Video Article

The α-test: Rapid Cell-free CD4 Enumeration Using Whole Saliva

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URL: http://www.jove.com/video/3999
DOI: doi:10.3791/3999

Keywords: Medicine, Issue 63, CD4 count, saliva, antitrypsin, hematopoiesis, T cells, HIV/AIDS, clinical

Date Published: 5/16/2012

Citation: Bristow, C.L., Babayeva, M.A., Modarresi, R., McArthur, C.P., Kumar, S., Awasom, C., Ayuk, L., Njinda, A., Achu, P., Winston, R. The α-test: Rapid Cell-free CD4 Enumeration Using Whole Saliva. *J. Vis. Exp.* (63), e3999, doi:10.3791/3999 (2012).

Abstract

There is an urgent need for affordable CD4 enumeration to monitor HIV disease. CD4 enumeration is out of reach in resource-limited regions due to the time and temperature restrictions, technical sophistication, and cost of reagents, in particular monoclonal antibodies to measure CD4 on blood cells, the only currently acceptable method. A commonly used cost-saving and time-saving laboratory strategy is to calculate, rather than measure certain blood values. For example, LDL levels are calculated using the measured levels of total cholesterol, HDL, and triglycerides[1]. Thus, identification of cell-free correlates that directly regulate the number of $CD4^+$ T cells could provide an accurate method for calculating CD4 counts due to the physiological relevance of the correlates.

The number of stem cells that enter blood and are destined to become circulating $CD4^+$ T cells is determined by the chemokine CXCL12 and its receptor CXCR4 due to their influence on locomotion[2]. The process of stem cell locomotion into blood is additionally regulated by cell surface human leukocyte elastase (HLE_{CS}) and the HLE_{CS}-reactive active $\alpha_1$proteinase inhibitor (α_1PI, $\alpha_1$antitrypsin, SerpinA1)[3]. In HIV-1 disease, α_1PI is inactivated due to disease processes [4]. In the early asymptomatic categories of HIV-1 disease, active α_1PI was found to be below normal in 100% of untreated HIV-1 patients (median=12 μM, and to achieve normal levels during the symptomatic categories[4, 5]. This pattern has been attributed to immune inactivation, not to insufficient synthesis, proteolytic inactivation, or oxygenation. We observed that in HIV-1 subjects with >220 CD4 cells/μl, CD4 counts were correlated with serum levels of active α_1PI (r^2=0.93, p<0.0001, n=26) and inactive α_1PI (r^2=0.91, p<0.0001, n=26) [5]. Administration of α_1PI to HIV-1 infected and uninfected subjects resulted in dramatic increases in CD4 counts suggesting α_1PI participates in regulating the number of $CD4^+$ T cells in blood [3].

With stimulation, whole saliva contains sufficient serous exudate (plasma containing proteinaceous material that passes through blood vessel walls into saliva) to allow measurement of active α_1PI and the correlation of this measurement is evidence that it is an accurate method for calculating CD4 counts. Briefly, sialogogues such as chewing gum or citric acid stimulate the exudation of serum into whole mouth saliva. After stimulating serum exudation, the activity of serum α_1PI in saliva is measured by its capacity to inhibit elastase activity. Porcine pancreatic elastase (PPE) is a readily available inexpensive source of elastase. PPE binds to α_1PI forming a one-to-one complex that prevents PPE from cleaving its specific substrates, one of which is the colorimetric peptide, succinyl-L-Ala-L-Ala-L-Ala-p-nitroanilide (SA^3NA). Incubating saliva with a saturating concentration of PPE for 10 min at room temperature allows the binding of PPE to all the active α_1PI in saliva. The resulting inhibition of PPE by active α_1PI can be measured by adding the PPE substrate SA^3NA. **(Figure 1)**. Although CD4 counts are measured in terms of blood volume (CD4 cells/μl), the concentration of α_1PI in saliva is related to the concentration of serum in saliva, not to volume of saliva since volume can vary considerably during the day and person to person[6]. However, virtually all the protein in saliva is due to serum content, and the protein content of saliva is measurable[7]. Thus, active α_1PI in saliva is calculated as a ratio to saliva protein content and is termed the α_1PI Index. Results presented herein demonstrate that the α_1PI Index provides an accurate and precise physiologic method for calculating CD4 counts.

Video Link

The video component of this article can be found at http://www.jove.com/video/3999/

Protocol

1. Prepare Fresh Working Solutions

1. **TBS:** 0.05 M Tris Buffered Saline, pH 7.8 (TBS). Prepare 60 ml TBS/microplate.

2. **PPE:** Porcine pancreatic elastase, type 1 (PPE) is provided as a suspension. Shake well and dilute PPE in TBS to approximately 0.01 U/ml. Prepare 6 ml working solution/microplate).
3. **SA^3NA:** Succinyl-L-Ala-L-Ala-L-Ala-p-nitroanilide (SA^3NA). Prepare 0.1 M stock SA^3NA solution in dimethyl sulfoxide (DMSO) and use immediately or store at -20 °C. Prepare 6ml working solution/microplate by diluting stock SA^3NA solution 1/100 in TBS.
4. **COOMASSIE BLUE:** Prepare a stock solution of 10% Coomassie Brilliant Blue R-250 (Coomassie Blue) in TBS and use immediately or store at -20 °C. Prepare 6 ml working solution by diluting stock solution 1/1000 in TBS.

2. Prepare Saliva

1. **SALIVA COLLECTION:** In the standing position, stimulate serum exudation into saliva by placing a sialogogue such as citric acid or chewing gum in the mouth. A sugar-free lemon drop is convenient for HIV-1 patients who are frequently edentulous and unable to chew gum. Swallow as needed for the first 3 min, and collect whole mouth saliva into a capped cup for the next 3 min. This will provide approximately 2 ml of saliva. Use immediately or store at -80 °C.
2. **SALIVA HANDLING:** If frozen, thaw specimen at 37 °C and place on ice. Store fresh or thawed specimen at 2-4 °C no more than 3 days. Do not vortex saliva and avoid pipetting mucin which contains no serum exudate. Use Standard Precautions when handling saliva http://www.cdc.gov/hicpac/pdf/isolation/Isolation2007.pdf .

3. Microplate Set-up

1. **BUFFER:** Measurements are performed in triplicate. To each well of a microtiter plate is added 130 µL TBS.
2. **SPECIMENS:** To each test well, is added 20 µL saliva for a final volume of 150 µl/well. Mix by pipetting without introducing bubbles.
3. **CONTROLS:** The positive control contains PPE, but no saliva. The negative control contains saliva, but no PPE.To the positive control well is added 20 µL TBS. To the negative control well is added 20 µL saliva.
4. **PPE INCUBATION:** Shake PPE working solution well. Add 50 µL PPE working solution to each well excluding the negative control wells. To the negative control wells, add 50 µL TBS. Incubate 10 min at 23 °C.
5. **SUBSTRATE INCUBATION:** Add 50 µL SA^3NA working solution to each well. Incubate 45 min at 23 °C.
6. **COOMASSIE BLUE:** Add 50 µL Coomassie Blue working solution.
7. **READ-OUT:** Read absorbance in microplate reader, $A_{405\ nm}$ to detect SA^3NA cleavage which represents active α_1PI and $A_{595\ nm}$ to detect Coomassie Blue which represents protein content.

4. Calculate α_1PI Index

1. **NORMALIZE THE AVERAGE $A_{405\ nm}$ AND $A_{595\ nm}$**: For each triplicate, calculate the average. Subtract the Negative Control average from the Specimen average. To minimize plate-to-plate variation, normalize the averages by dividing each Specimen average by the Positive control average as follows:

$$\text{Normalized Absorbance} = \frac{\text{(Specimen average - Negative Control average)}}{\text{(Positive Control average - Negative control average)}}$$

2. **CALCULATE THE α_1PI INEX:** Divide the normalized $A_{405\ nm}$ by the normalized $A_{595\ nm}$:

$$\alpha_1\text{PI Index} = \frac{\text{Normalized } A_{405nm}}{\text{Normalized } A_{595nm}}$$

5. Representative Results

To determine whether the α-test might be suitable for use as a point-of-care screening test to monitor CD4 counts in endemic, resource-limited regions, stimulated saliva was collected from 20 female and 11 male HIV-1 subjects attending clinic for routine care in Cameroon. Consistent with preliminary observations during development of the α-test using saliva collected in New York City, the α_1PI Index in saliva collected in Cameroon correlated with CD4 counts (r^2=0.91, p<0.0001. n=31). The relationship was defined by a 3-parameter sigmoidal curve which is consistent with a dose-response relationship **(Figure 2A)**. The 95% confidence and prediction intervals are depicted with blue and red lines, respectively **(Figure 2B)**.

In a previous study, we determined that $CD4^+$ T cells exhibit sinusoidal cycling with periodicity 23±3.5 days in HIV-1 subjects and in subjects with the inherited version of α_1PI deficiency (PIZZ)[3]. Depicted herein is a representative example of computer-generated sine curve analysis of CD4 cycling in a non-HIV-1 healthy control exhibiting 27 day periodicity **(Figure 3A)**.

Computer-generated sine curve analysis of the $CD4^+$ T cell changes in 6 subjects yielded values for peak-to-peak amplitude and axis of oscillation[3]. As would be expected, the axis of oscillation was correlated with amplitude (r^2=0.96, p=0.009, n=6) (**Figure 3B**). In patients will low CD4 T^+ cell counts, the cyclic changes in CD4 T cell count were small, and in patients with high $CD4^+$ T cell counts, cyclic changes were large.

Because the regression line depicted in **Figure 2** estimates the axis of oscillation and the axis of oscillation is correlated with amplitude, amplitude can be calculated for the regression line thereby estimating how far above and below the regression line $CD4^+$ T cells would be expected to vary due to cycling (**Figure 3C**). Overlaying the amplitude calculated in **Figure 3C** and the confidence interval calculated in **Figure 2B**, it was found that amplitude (green line) lies within the 95% the confidence interval (blue line) (**Figure 3D**).

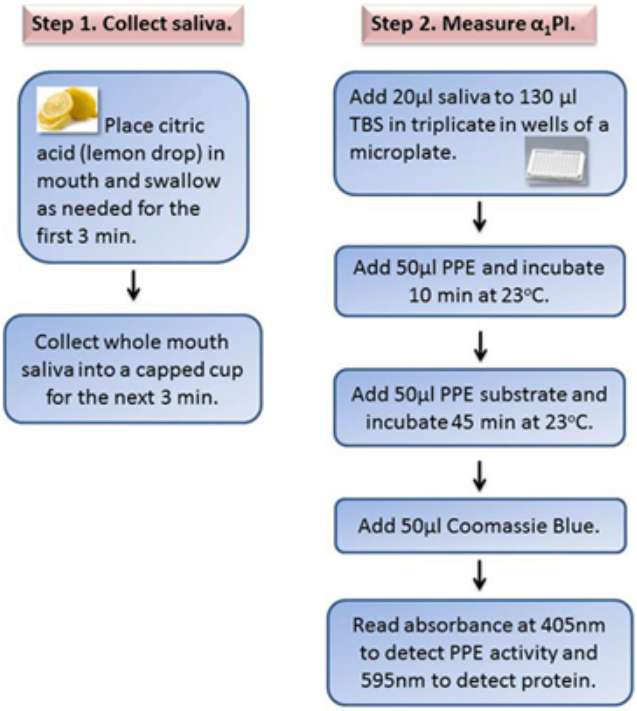

Figure 1. Procedure Summary: Place citric acid in mouth and collect saliva. Add diluted saliva to wells of a microplate in triplicate. Add PPE to each well and incubate for 15 min at 23 °C. Add the PPE substrate to each well and incubate for 45 min at 23 °C. Add Coomassie Blue and read at 405 nm to detect PPE activity and at 595 nm to detect protein.

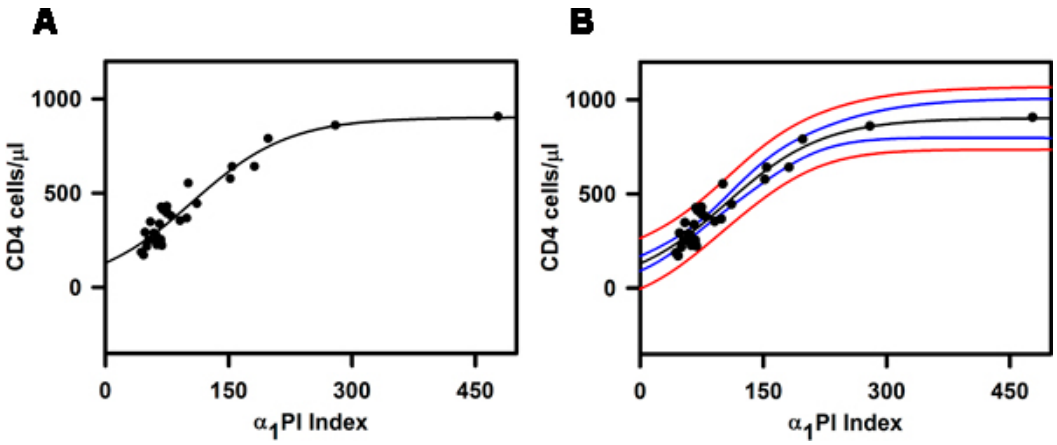

Figure 2. Comparison of the α-test with the standard CD4 enumeration method. A) The α-test was performed by quantitating the α_1PI Index in stimulated whole mouth saliva. CD4 counts were performed by an independent medical laboratory using standard flow cytometry. Blood for flow cytometry and saliva were collected at the same clinic visit. The relationship between the α_1PI Index and CD4 counts was defined by a 3-parameter sigmoidal curve (r^2=0.91, p<0.0001, n=31). **B)** The 95% confidence and prediction intervals are depicted in blue and red lines, respectively.

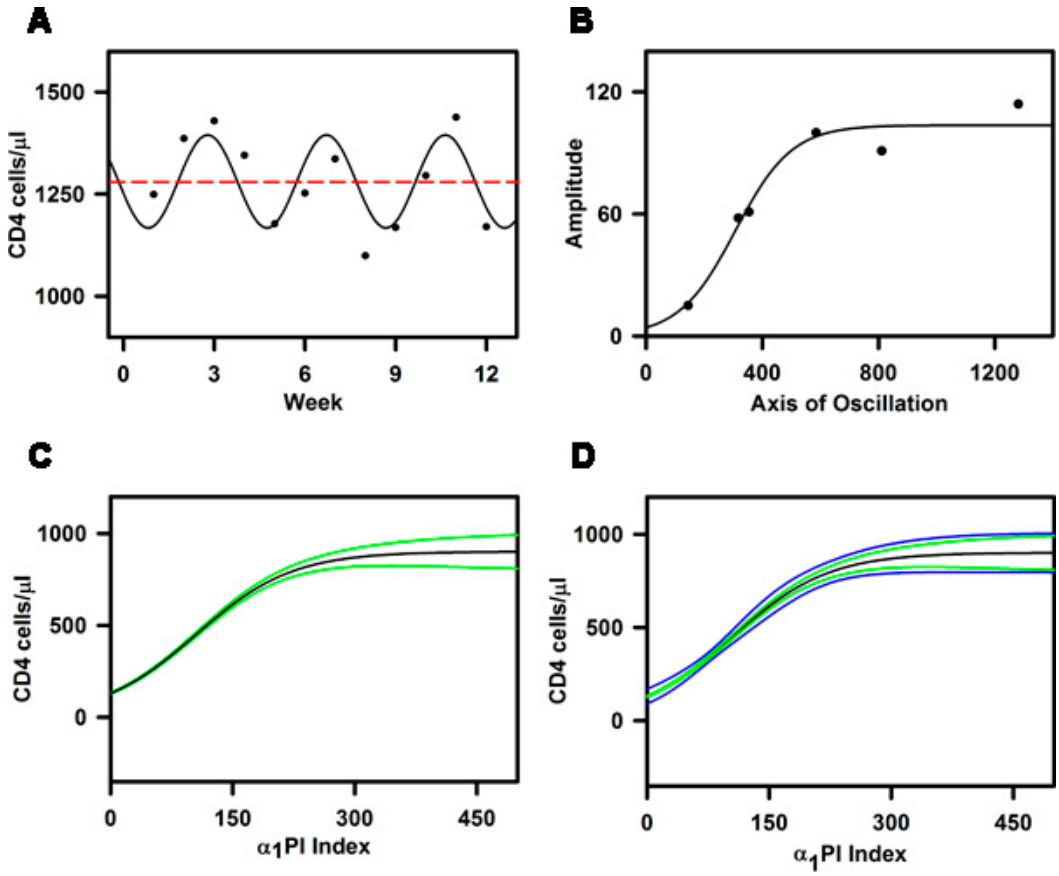

Figure 3. Accuracy of calculating CD4 counts using the α1PI Index. A) $CD4^+$ T cells exhibited sinusoidal cycling with 27 day periodicity in a non-HIV-1 healthy control. The axis of oscillation is depicted (red dotted line). **B)** Computer generated sine wave analysis of CD4 T cell cycling was used to calculate the peak-to-peak amplitude and axis of oscillation in 4 HIV-1 subjects, 1 non-HIV-1 PIzz subject, and the non-HIV-1 healthy control depicted in part A. The relationship between CD4 T^+ cell amplitude and axis of oscillation was defined by 3-parameter sigmoidal curve (r^2=0.96, p=0.009, n=6). **C)** The sigmoidal relationship between amplitude and axis of oscillation derived in part B) was used to calculate the expected amplitude (green lines) of points along the regression line (black line) from **Figure 2**. **D)** The expected amplitude interval (green lines) was significantly different from the 95% confidence interval (blue lines) as determined by the Mann Whitney Rank Sum Test (based on a comparison of the median difference between the 95% confidence interval and regression line (63 cells/µl) and the median difference between the amplitude line and regression line (37 cells/µl); p=0.001).

Discussion

Undiluted serum contains median 36 µM α_1PI and 3.6 µM $\alpha_2$macroglobulin (α_2M) a 10-fold difference [8]. Both proteins compete for binding to PPE. These two characteristics, concentration and PPE affinity, have been exploited to develop a method that is capable of specifically measuring α_1PI in normal serum in the presence of competing α_2M [8]. This method can detect as little as 50 nM μ_1PI in 0.3% serum. At a serum dilution of 2.5%, binding of α_2M to PPE becomes undetectable, and this dilution contains about 900 nM α_1PI and 90 nM α_2M.

In contrast to serum which is composed of 10-fold more α_1PI than α_2M, whole mouth saliva contains a 20-fold difference [9]. We found in the present study that undiluted, stimulated saliva contains approximately 30-fold less α_1PI than serum or approximately 1200 nM α_1PI and would be expected to contain approximately 60 nM α_2M. The optimal saliva dilution for the α-test was found to be 13% stimulated saliva, and this dilution would be expected to contain approximately 160 nM α_1PI and 8 nM α_2M, a concentration of α_2M that is below the threshold of detection in the protocol. Due to the low concentration of α_2M, it is possible to accurately determine the specific activity of α_1PI in saliva without concern for competition from α_2M. Measurements of active α_1PI and protein content in saliva allowed the calculation of the α_1PI Index which was found to correlate with the standard method for measuring CD4 counts, flow cytometry. Importantly, the results of regression analysis (r^2=0.91, p<.0.001, n=31) show that the α-test can be used to accurately calculate CD4 T cell counts.

CD4 enumeration methods have not previously taken into consideration the variation that is due to cycling. In practical terms, CD4 counts can vary by as much as 200 cells/µl between the nadir and apex with a periodicity of 23-27 days, and this is true regardless of the method for measuring $CD4^+$ T cells [3]. Thus, the sine wave axis of oscillation provides a more accurate measure of an individual's true $CD4^+$ T cell count than a single day's measure because the axis of oscillation is constant hour to hour, week to week. While it may not be feasible to directly measure the axis of oscillation for an individual's $CD4^+$ T cells, this can be estimated using an average of longitudinal $CD4^+$ T cell counts as long as variation in CD4 counts does not exceed the expected amplitude. Large variations in CD4 counts would indicate the influence of variables other than cycling.

The regression line depicted in **Figure 2** is an estimate of the axis of oscillation. Calculation of the expected amplitude above and below the regression line showed that amplitude lies within the 95% confidence interval. The median difference between the 95% confidence interval and regression line is 63 CD4 cells/µl, and the median difference between amplitude and the regression line is 37 CD4 cells/µl. This can be interpreted to mean that 95% of the CD4 counts calculated from the α-test measurements will be within approximately 63 CD4 cells/µl from the actual CD4 counts and that approximately 58% of this difference will be due to CD4 cycling. Thus, the precision of the α-test is the distance between the confidence interval and amplitude which is approximately 26 CD4 cells/µl, and the accuracy of the α-test is the correlation coefficient of the regression line which is 0.95 or 95%.

There are several limitations to the α-test as described herein, but solutions are readily available. For example, even though the α-test described uses a microplate format, the ability to perform the α-test using a single saliva dilution allows the α-test to be used with dipstick technology, an instrument-free, technologically simple system. Approximately 2% of the saliva samples collected in Cameroon were too viscous to manipulate, and this is consistent with saliva collected in New York City. The application of DNase to such samples can be used to ameliorate viscosity thereby permitting measurement [10]. An additional solution might be to use pharmacologic sialagogues that specifically stimulate saliva secretion via the parasympathetic versus sympathetic pathway thereby producing watery saliva. One such sialagogue is pilocarpine [11]. Finally, the α-test has not yet been validated, and this is necessary to determine whether performance is sufficiently discriminatory for critical levels of CD4 counts [12].

The critical levels of CD4 counts are in the linear region of the sigmoidal regression curve which allowed these values to be calculated by a linear algorithm. In this population, the linear regression for values <860 CD4 cells/µl (r^2=0.86, n=30, $p<2x10^{-7}$) was as good as the sigmoidal regression (r^2=0.88, n=30, p<0.0001). The linear regression for α_1PI Indices above 67 which corresponds to approximately 350 CD4 cells/µl (r_2=0.88, n=15, p=0.002) was as good as the sigmoidal regression (r^2=0.89, n=15, p<0.0001). However, correlation was not significant at α_1PI Indices below 67 which corresponded to <350 CD4 cells/µl (r^2=0.17, p=0.54, n=15). This is a critical value since WHO guidelines recommend a CD4 count <350 CD4 cells/µl to qualify for access to antiretroviral therapy [13]. To better determine the performance of the α-test and improve on the sensitivity at lower values, an additional 800 saliva samples are being collected in Cameroon from 4 groups: newborns-2 yrs, 3-5 yrs and 6-15 yrs and 16 yrs and older.

In conclusion, the α-test is physiologically relevant to the number of CD4 cells in blood yet can be performed using saliva thereby providing a noninvasive, accurate and precise point-of-care method for monitoring CD4 counts in endemic regions with no instrumentation at a cost-per- test that is less than a dollar.

Disclosures

No conflicts of interest declared.

Acknowledgements

Research was supported by the Harry Winston Research Foundation.

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