with both dosing recency and cumulative dosing time. Urine and plasma metrics of TFV (which both measure recent adherence) were modestly correlated with TFV-DP levels in DBS.

Participants with perfect (7 doses/week) and moderate (4 doses/week) had on average higher TFV-DP concentrations than those with low (2 doses/week) adherence, which is consistent with former studies that demonstrated dose-proportionality of DBS TFV-DP measurement ¹¹. Drug concentrations have been increasingly used to assess PrEP or ART adherence and corresponding outcomes ^{36–38}. However, most prior studies focused on only one aspect of adherence in relation to dosing time: either dosing recency or cumulative time. Also, biological samples tested in prior studies were usually collected at “convenient” times without regard to time since last dosing. Our study utilized directly observed dosing and pre-dose samples to assess the effect of both cumulative weeks of dosing and days since last dose on the concentrations of TFV-DP in DBS and TFV in urine/plasma among an Asian population.

Based on statistical models, previous studies estimated clinical meaningful TFV-DP thresholds of 350, 700, and 1,250 fmol/punch in DBS at steady state (week 8 to 12) for 2, 4, and 7 doses/week ^{11,28}. These benchmarks showed strong correlations with PrEP effectiveness among men who have sex with men (MSM) and have been used to assess gradients of adherence ^{22,39–41}. TFV-DP levels of > 700 fmol/punch has been considered equivalent to oral TDF-FTC dosing of more than 4 doses/week and correlated with a 100% reduction in HIV risk ²⁹. However, this benchmark was estimated with the rounded 25th percentiles of TFV-DP concentrations of participants taking over 4 doses/week. In other words, there were also at least 25% of participants taking over 4 doses/week but whom had TFV-DP levels $\leq$ 700 fmol/punch. For

such individuals, the clinical implication of TFV-DP benchmarks as proxies for sufficient weekly doses offering good enough protection is yet unclear.

Our benchmark TFV-DP concentrations were slightly higher but comparable to prior predicted estimates. Several reasons might have contributed to the observed differences. Firstly, the participants in the prior study were on 33% vs 67% vs 100% weekly dosing regimens. Predictions were made for dosing rates at 2 and 4 doses/week on average based on a mixed model with $\log(\text{TFV-DP})$ levels as a smooth, nonlinear function of time and weekly doses, while our benchmarks were directly observed from participants with 2, 4 and 7 doses/week. In addition, all DBS samples in the prior study were collected every 2 weeks during the 12-week dosing phase conveniently without regard to time since last dose. In the current analysis, trough TFV-DP concentrations were tested with pre-dose DBS samples collected before the second dose in week 1/3/5 and before the last dose in week 7. Secondly, our study population was entirely Asian from Thailand whereas the prior study included no Asian participants, and race was recently found to be associated with different TFV-DP concentrations, possibly driven by pharmacogenomics^{11,42}. Finally, our study used the drug concentrations at week 7 as steady state whereas the prior benchmarks were calculated with drug concentrations between week 8 to 12 (steady state). There was concern that the DBS TFV-DP levels at week 7 in our study had not yet reached steady state levels; however, the median TFV-DP concentration from daily dosing at week 7 in our study was 1528 fmol/3mm punch, which was comparable to median concentrations reported at week 8 (1493 fmol/punch) and during the entire steady state (1534 fmol/punch) in the prior study. Consistent with previous findings, our study also showed higher quantifiable median TFV-DP concentrations in females than males¹¹. More number of doses received per week among females than males might have contributed to the observed sex differences. In our study, a higher percentage of females were randomly assigned to the perfect adherence group (7 doses/week) than males. Females on average received 4.83 doses per week during the dosing phase while males only received 3.94 doses per week. Whether there would be higher drug concentrations among females than males with the same number of weekly doses warrants further research.

Pharmacologic measurements for PrEP adherence monitoring are generally grouped into short-term and long-term metrics based on their half-lives in different biological specimens. Though both set of metrics have demonstrated strong associations with PrEP efficacy/effectiveness, most prior studies have only assessed short-term metrics for “dosing recency” or long-term metrics for “cumulative dosing”. For instance, plasma TFV concentrations can inform drug adherence within the preceding 2 days up to a week ³⁰. Plasma TFV concentrations of at least 35.5, 4.2, 2.5, and 0.5 ng/ml also indicated adherence consistent with at least seven, four, two, and one dose in the prior week, respectively from the HPTN 066 study ⁴³. With similar half-life to plasma TFV, urine TFV concentrations are much higher and more variable. Urine TFV levels ≥ 1500 ng/mL have accurately classified 98% of participants who took their last dose within the past 24 hours ⁴⁴ and are gaining in interest as point-of-care urine-based tests become available. Although short-term metrics can be susceptible to the “white-coat” effect where non-adherent participants may appear to be adherent by ingesting a dose right before drug level testing, a recent analysis examining emtricitabine triphosphate (FTC-TP, short-term metric) and TFV-DP levels in study participants who knew their blood was being monitored for adherence testing did not show that effect⁴⁵. Long-term metrics such as TFV-DP are therefore increasingly preferred in clinical trials to evaluate established adherence patterns over a longer period of time. The resulting drug concentrations in DBS and peripheral blood mononuclear cells (PBMCs) ²² and hair samples^{46,47} provided important benchmarks for interpreting the adherence-drug concentration-efficacy relationship. For example, a TFV-DP concentration of 700 fmol/punch in DBS and a TFV level of 0.023 ng/mg in hair was found commensurate with a median adherence of 4.1 doses/week which is highly protective against HIV infection among MSM ⁴⁶.

Since the endorsement on ED-PrEP “2-1-1” regimen from The International Antiviral Society-USA and the WHO as an alternative to the daily regimen, there is growing acceptance of ED-PrEP among MSM⁴⁸. However, current adherence benchmarks for short or long-term metrics have mostly been based on established dosing patterns (one dose per day, 1-7 doses/week), their interpretations in the setting of ED-PrEP is unclear. Because ED-PrEP is normally taken at irregular intervals due to the sex-act dependent nature of ED PrEP, drug concentrations are very much likely not going to reach steady state levels as the daily regimen when tested for adherence. A recent study of ED-PrEP adherence in Amsterdam reported a

median TFV-DP concentration of 591 fmol/punch, suggesting half of the participants on ED PrEP that offered good protection were below the 700 fmol/punch threshold ⁴⁹. Hence, a metric to measure both dosing recency and cumulative dosing information may be critical when assessing ED-PrEP adherence. More data is urgently needed to assess the association between cumulative dosing time/time since last dose and the concentrations of both short/long-term metrics for PrEP adherence monitoring.

Contrary to expectations that TFV-DP in DBS, a long-term metric, can only provide information about cumulative dosing, our results showed that each additional day since last dose taken (dosing recency) was also associated with lower TFV-DP in DBS. Though the finding was not statistically significant, the association between TFV-DP and dosing recency was further supported by its moderate to strong correlations with urine and plasma TFV. Both urine and plasma TFV were associated with dosing recency but not necessarily cumulative dosing according to our data. The higher correlation coefficient between DBS TFV-DP & plasma TFV vs that between DBS TFV-DP & urine TFV may partly be explained by the much higher concentration and variability of TFV in urine than in plasma. Of note, the associations between drug concentrations and dosing time (either dosing recency or cumulative doses) in our study should be limited within the dosing phase only. Future studies with time since last dosing information available are needed to elucidate the relationship between long-term metrics such as TFV-DP in DBS and dosing recency.

In conclusion, urine/plasma TFV levels modestly correlate with TFV-DP concentrations in DBS. The latter metric provides valuable insight into both dosing recency and cumulative doses from variable adherence patterns. Our study provides new information on defining benchmarks of adherence from a direct-observed therapy study. Benchmarks of TFV-DP concentrations observed from our study will help guide PrEP adherence monitoring and interventions.

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2.6 Tables and Figures:

Figure 2.6.1. TFV-DP Concentrations in DBS (y axis) by Adherence Groups (Low vs. Moderate vs. Perfect) over Weeks into the Study for the Dosing and Washout Phase (Separated by the Dashed Vertical Line)

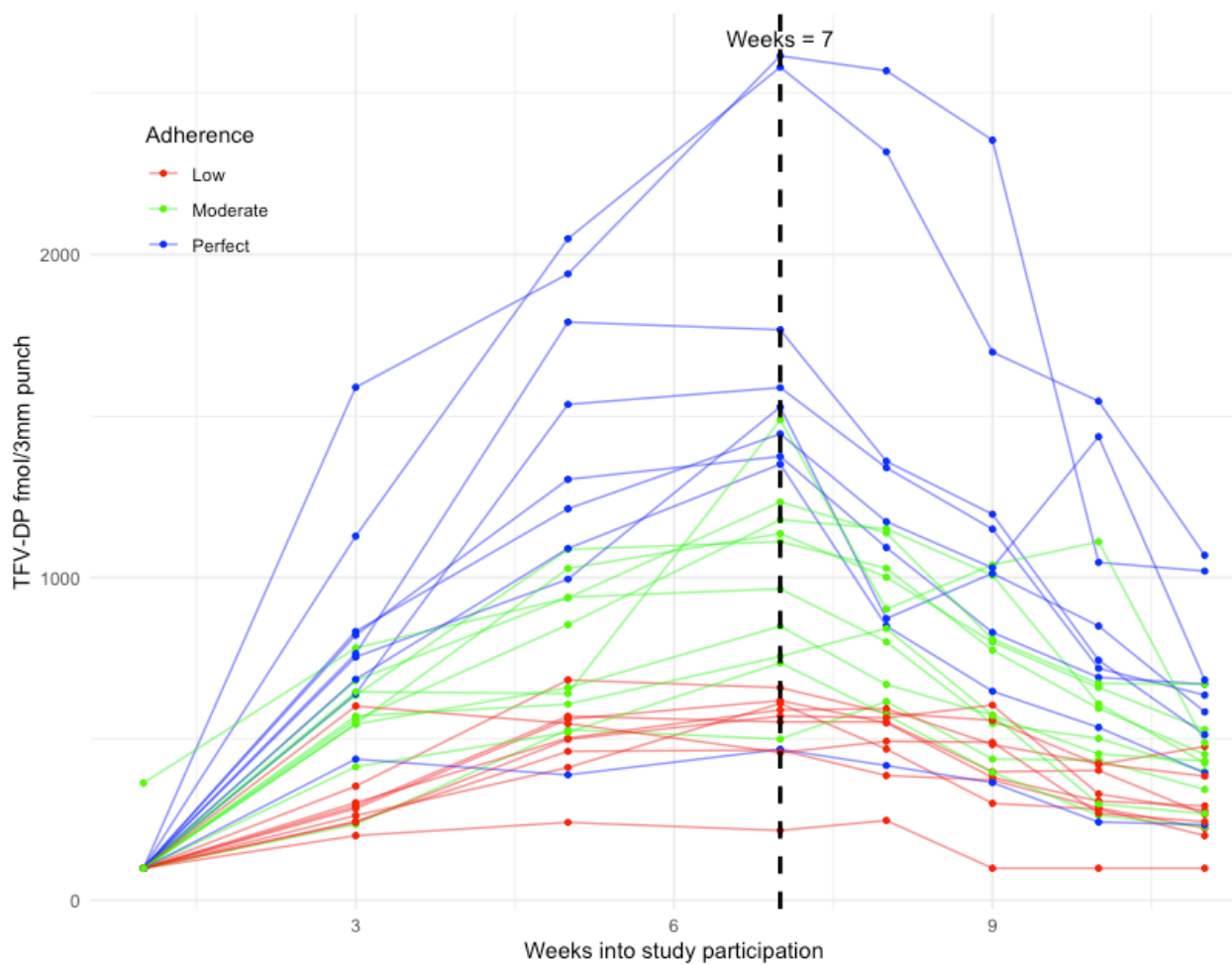


Table 2.6.1*. Baseline Characteristics by Adherence Groups

	Adherence Group [#]		
	Low (N=9)	Moderate (N=10)	Perfect (N=9)
Age, years	38 (21-45)	32 (20-49)	34 (25-44)
Body mass index, kg/m ²	23.1 (20.2-28.4)	24.0 (22.1-25.1)	20.2 (19.1-24.3)
Female, N. (%)	4 (44.4%)	2 (20%)	6 (66.7%)
Hemoglobin, g/dL	14.7 (9.9-15.4)	14.5(12.6-16.2)	12.4(10.1-15.8)
Hematocrit, %	43%(33%-47%)	44%(39%-48%)	38%(32%-48%)
eGFR [^] , ml/min	108(60-126)	124(97-149)	91(83-116)

*Data are presented as median (Interquartile Range) unless otherwise specified.

[#]TDF adherence groups: perfect: 7 doses/week; moderate: 4 doses/week; low: 2 doses/week.

[^]Estimated with the Cockcroft-Gault equation.

Table 2.6.2. TFV-DP Concentrations (fmol/3mm punch) by Adherence Groups

Dosing Phase	N*	Mean	SD [#]	Median	25 th Quantile	75 th Quantile
Low Adherence	36	359	202	329	176	555
Moderate Adherence	40	616	378	622	332	875
Perfect Adherence	36	988	734	914	317	1530
Week 7 (after the last dose)	N*	Mean	SD [#]	Median	25 th Quantile	75 th Quantile
Low Adherence	9	527	134	571	466	610
Moderate Adherence	10	995	290	1038	779	1168
Perfect Adherence	9	1635	655	1528	1375	1767
Washout Phase	N*	Mean	SD [#]	Median	25 th Quantile	75 th Quantile
Low Adherence	36	373	143	383	276	485
Moderate Adherence	40	638	259	585	437	803
Perfect Adherence	36	997	562	862	645	1179

*N: Total number of DBS samples.

[#]SD: Standard Deviation

Table 2.6.3: TFV-DP Levels in DBS by Sex

Dosing Phase	N*	Mean	SD [#]	Median	25 th Quantile	75 th Quantile
Male	64	636	521	557	241	800
Female	48	675	582	554	176	1088
Washout Phase	N*	Mean	SD [#]	Median	25 th Quantile	75 th Quantile
Male	64	667	390	560	419	844
Female	48	670	503	566	351	835

*N: Total number of DBS samples.

[#]SD: Standard Deviation

Chapter 3: Oral Fluid Concentrations of Tenofovir and Emtricitabine for Monitoring HIV Preexposure Prophylaxis Adherence: Findings from a Randomized Trial with Directly Observed Therapy (TARGET Study)

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3.1 Abstract

Background. PrEP adherence monitoring is crucial for the early detection of non-adherence and timely initiation of support interventions. This study evaluated the feasibility of detecting Emtricitabine/Tenofovir (FTC/TFV) in oral fluids to monitor daily oral PrEP adherence.

Methods. We collected oral fluids from HIV-negative adults randomized to 2 or 4 or 7 doses/week of oral PrEP with Emtricitabine/Tenofovir Disoproxil Fumarate (FTC/TDF) for directly observed therapy. Drug concentrations were measured using liquid chromatography tandem mass spectrometry. Generalized Estimating Equations were used to examine the associations between FTC/TFV detectability and the three dimensions of PrEP adherence: dosing recency, cumulative dosing time and dosing frequency. We also assessed the diagnostic accuracy of various benchmarks of drug concentrations in oral fluids for predicting daily oral PrEP non-adherence

Results. Among 165 oral fluids, 15 (9.0%) had detectable TFV, and 88 (53.3%) had detectable FTC. Compared to oral fluids collected within 24 hours of the last dose, those collected between 24 and 48 hours post-dose had significantly lower odds of detectable FTC (odds ratio: 0.21, 95% CI: 0.11-0.38). An FTC threshold of <7.5 ng/mL achieved a sensitivity of 90% (95% CI: 84%-94%) and a specificity of 65% (95% CI: 57%-74%) for identifying PrEP non-adherence (defined as having a time since last dose >24 hours).

Conclusions. FTC levels in oral fluids are a highly sensitive pharmacologic marker for identifying recent PrEP non-adherence as early as having a single missed dose. However, TFV is poorly diffused in oral fluids and is not informative for PrEP adherence monitoring.

3.2 Background

Daily oral pre-exposure prophylaxis (PrEP) demonstrated high efficacy against HIV-1 acquisition with more than 2.6 million users worldwide ⁷. The United Nations' political declaration on Ending AIDS by 2030 established an ambitious yet achievable target for national governments to ensure PrEP access for 10 million people by 2025 ⁵⁰. The fight against HIV has seen a significant progress with the growing availability and diversification of PrEP medications/regimens such as Long-Acting Cabotegravir (CAB-LA) or Event-Driven (ED) PrEP. These advancements now offer a wider range of effective options, catering to different individual needs/preferences of people at risk for HIV infections. While daily oral PrEP with Emtricitabine/Tenofovir Disoproxil Fumarate (FTC/TDF) remains the first-line option recommended by the World Health Organization (WHO) ⁵¹, its effectiveness is highly dependent on consistent and timely medication adherence ^{4,5}. Unfortunately, non-adherence continues to be a global challenge with studies showing high discontinuation rate (41% within six months) ⁶ and suboptimal adherence (only 29% maintaining ideal daily dosing) ⁴.

PrEP adherence monitoring is a critical tool for identifying individuals who struggle with the daily oral regimen. This information allows for targeted support interventions, such as electronic reminders or pharmacy-based PrEP services, to enhance adherence. Detecting non-adherence may also highlight the need to explore alternative PrEP options, such as CAB-LA, which may better meet users' need and improve adherence. Compared to traditional methods for adherence monitoring such as self-reporting, pill counts or pharmacy refills, direct pharmacologic measurement of biological samples provides a more accurate reflection of real-time drug concentrations and is less susceptible to social desirability or recall bias ⁸⁻¹⁰. Pharmacologic metrics of adherence not only can guide interventions but also predict key HIV prevention/treatment clinical outcomes, including future seroconversion events and virologic suppression, and has become critically important for understanding variations observed in trial efficacy ⁵²⁻⁵⁵.

Pharmacologic metrics of PrEP drug metabolites provide different insights into adherence based on their half-lives in various biological samples. Tenofovir-diphosphate (TFV-DP) in dried blood spots (DBS) has a long half-life (approximately 17-21 days) and is effective for assessing cumulative adherence (dosing frequency) over the preceding 6-12 weeks after steady state is achieved. TFV-DP thresholds in

DBS have been established as follows: <350 fmol/punch for less than 2 doses per week, 350-699 fmol/punch for 2-3 doses per week, 700-1249 fmol/punch for 4-6 doses per week, and ≥ 1250 fmol/punch for daily dosing of oral FTC/TDF ¹¹. However, this relationship is only valid for steady-state drug concentrations which require 4-5 half-lives to achieve with repeated dosing at relatively consistent intervals. In contrast, plasma and urine tenofovir (TFV) do not accumulate significantly due to their short half-life (~15 hours) but provide valuable information about when the last PrEP dose was taken (dosing recency) ^{12,13}. This can help understand adherence patterns before achieving steady state or when steady state is not maintained due to inconsistent dosing intervals such as sex-act dependent ED PrEP.

Compared to blood and urine, oral fluid is less invasive, easier-to-collect and requires minimal privacy. These characteristics make oral fluid a preferable biological sample, especially for high-risk populations where rapid HIV self-tests based on oral fluids are already highly accepted and recommended as an innovative alternative strategy to increase access to and effective use of PrEP ³. However, there is limited research on the detectability of FTC/TFV in oral fluids ⁵⁶⁻⁵⁸. The relationship between PrEP adherence and drug concentrations in oral fluids also remains underexplored, and it is unclear whether or at what thresholds FTC/TFV levels in oral fluids can accurately predict PrEP non-adherence. To address these gaps, we tested oral fluids collected from a randomized trial of FTC/TDF with directly-observed therapy (DOT). Our objectives were to (1) evaluate the relationships between drug concentrations in oral fluids and three dimensions of PrEP adherence (dosing recency, cumulative dosing time, and dosing frequency), (2) establish benchmarks and assess the diagnostic performance of FTC/TFV in oral fluids for predicting PrEP non-adherence, and (3) estimate the correlations between FTC/TFV concentrations in oral fluids and plasma.

3.3 Methods

Study Design

Our TARGET study, an open-label randomized pharmacokinetic (PK) trial, prospectively recruited healthy adults aged 18-49 years in Thailand for controlled levels of PrEP adherence. Details of the study protocol have been described elsewhere ³⁴. In brief, to represent low, moderate, and perfect PrEP adherence respectively, eligible participants were randomized (1:1:1) to receive 2 doses/week (Monday,

Thursday), 4 doses/week (Monday, Wednesday, Friday and Saturday), or 7 doses/week (once daily) of TDF 300 mg/ FTC 200 mg (Truvada, Gilead Sciences) for 6 weeks. We performed DOT to ensure medication adherence and recorded the time for each drug intake during the 6-week dosing phase (with the last dose at the start of week 7). All participants were followed for another 4 weeks (the washout phase) after their last dose on the Monday of week 7. Participants were excluded if they were pregnant, with other PrEP contradictions, or had been using PrEP. All participants were literate and provided written informed consent before study participation.

Sample Collections

Time-matched plasma and oral fluids samples were collected from participants before the next scheduled dose under DOT on the Mondays of week 1/3/5. During the washout phase, four collections were performed 0/1/4/12 hours after the last dose as well as on the mornings of Wednesday, Thursday and Friday of week 7. Of note, one additional collection of oral fluids was scheduled 24 hours after the last dose at the start of week 7. Oral fluids were collected using the ACCU.SAL™ (*Oasis Diagnostics*) oral fluid collection device that incorporated a sample volume adequacy indicator. The indicator changes color once sufficient oral fluids (1 mL) has been collected. Collected oral fluids were then transferred into the transport tube containing 2 mL of extraction buffer, resulting in a 1:3 dilution. Plasma samples were collected from a single venous blood draw (5 ml) after centrifugation.

Measurement of Drug Concentrations

Oral fluids and plasma samples were stored at -70°C until testing for PrEP drug levels. FTC and TFV concentrations in oral fluids and plasma were measured using validated high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) (Sciex, Foster City, California, USA; Shimadzu Scientific Instruments, Durham, North Carolina, USA) based on previously published methodology⁵⁹. Drug concentrations were estimated from a standard curve with a range of 0.5-2000 ng/mL. The limit of quantification (LOQ) for both TFV and FTC was 10 ng/mL in oral fluids and plasma. The limit of detection (LOD) was 10 ng/mL for both drug molecules in plasma, and 5 ng/mL in oral fluids. To minimize bias, all laboratory testing (except for the pilot testing) was conducted in a blinded fashion regarding the dosing time of each sample. In our pilot testing of 20 randomly selected samples, TFV and FTC was

undetectable (below corresponding LODs) in oral fluids with a time since last dose greater than 48 hours. Consequently, only the subset of matched samples with a time since last dose of 48 hours or less was selected for further drug level testing.

Statistical Analyses

Stratified by randomization arms, we calculated descriptive statistics (mean, median, interquartile range (IQR), standard deviation (SD)) for the baseline participant characteristics. Dosing recency (hours since last dose taken), cumulative dosing time (weeks since first dose), and dosing frequency (number of doses per week or randomization arm) were tabulated as three dimensions to describe PrEP adherence for oral fluids collected. Drug concentrations below corresponding LOD were considered as not detected (0 ng/mL). All readouts below LOQ but above LOD with discernable peaks were imputed as half of the corresponding LOQ for statistical calculations. To adjust for the 1:3 dilution in oral fluids collection, drug levels detected were multiplied by three.

To account for repeated measures from the same participant, we performed binomial Generalized Estimating Equations (GEE) with exchangeable correlations to assess the relationships between FTC and TFV detectability (drug levels detected > LOD) in oral fluids and the three dimensions of PrEP adherence. PrEP adherence was parameterized as binary for dosing recency (having a time since last dose ≤ 24 hours vs between 24 to 48 hours), a continuous variable for cumulative dosing time (weeks of dosing, between 1 and 6), and a categorical variable for dosing frequency (assigned randomization arm: 2 vs 4 vs 7 doses/week). We plotted bar-plots and scatterplots of oral fluids drug concentrations by dosing recency. Correlation coefficients for repeated measures were calculated between time-matched drug levels detected in oral fluids and plasma.

To establish benchmarks and assess the diagnostic accuracy of FTC/TFV in oral fluids for predicting PrEP non-adherence, we calculated sensitivities and specificities for recent non-adherence using ROC (Receiver Operating Characteristic) analysis of clustered data. Recent non-adherence was defined as either “having a time since the last dose of more than 24 hours (up to 48 hours)” or “missing one dose of daily oral PrEP”. This definition served as the positive condition for prediction in the ROC analysis. We computed the 95% confidence intervals (CI) with stratified bootstrap. Optimal cutoffs were

determined based on the Youden's Index. To expand our definition of recent non-adherence to more than one missed dose, we included all additional untested oral fluids with a time since last dose greater than 48 hours. We assumed TFV/FTC would be undetectable in these samples based on results from our pilot testing and previously published studies.

3.4 Results

Participant/Specimen Characteristics

We collected 241 oral fluids from 22 participants (41% female). Since no oral fluids with a time since last dose greater than 48 hours had detectable TFV or FTC in our pilot testing of 20 unblinded samples, 165 (68.5%) oral fluids with a time since last dose $\leq$ 48 hours were tested for TFV/FTC. Among those 165 oral fluids, 143 (86.7%) had time-matched plasma samples collected. Fifty (30.3%) of the 165 oral fluids had a time since last dose between 24 hours and 48 hours while 115 (69.6%) with a time since last dose within 24 hours (**Table S2**). Most of these oral fluids tested for FTC/TFV were collected from participants randomized to the moderate/perfect adherence arms (75.8%) or with six weeks of cumulative dosing time (75.2%).

Drug concentrations in oral fluids

Fifteen (9.0%) oral fluids had detectable TFV above the LOD, eight (4.8%) were above the LOQ (**Table S3**). The proportions of oral fluids with detectable TFV concentrations were 18.1%, 9.1%, 4.5%, 8.2% and 8.0% for samples at 1, 4, 12, 24 and 48 hour(s) since last dose taken respectively (**Figure S1**). Detectability of TFV/FTC in oral fluids by the three dimensions of adherence was summarized in **Table 1**. We detected TFV only in 10% of oral fluids with a time since last dose $\leq$ 24h, 5% of samples from participants randomized to the perfect adherence group (7 doses/week), and 10% of those with six weeks of cumulative dosing time.

Unlike TFV, more oral fluids had detectable FTC concentrations. FTC levels in oral fluids were detected above the LOQ in 64 (38.8%) samples. Another 24 (14.5%) oral fluids had detectable FTC concentrations below the LOQ. The median(IQR) FTC concentrations were 15 (0-141) ng/mL for 115 oral fluids with time since last dose $\leq$ 24h, and 0 (0-11) ng/mL for 50 oral fluids with time since last dose

between 24 and 48 hours. FTC was detectable in 90.9%, 100%, 59.1%, 40.8% and 26% of oral fluids with a time since last dose at 1, 4, 12, 24, 48 hour(s) respectively. There was also a declining trend of mean FTC levels by dosing recency (**Figure 1**). Compared to samples with a time since last dose between 24 and 48 hours, we found a higher proportion of oral fluids with a time since last dose ≤ 24 h had FTC detected (65% vs 26%).

Among 143 pairs of time-matched samples, FTC in oral fluids showed moderate correlation with FTC in plasma (0.66, 95%CI(0.55 - 0.75)). However, TFV in oral fluids was not correlated with TFV in plasma (0.15, 95%CI(-0.03 - 0.32)). We also observed strong correlation between TFV and FTC in plasma (0.95, 95%CI(0.89 - 0.95)) but not in oral fluids (0.15, 95%CI(-0.02 - 0.30)).

TFV/FTC in Oral Fluids for PrEP Non-adherence

Detectability of TFV and FTC in oral fluids was not significantly associated with having more frequent doses per week or one more week of cumulative dosing time (**Table S4**) based on the GEE analyses. However, compared to oral fluids with a time since last dose ≤ 24 h, we found that a time since last dose between 24 and 48 hours was on average associated with 79% less odds of having detectable FTC in oral fluids (OR (odds ratio): 0.21, 95%CI(0.11-0.38), $P < 0.01$) with the same dosing frequency and cumulative dosing time. Given the significant association between FTC detectability and dosing recency, further ROC analyses were performed to estimate the diagnostic accuracy of oral fluids FTC for detecting PrEP non-adherence.

For ROC analyses among 165 oral fluids with a time since last dose ≤ 48 h (**Table S5**), the optimal FTC threshold at 7.5 ng/mL has accurately classified 74% (sensitivity) samples with one missed dose (having a time since last dose > 24 h but ≤ 48 h) as non-adherent while maintaining a moderate specificity for sample with a time since last dose ≤ 24 h (65%, 95%CI(57%-74%). To better reflect the real-world definition of PrEP non-adherence beyond “with one missed dose (having a time since last dose > 24 h but ≤ 48 h)”, we included the other 76 untested oral fluids (with 30 and 46 collected at 72h and 96h since last dose respectively). Assuming those samples had no detectable FTC, the threshold at 7.5 ng/mL achieved the same specificity (**Table 2**) but an improved sensitivity for recent non-adherence (90%, 95%CI(84%-94%)) and an AUC (area under the curve) of 0.79 (95%CI: 0.74-0.84).

3.5 Discussion

Pharmacologic PrEP adherence monitoring is essential for early detection of non-adherence to the daily regimen, and enabling timely initiation of individualized support interventions. Additionally, it may serve as an objective clinical measure that justifies the transition to long-acting regimens, particularly when insurance coverage requires evidence of medical necessity for the regimen switch. In this randomized trial of directly observed FTC/TDF administration, we found that FTC detectability in oral fluids is associated with dosing recency. A threshold of FTC <7.5 ng/mL achieved high sensitivity by accurately classifying 90% of PrEP non-adherence (as early as missing one dose or with time since last dose >24h). TFV however is poorly diffused in oral fluids, and its detectability is not associated with dosing recency, frequency or cumulative dosing time.

Due to improved adherence for people struggles with daily intake, long-acting injectables like CAB-LA or lenacapavir have demonstrated superiority over daily regimens in clinical trials ^{27,60,61}. However, it remains uncertain whether most PrEP users, who adhere well to daily oral PrEP (taking ≥ 4 doses/week), would still benefit from long acting injectables for better HIV prevention. Therefore, the WHO recommends CAB-LA not as a replacement for daily oral PrEP but as an additional prevention option for people at substantial HIV risk ⁵¹. Even for those facing challenges with the daily regimen, it is also unclear if CAB-LA would outperform oral daily PrEP that combined with adherence monitoring and targeted interventions. Pharmacologic adherence monitoring with prompt feedback was found highly acceptable and associated with improved adherence to daily oral PrEP ⁵². For example, in a cohort study among men who have sex with men, plasma TFV level feedback demonstrated modest adherence improvement ⁶². Females receiving urine TFV adherence counseling also showed better adherence with higher proportions of positive urine assay results and higher levels of TFV in hair ⁶³. Given its wider availability, lower cost, and flexibility, daily oral of FTC/TDF, when combined with effective adherence monitoring, may achieve greater public health impact in the real-world with similar effectiveness as long-acting injectables.

With a long half-life of 17-21 days that translates to 25-fold accumulation from first dose to steady state, TFV-DP in DBS provides a significant dynamic range to differentiate varying levels of adherence

over the proceeding 1-3 months ^{11,15}. This characteristic makes TFV-DP less susceptible to “white-coat” adherence, where consistently nonadherent users may take a dose immediately before pharmacologic testing to appear compliant ^{64,65}. Therefore, TFV-DP benchmarks in DBS were often preferred for predicting cumulative adherence and explaining PrEP outcomes in trials ^{4,5,66}. However, these established benchmarks are only applicable when steady-state drug concentrations are achieved, which could be challenging in real-world settings among non-adherent users due to irregular dosing patterns ^{67,68}. Without repeated dosing at consistent intervals over 4-5 half-lives, steady-state concentrations of TFV-DP for different gradients of adherence (2/4/7 doses/week) are not guaranteed. Also, the conservative estimation of TFV-DP benchmarks ¹¹, using the rounded 25th percentiles of participants at each adherence level, may result in the misclassification of about 25% of participants as having lower adherence than they actually do. These limitations could partly explain the variability in observed PrEP adherence-efficacy relationships across different studies ^{5,17,69}. Furthermore, the measurement of long half-life metrics like TFV-DP in DBS relies heavily on LC-MS/MS. The process is expensive, labor-intensive, and time-consuming, limiting its ability to provide real-time feedback to PrEP users. This delay would hinder timely identification of non-adherence and early interventions.

Like TFV detected in urine, FTC has a short half-life (9-10h) in oral fluids ⁵⁸. These pharmacologic metrics do not accumulate appreciably with repeated dosing and thus only indicating recent drug ingestion. While “white-coat” dosing may sometimes mask non-adherence, short half-life metrics provide valuable insights into dosing recency that does not require the attainment of steady-state. The timing of last dose relative to high-risk behavior is more informative than averaged dosing frequency over previous weeks for ED PrEP. Moreover, short half-life metrics offer advantages for point-of-care (POC) testing due to their simple sample collection and minimal laboratory processing requirements. This enables real-time counseling and prompt intervention initiation for improved PrEP adherence and outcomes. A POC lateral flow assay (LFA) for urine TFV, with a cutoff of 1500 ng/mL, has shown high a specificity of 98% for accurately classifying users who took a dose within 24 hours as adherent ¹⁶. It also has a high sensitivity of 86% for identifying non-adherence in users who missed > 3 missed doses (last dose taken > 96 hours ago). However, its sensitivity ranges from 16%-52% for detecting non-adherence with 1-2 missed doses (last dose taken between 48-72h ago). To identify more non-adherence as early as possible for timely

interventions, further efforts are needed to enhance sensitivity for better detection of non-adherence within the prior 24-96h (equivalent to missing 1-3 doses).

Our data demonstrated that FTC in oral fluids, using a threshold of 7.5 ng/mL, is highly sensitive for detecting PrEP non-adherence as early as one dose missed. It accurately classified 90% of users with time since last dose >24h as non-adherent. However, its modest specificity of 65% may lead to false positives among some adherent individuals, potentially causing unnecessary concerns. To address this limitation, a follow-up test with high specificity, such as the POC LFA for urine TFV¹⁶, could confirm their adherent status and alleviate stress. In the development of tests for non-adherence detection, the primary goal should be to identify non-adherent users as many and as early as possible to facilitate timely interventions or regimen changes. Therefore, sensitivity should be prioritized over specificity. A highly specific but less sensitive test might misclassify non-adherent users as adherent, inadvertently reinforcing non-adherent behaviors and exposing them to increased HIV risk due to suboptimal PrEP protection. Oral fluids are already widely accepted as a convenient and confidential specimen type for HIV self-testing. Given the high sensitivity observed in our study, FTC in oral fluids presents a promising pharmacologic metric for developing future POC tests to monitor PrEP dosing recency. To better understand the three dimensions of adherence patterns (dosing recency, dosing frequency and cumulative dosing time), more research is needed to assess the performance of a combination of pharmacologic metrics with short and long half-lives in the real-world settings for identifying non-adherence and improving outcomes.

Our study had several strengths and was one of the first randomized PK trials that comprehensively investigated the associations between oral fluids TFV/FTC and the three dimensions of adherence. However, one limitation of our analyses is that we assumed all oral fluids with a time since last dose > 48h had undetectable FTC without actually testing them all. This decision was based on our pilot testing results. Considering 26% of the oral fluids with a time since last dose at 48h had detectable FTC, it is possible that some samples, particularly those with a time since last dose close to 48h, would have detectable FTC. This might lead to slightly overestimated sensitivity. Nonetheless, the effect should be minimum, since all of our untested samples were collected either 72h or 96h since the last dose, and

prior research found FTC levels to be lower than the LOQ among all oral fluids with a time since last dose $\geq 72\text{h}$ ⁵⁸. Another limitation was the three-fold dilution of FTC/TFV in oral fluids using our collection devices. This may have resulted in undetectable drug concentrations in a few samples with low FTC/TFV levels before dilution. To account for this dilution effect, we adopted a lower LOD for detecting FTC/TFV in oral fluids.

In conclusion, we assessed the feasibility of using FTC/TFV in oral fluids to detect PrEP non-adherence. Our study found that the detectability of FTC in oral fluids is closely associated with dosing recency. A threshold of FTC $<7.5\text{ ng/mL}$ in oral fluids demonstrated high sensitivity for identifying recent non-adherence (having a time since last dose $> 24\text{h}$). These findings will help establish adherence benchmarks of FTC in oral fluids and guide the development of POC tests for adherence monitoring.

Notes

We thank the participants in the studies for their contribution and the staff who worked on the trial.

Financial support. This work was supported by research grants from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH; grant numbers R21AI127200 and R01AI143340). Gilead Sciences donated Truvada.

Potential conflicts of interest. All authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Patient consent. The participants' written consent was obtained. The design of the work was approved by ethics committees at the Institute for the Development of Human Research Protections at the Medical Sciences Department, Thai Ministry of Public Health (TARGET/ IHRP); the ethics committee of Sanpatong Hospital (SPT REC 007/60); the Faculty of Associated Medical Sciences, Chiang Mai University (AMSEC-FB-009); and the University of Washington Institutional Review Board in Seattle (STUDY00000058).

3.6 Tables and Figures

Table 3.6.1. Detectability of Tenofovir and Emtricitabine in Oral Fluids by Three Dimensions of Adherence Patterns (Dosing Recency vs Dosing Frequency vs Cumulative Dosing time)*

TFV Detected in Oral Fluids ($\geq$ LOD)		
<i>Dosing Recency</i>	Yes	No
Time Since Last Dose $\leq$ 24h	11(10%)	104(90%)
24h<Time Since Last Dose $\leq$ 48h	4(8%)	46(92%)
<i>Dosing Frequency</i>	Yes	No
Low(2 doses/week)	4(10%)	36(90%)
Moderate(4doses/week)	8(12%)	55(87%)
Perfect (7 doses/week)	3(5%)	59(95%)
<i>Cumulative Dosing Time (weeks)</i>	Yes	No
<One	1(8%)	12(92%)
Two	1(7%)	13(93%)
Four	1(7%)	13(93%)
Six	12(10%)	112(90%)
Total	15(9%)	150(91%)
FTC Detected in Oral Fluids ($\geq$ LOD)		
<i>Dosing Recency</i>	Yes	No
Time Since Last Dose $\leq$ 24h	75(65%)	40(35%)
24h<Time Since Last Dose $\leq$ 48h	13(26%)	37(74%)

Dosing Frequency	Yes	No
Low(2 doses/week)	23(58%)	17(42%)
Moderate(4doses/week)	28(44%)	35(56%)
Perfect (7 doses/week)	37(60%)	25(40%)
Cumulative Dosing Time (weeks)	Yes	No
<One	3(23%)	10(77%)
Two	7(50%)	7(50%)
Four	6(43%)	8(57%)
Six	72(58%)	52(42%)
Total	88(53.3%)	77(46.7%)

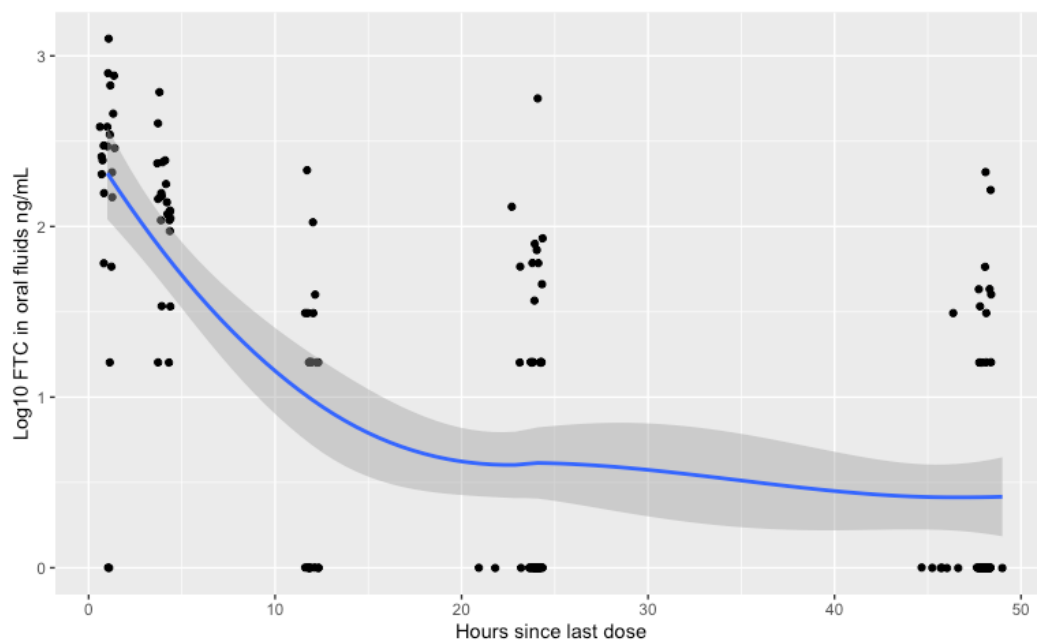
Table 3.6.2. Diagnostic Accuracy of FTC in Oral Fluids for Detecting PrEP Non-Adherence (with a time since last dose >24 hours)*

FTC thresholds in oral fluids (ng/mL)	Sensitivity (95%CI)	Specificity(95%CI)
7.5	90%(84%-94%)	65%(57%-74%)
22.5	93%(88%-97%)	48%(38%-57%)
31.5	94%(90%-98%)	45%(36%-54%)
34.4	95%(91%-98%)	44%(34%-52%)
43.5	98%(94%-100%)	42%(33%-50%)
58.5	98%(96%-100%)	39%(30%-48%)

169.5	99%(97%-100%)	20%(13%-28%)
210	100%(100%-100%)	17%(0%-24%)

Figure 3.6.1. Scatterplot Emtricitabine Concentrations (Log 10 transformed)

in Oral Fluids by Dosing Recency (Hours Since Last Dose)*



*LOESS smoothed line added with 95% confidence interval

3.7 Supplementary Data

Table 3.7.S1. Participant Baseline Characteristics by Randomization Arms*

	Low	Moderate	Perfect	All
Adherence Arm	(2 doses/week)	(4 doses/week)	(7 doses/week)	
Baseline Characteristics	(N=8)	(N=7)	(N=7)	(N=22)
Age, years	33.5(9.3)	31.1(9.4)	34.4(6.6)	33.1(8.1)
Female, N(%)	3(38%)	1(14%)	5(71%)	9(41%)
Body Mass Index, kg/m ²	23.4(20.1-28.5)	23.5(21.4-24.9)	20.3(19.3-24.0)	23.3(19.8-24.9)
Hemoglobin, g/dL	14.7(13.7-15.0)	14.4(14.4-14.9)	12.3(11.2-12.7)	14.2(12.5-14.7)
Hematocrit, %	43.6%(41.5%-45.4%)	44.1%(42.4%-45.1%)	37.1%(34.6%-38.7%)	42.2%(38.3%-44.7%)
eGFR [#] , mL/min	109(100-116)	121(104-133)	91(87-97)	106(91-117)
BUN, mg/dL	10.6(9.4-12.7)	9.7(7.7-11.3)	9.3(9.1-10.2)	10.0(8.8-12.0)
Creatinine (plasma), mg/dL	0.91(0.82-1.03)	0.82(0.78-0.91)	0.73(0.68-0.88)	0.83(0.74-0.96)
Total bilirubin, mg/dL	0.67(0.47-0.80)	0.97(0.82-1.24)	0.68(0.44-0.78)	0.73(0.52-0.97)

Abbreviations: eGFR, estimated glomerular filtration rate.

*Data are presented as median (interquartile range or standard deviation) unless specified otherwise.

#Estimated with the Cockcroft-Gault equation.

Table 3.7.S2. Three Dimensions of PrEP Adherence Patterns (Dosing Recency vs Dosing Frequency vs Cumulative Dosing time) for Oral Fluids Tested

Adherence Arm (Dosing Frequency)	Hours Since Last Dose (Dosing Recency)					N
	1 hour	4 hours	12 hours	24 hours	48 hours	
Low (2 doses/week)	8	8	8	8	8	40(24.2%)
Moderate (4 doses/week)	7	7	7	7	35	63(38.2%)
Perfect (7 doses/week)	7	7	7	34	7	62(37.6%)
N	22 (13.3%)	22(13.3%)	22(13.3%)	49(29.7%)	50(30.3%)	165(100%)

Weeks of Dosing (Cumulative Dosing Time)	Hours Since Last Dose (Dosing Recency)					N
	1 hour	4 hours	12 hours	24 hours	48 hours	
<One	0	0	0	6	7	13(7.9%)
Two	0	0	0	7	7	14(8.5%)
Four	0	0	0	7	7	14(8.5%)
Six	22	22	22	29	29	124(75.2%)
N	22 (13.3%)	22(13.3%)	22(13.3%)	49(29.7%)	50(30.3%)	165(100%)

Table 3.7.S3. Tenofovir and Emtricitabine Detected in Oral Fluids by Dosing Recency and the Limit of Detection and the Limit of Quantification *

<i>TFV in oral fluids</i>			
Time since last dose taken (hours)	<LOD	≥LOD but <LOQ	≥LOQ
1	18(81.8%)	3(13.6%)	1(4.5%)
4	20(90.9%)	0(0%)	2(9.1%)
12	21(95.5%)	0(0%)	1(4.5%)
24	45(91.8%)	2(4.1%)	2(4.1%)
48	46(92.0%)	2(4.0%)	2(4.0%)
All	150(90.9%)	7(4.2%)	8(4.8%)
<i>FTC in oral fluids</i>			
Time since last dose taken (hours)	<LOD	≥LOD but <LOQ	≥LOQ
1	2(9.1%)	1(4.5%)	19(86.4%)
4	0(0%)	2(9.1%)	20(90.9%)
12	9(4.1%)	7(31.8%)	6(27.3%)
24	29(59.2%)	10(20.4%)	10(20.4%)
48	37(74.0%)	4(8.0%)	9(18.0%)
All	77(46.7%)	24(14.5%)	64(38.8%)

Table 3.7.S4. GEE Model Results for Tenofovir/Emtricitabine Detected in Oral Fluids and Three Dimensions of Adherence*

	24h<Time since last dose ≤48h (vs Time since last dose ≤ 24h)	Moderate Adherence(vs Low)	Perfect Adherence(vs Low)	One more week of cumulative dosing
FTC detected in oral fluids	-1.58(-2.19 - -0.97)	0.17(-0.88 - 1.21)	0.12(-1.11 - 1.36)	0.15(-0.01 - 0.31)
TFV detected in oral fluids	-0.64(-2.34 - 0.41)	0.53(-1.17 - 2.25)	-0.88(-2.57 - 0.81)	0.0009(-0.20 - 0.21)
	24h<Time since last dose ≤48h (vs Time since last dose ≤ 24h)	Moderate Adherence(vs Low)	Perfect Adherence(vs Low)	One more week of cumulative dosing
FTC detected in oral fluids	0.21(0.11-0.38)	1.18(0.41 - 3.37)	1.13(0.33 - 3.89)	1.16(0.99 - 1.36)
TFV detected in oral fluids	0.53(0.10 - 1.51)	1.69(0.31 - 9.46)	0.41(0.08 - 2.24)	1.00(0.81 - 1.23)

Table 3.7.S5. Diagnostic Accuracy of FTC in Oral Fluids for Differentiating Samples “with a time since Last dose ≤24 hours” from Samples “with a time since last dose <48 but > 24 hours”

FTC threshold in oral fluids (ng/mL)	Sensitivity (95%CI)	Specificity(95%CI)
7.5	74%(62%-86%)	65%(57%-74%)
22.5	82%(70%-92%)	48%(38%-57%)
31.5	86%(76%-94%)	45%(36%-54%)

34.4	88%(78%-96%)	44%(34%-52%)
43.5	94%(86%-100%)	42%(33%-50%)
58.5	96%(90%-100%)	39%(30%-48%)
169.5	98%(94%-100%)	20%(13%-28%)
210	100%(100%-100%)	17%(0%-24%)

Figure 3.7.S1. Detectability/Quantifiability of Tenofovir and Emtricitabine

in Oral Fluids by Dosing Recency*

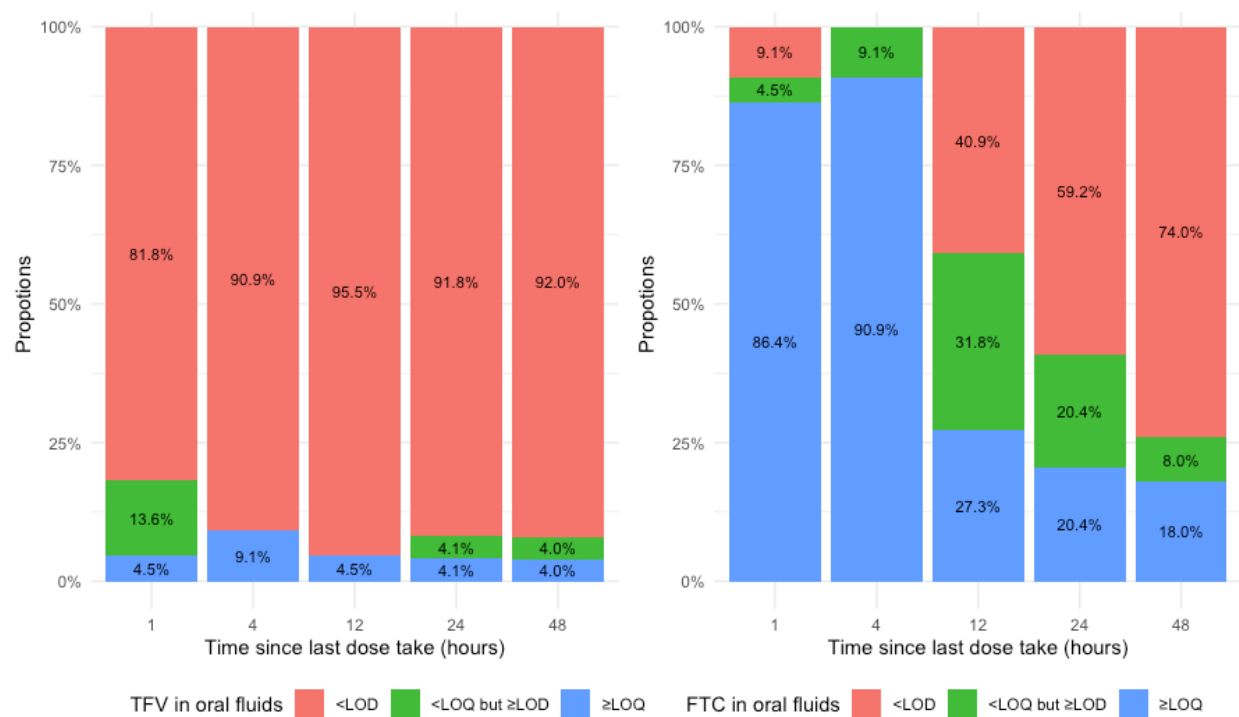


Figure 3.7.S2. Detectability/Quantifiability of Tenofovir and Emtricitabine

in Oral Fluids by Dosing Frequency*

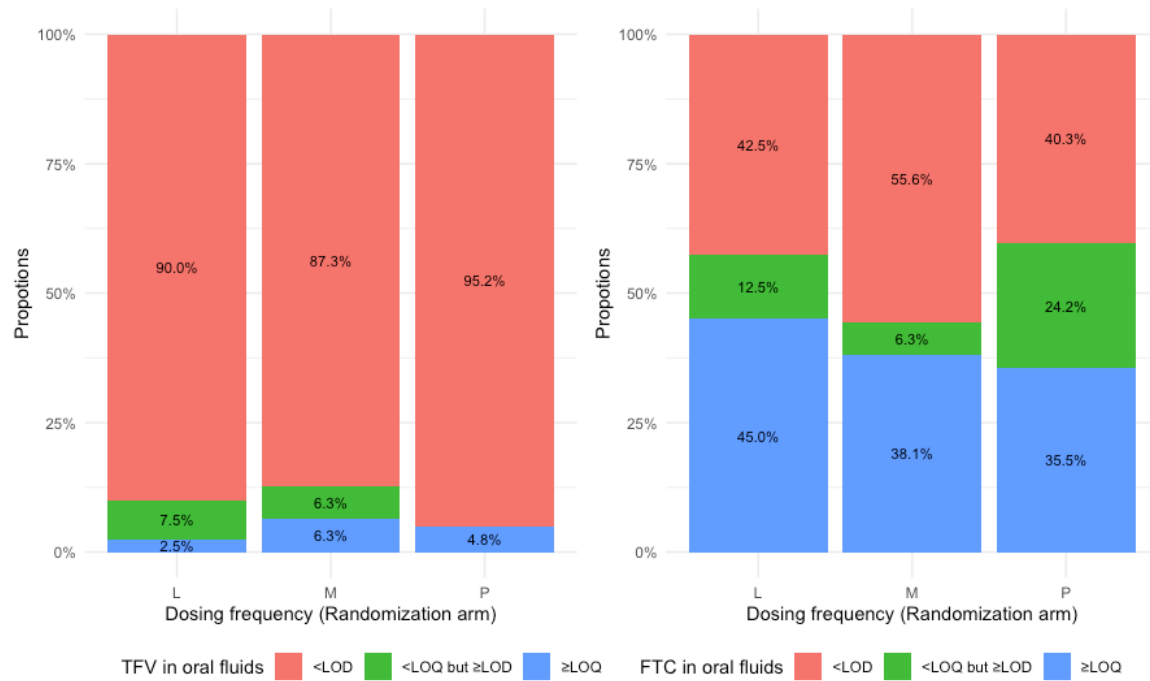
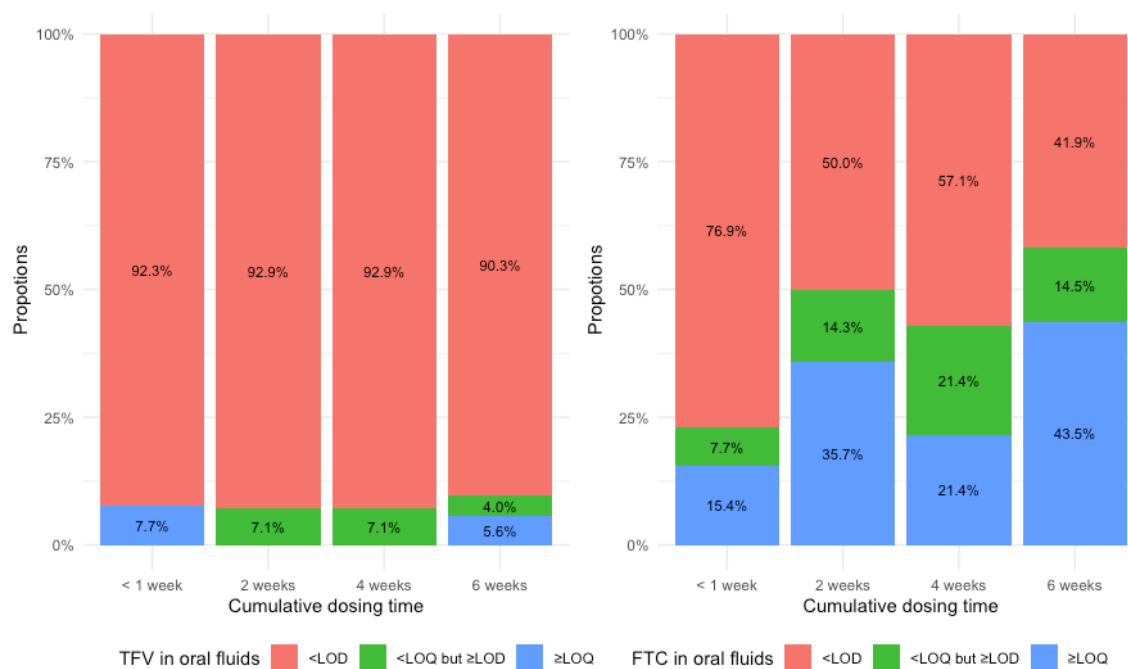


Figure 3.7.S3. Detectability/Quantifiability of Tenofovir and Emtricitabine

in Oral Fluids by Cumulative Dosing Time*



Chapter 4. Are Sex-Specific Cutoffs Needed for Urine Tenofovir Lateral Flow Assays to Measure Antiretroviral Drug Adherence?

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Keywords. HIV; PrEP; ART; adherence; tenofovir; urine; lateral flow assay; sex.

4.1 Abstract

Though females had lower urine tenofovir detected than males, we found similar sensitivities/specificities between two sexes using the same cutoffs for the lateral flow assay (LFA) readouts. Our next-gen LFA achieved high accuracy for non-adherence (including whitecoat dosing) detection with 92% sensitivity and 96% specificity.

4.2 Background

Despite the increasing availability of effective oral antiretroviral treatment (ART) and pre-exposure prophylaxis (PrEP), an estimated 1.3 million new HIV infections were reported globally in 2022 ⁷.

Inconsistent adherence remains a major global challenge with only 65% of people living with HIV on ART achieved virologic suppression and 29% of PrEP users maintained optimal daily dosing ^{4,6}. To meet the 95-95-95 UNAIDS targets, substantial improvements in both ART and PrEP adherence are needed.

According to World Health Organization guidelines, oral tenofovir (TFV)-based regimens remain the first-line therapy for both ART and PrEP. Point-of-care testing for urine TFV may allow for better monitoring and interventions to enhance adherence ⁵². One urine tenofovir lateral flow assay (LFA) was developed based on a TFV threshold at 1500 ng/mL to have high specificity for detecting PrEP non-adherence (accurately classified 98% of users who took a dose within 24 hours as adherent) ¹⁶. However, females may require higher adherence to achieve equivalent therapeutic drug levels compared to males ¹⁷, which suggests sex-specific cutoffs of urine TFV LFAs may be necessary. Further efforts are also needed to improve the sensitivity of the urine LFA in detecting non-adherence during the 24 to 96-hour window since the last dose.

We developed a next-gen urine TFV LFA that does not have a predetermined threshold for urine TFV concentration, and evaluated the quantified results (optical readings & visual scores) using urine specimens from a directly-observed PrEP adherence randomized trial ¹³. This study aimed to (1) determine if LFA results were different between females and males with the same observed adherence levels, (2) evaluate the diagnostic performance of the next-gen TFV LFA for detecting PrEP non-adherence by sex, and (3) assess the ability of the next-gen TFV LFA to detect non-adherence with a more stringent definition that includes whitecoat dosing.

4.3 Methods

We conducted a randomized controlled pharmacokinetic trial of adults receiving varying adherence levels of oral PrEP in Thailand, with directly-observed-therapy (DOT) for each drug intake (ClinicalTrials.gov #NCT0301260) ¹². To approximate groups with low, moderate and perfect adherence,

healthy adults were randomized to receive either 2, 4, or 7 doses/week of Tenofovir Disoproxil Fumarate (TDF) 300mg/Emtricitabine (FTC) 200mg for 6 weeks. All participants were followed for another 4 weeks after the last dose during a washout period. We recorded the time since last TDF/FTC pill for each urine sample collected.

We tested the urine samples using the next-gen urine TFV LFA with three quantified readouts. Since this is a competitive assay, higher concentrations of urine TFV will prevent more TFV-specific antibody to conjugate and appear at the LFA test line. Therefore, higher drug concentrations in urine result in weaker color intensity on the test line. Two lab technicians performed independent visual scoring of the test line against the same color intensity reference card, which had a graduated scale from 0 (not visible) to 5 (very dark). Averaged visual scores from two lab technicians were used to account for variations between users. Quantified LFA readouts were also obtained using a commercial strip reader (Axxin Ax-2x-S; Fairfield, Australia) for the peak color intensity of the test line. Ratios of the test versus control line peak color intensity were calculated as another comprehensive quantified optical readout.

We compared the next-gen LFA readouts (averaged visual and optical readings) by sex among samples with a last dose taken in the prior day. We performed Generalized Estimating Equations (GEE) with unstructured correlations to assess the effect of sex on the next-gen LFA readouts. Hours since last dose was included as the time variable while the randomized adherence arm was adjusted as a confounder.

We calculated overall and sex-specific sensitivities and specificities for recent non-adherence (defined as not having taken a dose within the prior 24h) using ROC (Receiver Operating Characteristic) analysis of clustered data. We computed the 95% confidence intervals (CI) with stratified bootstrap. Optimal cutoffs were determined based on the Youden's Index. To reduce potential impact from whitecoat dosing, ROC analyses were also performed for recent non-adherence with a more stringent definition (not having taken two consecutive daily doses within the prior 48 hours).

4.4 Results

Participant/Specimen Characteristics

Twenty-eight participants (43% female) with a median (interquartile range,[IQR]) age at 33 (28-40) years old were enrolled (**Table S1**). The perfect adherence group had more females (6/9, [67%]) than the low (4/9, [44%]) and moderate (2/10,[20%]) adherence groups. Seventy-six percent (447/586) of urine samples had a time since last dose longer than 24 hours and thus were classified as “non-adherent”. Using the more stringent definition of non-adherence, 453 urine samples were collected when the participants did not take two consecutive daily doses in the prior 48 hours. Six urine samples were “whitecoat dosing” samples from participants who took a dose in the prior day but not two consecutive daily doses in the prior two days.

Sex Differences in Descriptive Statistics

Among 139 urine samples with a time since last dose ≤ 24 hours, the medians for the test line peak color intensity were 385 and 340 among females and males, respectively. Compared with males, we also observed 0.5 unit higher median averaged visual score for urine samples from females (**Table S2**). Among samples with a time since last dose between 10 to 24 hours, scatterplots show females having higher values for all three LFA readouts (indicating lower urine TFV levels) than males (**Figure S1a-S1c**). Overall, males had 33% percent less peak color intensity of the LFA test line ($p\text{-value} < 0.01$), and a 1.82 unit lower averaged visual score of the LFA test line ($p\text{-value} < 0.01$), when compared to females (**Table S3**).

ROC Analysis for the Total Population

For recent non-adherence defined as “no dosing in the prior day”, the next-gen LFA demonstrated the highest sensitivity (93%, 95%CI: 91%-95%) with a ratio of the test/control line color intensity > 0.095 , while maintaining a high specificity (96%, 95%CI: 93%-99%) in the total population (**Table 1**). By using the more stringent definition for recent non-adherence (not having two consecutive daily doses in the prior 48 hours), we observed similarly high specificities across all LFA readouts but slightly lower sensitivity from the averaged visual score (78%) compared with the quantified test line color intensity (85%).

Sex Specific ROC analysis

The next-gen LFA had minor differences for diagnostic sensitivity and specificity among males and females (**Table 1**). Females shared the same optimal cutoffs for all three LFA readouts as the total population, while males had lower optimal cutoffs for the test line peak color intensity and averaged visual score. With the same optimal cutoffs (>0.095) for the test/control line color intensity ratio, we found similar sensitivities (93% vs 93%) and specificity (99% vs 94%) among males versus females. Comparable sensitivities (86% vs 86%) and specificities (100% vs 95%) by sex were also shown when using the same cutoff (>705) for the test line color intensity. However, the sensitivity among females was slightly better than that among males (82% vs 76%) when using the same cutoff (>1.25) for the averaged visual score.

4.5 Discussion

In a randomized directly-observed PrEP adherence trial, we found higher LFA readouts (indicating lower urine TFV levels) among females, as compared to males, using both averaged visual score and quantified peak color intensity by an optical reader. However, these differences had a small impact on diagnostic accuracy of the next-gen LFA when using the same cutoffs between sexes. The optimal results were obtained using an optical reader to measure the ratio of the test/control line peak color intensity. When using an optical reader, sex differences were mitigated and our next-gen LFA achieved improved sensitivity (92%) while preserving a high specificity (96%) in identifying non-adherence, including whitecoat dosing, among both males and females.

Sex differences in antiretroviral drug concentrations and effectiveness have been described. PrEP DOT clinical trials have revealed higher tenofovir-diphosphate concentrations in dried blood spots among females when compared to males with the same adherence levels^{11,12}. Pharmacokinetic studies have reported lower TFV concentrations in vaginal tissues compared to rectal tissue^{70,71}. Compared to cisgender men who have sex with men (MSM), the minimum PrEP adherence required to achieve sufficient HIV protection has not been well characterized for cisgender females. The HIV Prevention Trial Network (HPTN) 083 & 084 studies indicated that females may require higher adherence to achieve the same level of risk reduction as males¹⁷. However, a recent pooled analysis estimated a low HIV incidence (0.13/100 person-years) for females with 4-6 doses/week, suggesting comparable PrEP

adherence benchmarks that were established for cisgender MSM ⁵. But due to diverse characteristics across different adherence groups, the pooled analysis only compared HIV incidences observed in each group but did not assess relative risk reduction or PrEP effectiveness. These findings highlight the importance of understanding the sex differences in measuring drug concentrations for monitoring antiretroviral drug adherence.

Consistent with prior pharmacokinetic studies, our study found sex differences in urine TFV detected by the LFA. However, unlike the higher intracellular TFV-DP in females, urine TFV was lower among females (as indicated by higher LFA readouts) than males who took a dose in the prior day. These opposing sex differences can be explained by faster drug clearance among males, leading to higher levels of TFV in urine but lower intracellular TFV-DP concentrations compared to females. Nevertheless, this statistically significant difference had a minor impact on sex differences in the diagnostic performance for non-adherence detection. Our next-gen LFA also represents a significant advancement in sensitivity for detecting recent non-adherence (including missing a dose 24 to 96 hours before testing or whitecoat dosing). Even in resource-limited areas where optical readers may not be readily available, averaged visual scores from our next-gen LFA accurately classified 95% of PrEP users who took two consecutive daily doses in the prior two days as adherent. To further reduce cost and turnaround time, ongoing efforts are directed towards developing and validating smartphone applications for image analysis and self-effectiveness-monitoring, eliminating the need for optical readers and color intensity reference cards.

In summary, while females showed higher LFA readouts (indicating lower urine TFV levels) than males, sex-specific cutoffs for a next-gen LFA may not be necessary to measure antiretroviral drug adherence. Our next-gen LFA achieved high accuracy for non-adherence (including whitecoat dosing) detection, and holds promise for applications in real-time ART and PrEP adherence monitoring.

Notes

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Potential conflicts of interest. All authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

4.6 Tables and Figures

Table 4.6.1. Diagnostic Performance of the Next-Gen Lateral Flow Assay (LFA) for Non-Adherence Detection*

Non-Adherence +		Average Visual		Test Line Peak Color		Test/Control Line Peak	
		Score >1.25		Intensity >705		Color Intensity	
						Ratio >0.095	
Less than one dose in the prior day + (Total: N=586)							
Sensitivity	79%(75%-82%)	LR+ : 16	86%(83%-89%)	LR+ : 43	93%(91%-95%)	LR+ : 23	
Specificity	95%(91%-95%)	LR- : 0.22	98%(95%-100%)	LR- : 0.14	96%(93%-99%)	LR- : 0.07	
Less than one dose in the prior day + (Males: N=341)							
Sensitivity	76%(71%-81%)	LR+ : 25	86%(82%-91%)	LR+ : NA	93%(90%-96%)	LR+ : 93	
Specificity	97%(93%-100%)	LR- : 0.25	100%(100%-100%)	LR- : 0.14	99%(96%-100%)	LR- : 0.07	
Less than one dose in the prior day + (Females: N=245)							

	Sensitivity	82%(77%-88%)	LR+ : 10.3	86%(81%-91%)	LR+ : 17	93%(89%-96%)	LR+ : 16
	Specificity	92%(86%-98%)	LR- : 0.09	95%(89%-100%)	LR- : 0.15	94%(88%-98%)	LR- : 0.07
Less than two consecutive daily dose in the prior two days + (Total: N=586)							
	Sensitivity	78%(74%-81%)	LR+ : 16	85%(82%-88%)	LR+ : 85	92%(89%-94%)	LR+ : 23
	Specificity	95%(91%-95%)	LR- : 0.23	99%(96-100%)	LR- : 0.15	96%(93%-99%)	LR- : 0.08
Less than one dose in the prior day + (Males: N=341)							
		<i>Average Visual Score>0.75</i>		<i>Test Line Peak Color Intensity >615</i>		<i>Test/Control Line Peak Color Intensity Ratio >0.095</i>	
	Sensitivity	92%(89%-95%)	LR+ : 5	89%(85%-92%)	LR+ : 89	93%(90%-96%)	LR+ : 93
	Specificity	83%(73%-91%)	LR- : 0.10	99%(96%-100%)	LR- : 0.11	99%(96%-100%)	LR- : 0.07

*(95% Confidence Interval) if not specified; LR+: Positive Likelihood Ratio; LR-:Negative Likelihood Ratio;

NA: not applicable.

4.7 Supplementary Data

Table 4.7.S1. Baseline Participant Characteristics by Randomized Adherence Arm*

	Low (2 doses/week)	Moderate (4 doses/week)	Perfect (7 doses/week)
Baseline Characteristics	(N=9)	(N=10)	(N=9)
Age, years	38(27-40)	32(28-33)	34(31-39)
Female, N(%)	4(44%)	2(20%)	6(67%)
Body Mass Index, kg/m2	23.1(20.2-28.4)	24.0(22.3-25.0)	20.2(19.1-24.3)
Hemoglobin, g/dL	14.7(13.3-15.0)	14.5(14.4-15.1)	12.4(11.9-13.2)
Hematocrit, %	43.0%(39.2%- 45.1%)	43.8%(41.9%-45.2%)	38.0%(36.2%-39.3%)
eGFR, mL/min	108(102-115)	124(101-132)	91(87-108)
BUN, mg/dL	10.5(10.2-12.3)	10.4(8.7-13.0)	9.3(9.0-11.0)
Creatinine (plasma), mg/dL	0.86(0.75-1.01)	0.84(0.78-.96)	0.73(0.68-0.81)
Total bilirubin, mg/dL	0.58(0.47-0.79)	0.84(0.55-1.07)	0.68(0.35-0.76)

*Median(Interquartile range) if not specified

Table 4.7.S2. Next-Gen LFA Readouts among Urine Samples with Time Since Last Dose $\leq$ 24h Stratified by Sex

Urine Samples	Male	Female
(one dose in the prior day)	(N=75)	(N=64)

	Median (interquartile range)	Mean (min-max)	Median (interquartile range)	Mean (min-max)
Test Line Peak Color Intensity	340 (270-452)	359 (160-680)	385 (290-568)	432 (160-1120)
Test/Control Line Peak Color Intensity Ratio	0.06 (0.05-0.07)	0.06 (0.03- 0.10)	0.06 (0.05-0.08)	0.06 (0.02-0.15)
Test Line Averaged Visual Score	0 (0-0.5)	0.29 (0-2.0)	0.5 (0-1.0)	0.49 (0-2.0)

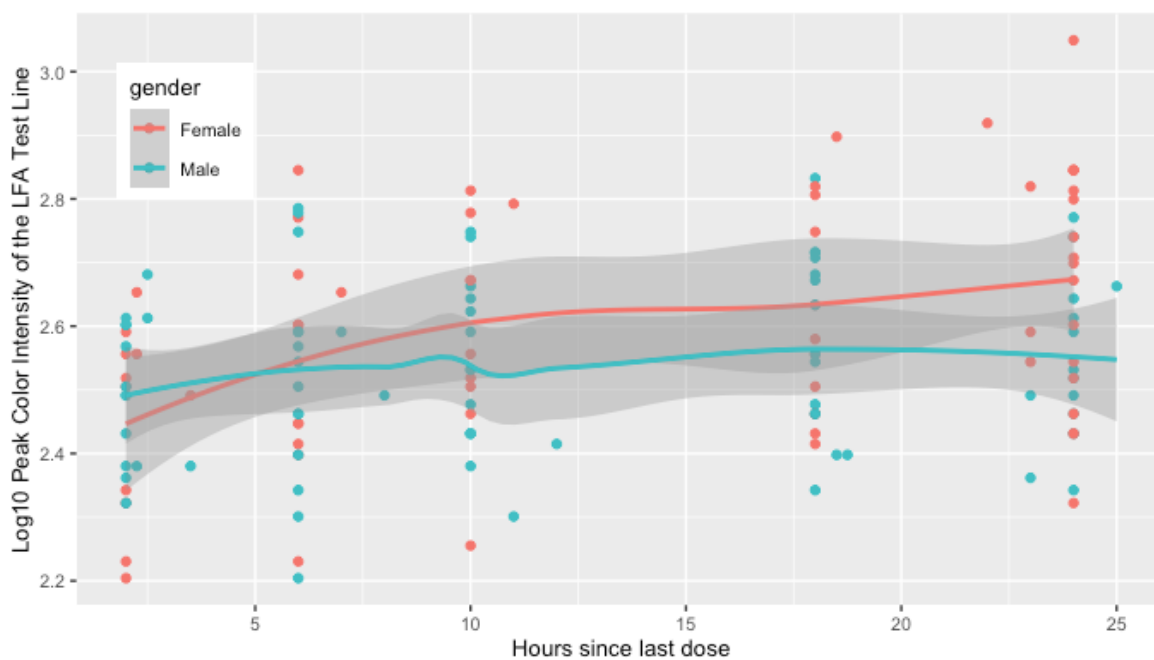
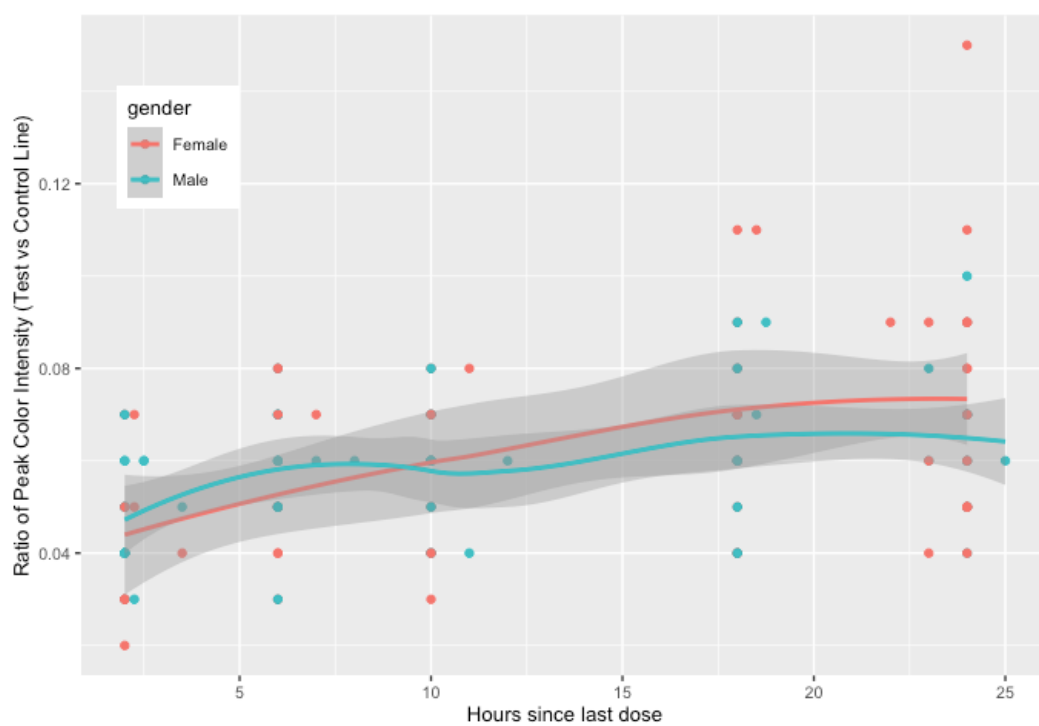
Figure 4.7.S1a: Scatterplot of Log₁₀ Test Line Peak Color Intensity by Hours Since Last Dose**Figure 4.7.S1b:** Scatterplot of Test/Control Line Peak Color Intensity Ratio by Hours Since Last Dose

Figure 4.7.S1c: Scatterplot of Test Line Averaged Visual Score by Hours Since Last Dose

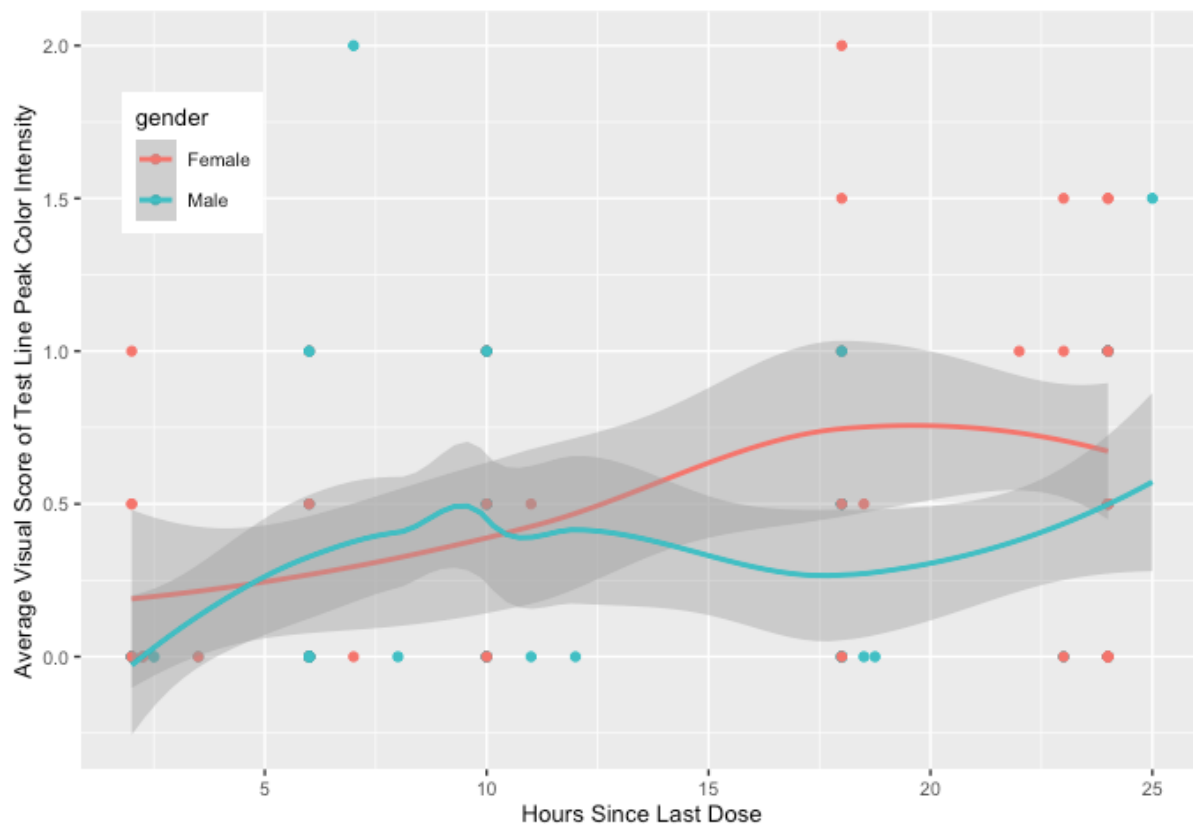


Table 4.7.S3. Next-Gen LFA Readouts among Urine Samples with Time Since Last Dose ≤ 24 h Stratified by Sex*

	Log 10 Test Line Peak Color Intensity	Test/Control Line Peak Color Intensity Ratio	Averaged Visual Score
Male (vs Female)	-0.17 (-0.258 to -0.082)	0.04 (-0.025 to 0.11)	-1.82 (-2.70 to -0.94)
One additional hour since last dose	0.0034 (-0.0001 to 0.0069)	0.0017 (0.0012 to 0.0022)	-0.31 (-0.47 to -0.15)

*(95% Confidence Interval) if not specified

Chapter 5. *Conclusions*

This dissertation evaluated the feasibility and performance of measuring TFV-DP in DBS, TFV/FTC in oral fluids, and TFV in urine for PrEP adherence monitoring. Our observed steady state adherence benchmarks for TFV-DP in DBS were found slightly higher but comparable to prior predicted estimates. Besides dosing frequency, TFV-DP levels in DBS were also associated with cumulative dosing time and dosing recency. Additionally, we demonstrated that FTC in oral could be a sensitive marker for identifying early non-adherence. Furthermore, our study revealed significant sex differences in urine TFV levels detected by the next-gen LFA. Despite these differences, the high accuracy of the next-gen LFA in identifying non-adherence was maintained for both males and females. Together, these findings provide valuable insights into the potential applications of measuring antiretrovirals for daily oral PrEP adherence. They will also aid in developing future POC tests for PrEP adherence monitoring with real-time feedback. More research on these adherence monitoring tools is warranted to examine their real-world impact on PrEP adherence and outcomes.

Long-acting injectables, such as CAB-LA or lenacapavir, have shown superior efficacy over daily oral regimens in trials, likely due to easier adherence. However, it remains uncertain if this superiority would persist among individuals without adherence issues to daily oral PrEP. Future studies should also assess if long-acting injectables would outperform daily oral PrEP when combined with effective adherence monitoring and follow-up interventions. Given its wider availability, lower cost, and flexibility, daily oral FTC/TDF, when combined with effective adherence monitoring, may achieve a greater public health impact in the real world, with similar effectiveness to long-acting injectables.

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