

# **Evaluation of a CD4-aptamer for Flow Cytometry-based Enumeration of T-cell Subsets**

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## **Disclosures**

Aptamer reagents used in this thesis were generously provided in collaboration by SomaLogic, Boulder, CO. Additional general laboratory and flow cytometry reagents were provided by the University of Washington, Hematopathology Laboratory.

The conclusions, results, and data interpretation detailed herein were solely the work of the author and thesis committee members with no specific influence by SomaLogic

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## Introduction

### Role of CD4 T cells in HIV infection

Human immunodeficiency virus (HIV) infection, a sexually transmitted infection caused by Lentivirus, has become a pandemic since it was first reported in 1981 [1]. The U.S. Center for Disease Control (CDC) estimates that more than one million people are living with HIV in the United States. A global summary of HIV prevalence by WHO reported that approximately 34 million of people are living with HIV, and that AIDS deaths total 1.7 million in 2011.

CD4 T cells, also known as T helper ( $T_H$ ) cells, have a critical role in coordinating the immune system by helping the activation of B cells, cytotoxic T cells, macrophages, and various other cells that involve in the immune response [2]. These cells are also the primary targets of HIV, which selectively infects cells expressing surface CD4 molecule. The CD4 molecule serves as a primary receptor for the virus, helping the virus to bind to host cells as the initial step in viral infection.

Persistent viral infection directly and indirectly destroys CD4 T cells, which leads to a progressive decline in the CD4 T cell population. Such depletion causes substantial defects in both the cellular and humoral immune responses. In the last phase of infection, the number of CD4 T cells decreases to a point ( $< 200$  cells/ $\mu$ l)

that ultimately leads to acquired immunodeficiency syndrome (AIDS), in which patients develop increasing susceptibility to a broad spectrum of opportunistic infections and neoplasms due to the collapse of the immune system.

### **Role of Absolute CD4 T-cell Counts**

A decrease in CD4 T cells is the hallmark of acquired immunodeficiency syndrome (AIDS). Accordingly, measurement of the absolute CD4 T-cell count is an effective marker for monitoring HIV disease progression, an important indicator for initiating anti-retroviral therapy (ART), and a tool for evaluating the effectiveness of clinical treatment. Measurement of CD4 T-cell count has remained the most robust method for monitoring immune competence in HIV patients.

### **Absolute CD4 T-cell Counts by Flow Cytometry**

Flow cytometry-based methods are the gold standard for measuring absolute CD4 T-cell counts. With precision and accuracy, multicolor flow cytometric analysis has been widely used in clinical settings for determining absolute CD4 T-cell counts, especially in high-volume laboratories [3].

Dual-platform and single-platform are the two common approaches for CD4 T-cell measurement in flow cytometry assay. For the dual-platform approach, absolute CD4 T-cell counts are derived from the CD4 T-cell percentage as measured by flow cytometry and the absolute lymphocyte count as determined by a hematology analyzer. For the single-platform method, absolute CD4 T-cell counts can be assessed by flow cytometry alone, with the use of either built-in volumetric system or the use of reference beads for quantitation [4]. The latter, single-platform approach has been considered to be the preferred method since studies have reported that the single-platform method has less variation than the dual-platform approaches [3].

### **Alternatives for an Affordable Absolute CD4 T-cell Counts**

Anti-retroviral therapy (ART) has been considered a promising intervention strategy to control HIV/AIDS with proven clinical outcome and relative cost-effectiveness due to decreasing costs of these therapies. Substantially decreasing costs in these therapies are especially beneficial for under-developed and developing countries, as many of these countries have substantial proportions of their population infected by HIV.

The measurement of absolute CD4 T-cell counts by flow cytometry has remained the most reliable and important measurement for monitoring HIV-infected patients in resource-limited settings. However, the costs of this laboratory-based measurement remain relatively high, which can be challenging to perform in resource-poor countries where a large population of patients need to be screened. A cost-effective monitoring method is in urgent demand because a cost-effective, accurate laboratory monitoring is essential for leading successful ART programs in resource-constrained countries [3].

## **Aptamer**

Aptamers are short single-stranded oligonucleotides synthetically generated from large random sequence libraries with specific ligand binding properties [5]. Synthesized aptamers are approximately 20 to 80 bases in size (6 to 26kDa) [6]. Aptamers usually form various three-dimensional structures by base-pairing and intra-molecular hybridization [7], and are able to bind to target ligands similar to antibodies with high binding specificity and affinity, and with low pico- to nanomolar equilibrium binding constants ( $K_d$ ) [8]. In addition to comparable sensitivity and specificity as compared to monoclonal antibodies, aptamers may be generated to bind

to a wide range of target molecules, including small organic molecules, peptides, proteins, etc.

### **SELEX (Systematic Evolution of Ligands by Exponential enrichment)**

In a process referred to as *Systematic Evolution of Ligands by Exponential enrichment* (SELEX), aptamers may be selected and generated by an *in vitro* process to enhance binding specificity and affinity to target antigens or ligands, as illustrated in **Image 1**. This process starts with a large, typically random, oligonucleotide library pool consisting of at least  $10^{12} \sim 10^{15}$  single-stranded DNA or RNA sequences fixed with constant regions at both ends [9]. The pool is incubated with the target of interest for positive selection so that aptamer sequences that specifically bind to the target (such a chemical ligand or a cell surface molecule) are separated from the unbound sequences by a partitioning technique, e.g. chromatography, magnetic beads, or via a filter [6]. Negative selection of aptamers can also be performed to eliminate the sequences that bind to closely related analogs of the target (or for example, control cell), enhancing the specificity of the aptamers of interest. The bound sequences are then amplified by PCR and in repeated rounds of selection with increasing stringency, to increase binding affinity [10]. The selected aptamer pool may be highly enriched

after 5 to 20 rounds, and the resulting sequences are then cloned into vectors and sequenced to identify individual aptamers. A traditional SELEX technique can produce aptamers in a large scale within a short time, typically 2 to 3 months [11].

### **Potential Value for Clinical Use**

Antibodies have been one of the most valuable and widely used reagents in the clinical diagnostic laboratory. Despite excellent performance characteristics in clinical assays, antibodies have certain limitations due to their biological nature. Aptamers, on the other hand, may not have similar limitations and have therefore been considered a potential alternative for clinical diagnostic assays. Unlike antibodies, which depend on either animals or cell lines in production and discovery, aptamers are raised by an *in vitro* process. Accordingly, aptamers, in contrast to antibodies, may be developed for ligands that are poorly immunogenic or that are toxic. Furthermore, permutations of the SELEX technique have been developed by various investigators such that whole cells (such as human tumor cells) can be used in the selection process to identify unique, cell-specific aptamers even without prior knowledge of target molecules [12]. Some investigators have demonstrated the utility of SELEX for developing aptamers against leukemia cells and microorganisms

[13]. Because of the potentially wide range of antigens that can be detected using aptamers, the application of aptamers to the clinical diagnostic laboratory is a promising approach for new disease-related biomarker discovery.

Aptamers have additional benefits related to their derivation from synthetic materials. For instance, aptamers can be chemically modified or labeled without affecting the binding affinity to the targets to enhance functionality [14]. For example, nuclease-resistant RNA aptamers can be synthesized by modifying the ribose backbone to enhance stability of these reagents. Alternatively, to enhance the utility of aptamers in diagnostic assays, different chemical (or fluorescent) reporters can be attached to aptamers at precise locations without decreasing aptamer's binding affinity. These characteristics not only make aptamers versatile analyte-specific reagents for diagnostics assays, but these traits also permit clinical application as targeted therapeutics. Specifically, aptamers may be directly conjugated with drugs or with carriers as part of drug delivery devices [15].

For biologics like antibodies, there may be substantial between-batch variation and immunoassays may need to be re-optimized when a new batch of antibody reagents is used. On the other hand, there is little or no batch-to-batch variation in aptamer production because the chemical synthesis process is highly controlled. Last, but not least, unlike antibodies which may be temperature sensitive and undergo

irreversible denaturation, aptamers are generally extremely temperature-stable with nearly indefinite shelf-lives when stored lyophilized.

Due to the potential of aptamers to serve as an alternative for antibodies, many investigators have explored the use of aptamers in clinical diagnostic assays. It has been suggested that an aptamer assay may be approximately 1,000 fold less costly than a traditional antibody assay [16]. This feature would be significantly valuable for laboratories in resource-limited countries, particularly where flow cytometry-based assessment of CD4 T-cell subsets is frequently performed.

In this study, we designed an aptamer flow cytometric assay by combining a commercially provided, biotinylated CD4 aptamer and PE-Streptavidin with multicolor antibodies for T cell subset enumeration. We evaluated the clinical value of the CD4 aptamer in CD4 T cell quantitation of patient specimens, and we demonstrate the potential to adopt these reagents for routine T-cell subset enumeration.

## Materials and Methods

### Method Development

The routine manual procedure for sample preparation in the UW Hematopathology Laboratory is as follows: 100µl of a patient's sample, typically peripheral blood, is added to the antibody cocktail of choice and incubated in the dark for 15 minutes. 400 µl lyse-fix reagent composed of lysing buffer (Sigma reagent: 8.02 g/L, 0.84g/L NaHCO<sub>3</sub>, 0.37 g/L EDTA) and 0.25% paraformaldehyde (10% Formaldehyde Ultrapure, manufactured by Polysciences) is then added for another 15-minute incubation. After centrifugation for 5 minutes at 1700 rpm, the supernatant is decanted, and 3 ml of wash buffer is added. The sample is centrifuged again with the second wash also decanted. The now washed cell pellet is re-suspended in 100µl of wash buffer and is ready for subsequent flow cytometry evaluation of cell surface antigen expression (LSR-II, BD Biosciences).

In order to develop an optimal working procedure for the CD4 aptamer assay, multiple studies described below were performed to determine the optimal working conditions for the study assay.

## **Specimen processing**

All samples used in the study group were residual K3-EDTA anti-coagulated clinical samples submitted for T cell subset testing. The peripheral blood specimens used in study assay were one day old on average and were kept at room temperature. The samples used were de-identified clinical material. A lymphocyte count was obtained by a hematology analyzer (Horiba ABX Pentra 60) for each sample before specimen processing. Three specimen processing procedures were compared to determine the optimal procedure: the routine method, PBMC isolation, RBC lysis prior to cell staining.

For PBMC isolation, 4 ml of peripheral blood was added to 4 ml of PBS in a 15 ml tube. The mixture was then gently transferred to another tube containing 4 ml of Ficoll-Paque, without disturbing the interface. Sample was then spun at 1700 rpm for 25 minutes. After centrifugation, plasma was pipetted off and the buffy coat layer which contains PBMC was carefully aspirated and transferred to a new tube. Wash buffer was then added to the sample to a total volume of 15 ml and spun down for 10 minutes. The wash was decanted and the pellet was washed again and then spun down. The supernatant was decanted and cell pellet was re-suspended in wash buffer to a total volume of 1 ml.

For the RBC lysis procedure, 1 ml aliquot of whole blood was added to 6 ml of RBC lysis buffer and incubated in room temperature for 15 minutes. The sample was spun down for 10 minutes at 1700 rpm and decanted. 15 ml of wash buffer was added and the sample was spun, decanted and washed again. The wash was then decanted and the cell pellet is re-suspended in 500  $\mu$ l of wash buffer, ready to be incubated with CD4 aptamer and antibodies.

The results as seen in **Image 3** show that PBMC isolation and RBC pre- lysis procedures are both suitable methods to remove erythrocytes in the samples without influencing the CD4 aptamer binding to CD4 T cells. However, the pre- lysis method is less labor-intensive and time-consuming than collecting PBMC by Ficoll-Paque. Therefore, RBC lysis prior to cell staining was determined as the sample processing procedure for the study assay.

### **Binding condition**

Antibodies used in the study were obtained from Beckman Coulter, Becton Dickinson, and BioLegend as listed in **Table 1**. In the preliminary study, a combination of antibodies consisting CD3-PC5 and CD8-ECD was added in the study assay. 100  $\mu$ l of sample was incubated with the antibody cocktail along with 100  $\mu$ l

of biotinylated CD4 aptamer reagent in each tube. After the cell-binding reaction, the mixture was washed twice with 3ml wash buffer, and the supernatant was decanted. 10  $\mu$ l streptavidin-PE solution (0.2 mg/ml) was then added in the tube and incubated with the sample at 4°C for 5 minutes. 400  $\mu$ l of fix buffer (0.25% formaldehyde in 1X PBS) was added and the tube was incubated at room temperature for 15 minutes in the dark. The sample is then centrifuged for 5 minutes at 1700 rpm and decanted. 3 ml of wash buffer was added and the sample was centrifuged and decanted again. The cell pellet was re-suspended in 100  $\mu$ l of wash buffer and ready for detection on BD LSR II flow cytometer.

To determine the optimal cell-binding buffer for the study assay, CD4 aptamer reagent was prepared by two solutions respectively: wash buffer (PBS+0.3%BSA) and 1X SB-18 (SomaLogic, Boulder, CO), the selection buffer used in the production of the CD4 aptamer. As shown in **Image 6**, the CD4 aptamer did not bind to CD4 T cells under wash buffer condition. 1X SB-18 was found to be the ideal binding buffer for CD4 aptamer assay and was used throughout this study.

### **Reagent concentrations and incubation times**

Varying concentrations of CD4 aptamer reagents, 100nM, 200nM, 400nM, and

800nM were incubated with aliquots from the same sample to determine the optimal working concentration. As seen in **Image 5**, the optimal aptamer concentration was found at 800 nM. This was the lowest concentration which adequately separate CD4+ T cell populations were constantly given. Based on the provided affinity constant for this synthesized aptamer (SomaLogic, Boulder CO; personal communication), this concentration also is readily at least ~20 fold  $\times$  Kd, permitting binding of binding saturation at equilibrium.

A time-course study was performed as well to determine the incubation time for the aptamer assay. 800 nM of CD4 aptamer was incubated with aliquots of the same sample at room temperature for 10, 20, and 60 minutes, respectively. The antibody cocktail CD3-PC5 and CD8-ECD was added in each tube at the same time except for the 60 minute-incubation, in which antibodies were added in the last 20 minutes of incubation to hold the antibody incubation time consistent at less than 20 minutes. The results showed that 60 minute-incubation gave the best separation of CD4+ T cell population by the CD4 aptamer and was used throughout the study, as shown in **Image 7**.

Concurrently, another time-course study was also conducted to determine the most efficient incubation time for streptavidin-PE. 10  $\mu$ l of streptavidin-PE was incubated with the sample in 4°C after its incubation with antibodies and the biotinylated CD4

aptamer. 5 minutes, 10 minutes, 15 minutes, and 20 minutes of streptavidin-PE incubation were tested. As seen in **Image 8**, the optimal working condition of streptavidin-PE was found at 5-minute incubation at 4°C.

### **Multiplexing with CD4-FITC antibody**

In order to establish a CD4 aptamer assay for T-cell subset analysis, CD45-FITC antibody, a pan-leukocyte marker, was added to the panel. CD45-FITC (clone J.33), the routine clone used in the UW Hematopathology Laboratory, was combined with CD3-PC5, CD8-ECD, and CD4 aptamer for the detection of CD4+ population. The cell-binding patterns were then compared with that of the aptamer panel without CD45-FITC, as shown in **Image 9**. The results showed, however, that non-specific binding interaction of CD4-PE aptamer to CD8 T cells was evident in the presence of J.33 clone of CD45-FITC.

Due to the non-specific binding shown in the panel, another clone of CD45-FITC, HI30, was substituted for clone J.33 and tested. In these results, no cross-binding reaction was found in this combination—specifically, the CD4 aptamer had identical CD4 T cell-binding pattern to that from the aptamer panel without CD45-FITC antibody (see **Image 9**). It was then determined that a suitable multicolor panel for

the CD4 aptamer flow cytometry based assay could include CD3-PC5, CD8-ECD, CD4 aptamer, and HI30 clone of CD45-FITC antibody without non-specific interaction. This reagent combination was used throughout the remaining studies.

### **Multiplexing with CD4-A594 antibody**

To further characterize the CD4 aptamer, a competition assay was performed to study the binding capacity of the CD4 aptamer in the presence of a CD4 antibody. Samples were simultaneously stained with the multicolor CD4 aptamer panel and CD4-A594 antibody. Interestingly, the binding of CD4 aptamer to CD4 T cell subset was not affected by the presence of the CD4-A594 antibody, suggesting that the aptamer and the CD4-A594 antibody bind to different epitopes and do not sterically hinder each other. CD4<sup>+</sup> T-cell binding patterns were the same when the aptamer and the CD4 antibody were incubated together, as shown in **Image 10**. This result showed no substantial evidence of competition between the CD4 aptamer and the CD4 antibody-A594. Consequently, this CD4-A594 antibody was also combined with the CD4 aptamer reagent panel, to serve as an internal antibody control for CD4 aptamer in a single tube for each sample throughout the remaining studies. This reagent combination including both a CD4-aptamer (PE fluorochrome labeled) with a

CD4-antibody (A594 fluorochrome labeled) further allowed us to minimize the reagents used for setting up a control in a second tube.

### **T cell Phenotyping**

The use of CD4-PE aptamer, CD3, CD8, CD45, and CD4-A594 antibodies permitted the quantification of the CD4<sup>+</sup> T cell population using a gating protocol as follows: (See **Image 2**)

First, singlet cells are gated by using the flow cytometry software (Woodlist, version 2.6.7) using a plot of forward scatter height (FSC-H) versus forward scatter area (FSC-A), to aggregated cells in the analysis. A sequential created lymphocyte gate is then set around lymphocyte population, which is defined as CD45<sup>bright</sup> with low SSC-H. CD3<sup>+</sup> T cells are then subsequently selected from the lymphocyte population. CD4<sup>+</sup> T cells are then further identified as CD4<sup>+</sup>CD8<sup>-</sup> by separate gating of CD4 and CD8 subsets of the CD3<sup>+</sup> T-cell population.

### **Data Collection and Analysis**

Each sample was prepared using the optimized preparative procedure as previously detailed. Samples were analyzed using a Becton-Dickinson LSRII flow

cytometer, with 50,000 events collected in each sample. Analysis was performed using in-house computer software (WoodList, version 2.6.7).

To quantitate CD4<sup>+</sup> T cells subset, both the proportion of CD4 T cell and absolute CD4 T cell count were analyzed. The percentage of CD4 T cells over all lymphocytes was obtained by multiplying the CD3 percentage of total lymphocytes by the CD4 T-cell percentage in the CD3 lymphocyte population. To calculate the absolute CD4 count, CD4% of total lymphocytes from the flow cytometer was multiplied by the lymphocyte count using an ABX hematology analyzer.

In order to evaluate the performance characteristics of the CD4 aptamer for multicolor flow cytometric analysis of CD4 T cell subsets, the proportion of CD4 T cell and absolute CD4 T cell count from the study assay were compared with those from the standard CD4 T cell subset assay in the lab, defined as the predicate method. Correlation between the measurements obtained by the reference and the study methods were established by linear regression. The degree of agreement between two methods was illustrated by Bland-Altman analysis. The differences in CD4 percentages between two methods (CD4 values from the study method - CD4 values from the predicate method) were plotted against the mean difference, the bias. Limits of agreement (bias $\pm$ 2SD) were also calculated, which represent the 95% CI of the mean difference. If the range is not clinically significant, the two methods can

be used interchangeably [17].

To further evaluate the binding capacity of the CD4 aptamer to CD4 T cells as compared to antibody under the same condition, the measurements of CD4 T-cell percentage and CD4 absolute count determined by the CD4 aptamer were compared with those by the corresponding internal CD4-A594 antibody as well, by the same statistical analysis.

### **Predicate Method**

In general, clinical specimens submitted for routine T-cell subset evaluation in the UW Hematopathology Laboratory is performed using the Prep-Plus automated instrument (Beckman Coulter TQ-Prep™ Workstation) using a lyse no-wash sample preparation method. In the sample prep station, 100µl of whole blood is stained with 10 µl of CYTO-STAT tetraCHROME reagent (CD45F/CD4RD1/CD8ECD/CD3PC5) in a TruCOUNT Tube coupled with lyophilized reference beads and processed by T-Q-prep reagents. The processed sample is then run on the FC-500 flow cytometer for automatically calculating the percentages and absolute counts of CD4 T cells.

**Table 1. Reagents**

<b>Antibody</b>	<b>Clone</b>	<b>Isotype</b>	<b>Product#</b>	<b>Manufacturer</b>
CD3-PC5	UCHT1	IgG1, Mouse	IM2635U	Beckman Coulter
Anti-human CD4-PE	13B8.2	IgG1, Mouse	IM0449U	Beckman Coulter
CD4-A594			339403	BD
Anti-human D8-ECD (Phycoerythrin-Texas Red-X)	SFCI21Thy2D3	IgG1, Mouse	6604728	Beckman Coulter
Anti-human CD45-FITC	HI30	IgG1, Mouse	304006	BioLegend
Anti-human CD45-FITC	J33	IgG1, Mouse	IM0782U	Beckman Coulter
Cytostat TetraChrome CD45FITC/CD4RD1 /CD8ECD/CD3PC5	B3821F4A/ SFCI12T4D11/ SFCI21Thy2D3/ UCHT1	IgG2b, Mouse/ IgG1 Mouse/ IgG1 Mouse/ IgG1 Mouse	6607013	Beckman Coulter
Strept avidin-PE			405203	BioLegend
<b>Aptamer</b>	<b>Kd</b>	<b>Modified base</b>	<b>Product#</b>	
CD4 aptamer	5 nM	BndU	ABDC-3143-3	SomaLogic
CD4 aptamer	10 nM	TrpdU	ABDC-3153-45	SomaLogic
CD4 aptamer	60 nM	NapdU	ABDC-3164-59	SomaLogic

**Table 2. Buffer**

Buffer	Ingredients
10X Selection buffer 18 (SB18)	40mM HEPES 101mM NaCl 5mM KCl 5mM MgCl <sub>2</sub>
10X RBC Lysing buffer (1L in DH <sub>2</sub> O)	80.2g NH <sub>4</sub> Cl 8.4g NaHCO <sub>3</sub> 3.7g EDTA
Fix buffer (in 1X PBS)	0.25% formaldehyde (0.375ml 10% formaldehyde/15ml PBS)
Wash buffer	PBS+0.3%BSA

## Results

As shown in **Image 10**, the immunophenotypic pattern of CD4 T cells by the CD4 aptamer was with similar to that as assessed by the CD4-A594 antibody. In addition, the combination of CD4 aptamer in the multicolor antibody panel did not affect the cell binding characteristics of antibodies for CD3, CD8, and CD45. However, non-specific binding with the CD4 aptamer-PE was noted in CD8<sup>+</sup> T cells in some samples, as shown in **Image 11**.

### Evaluation of accuracy in CD4 T cell percentages

20 samples submitted for the clinical T cell subset test were randomly selected after routine testing, and tested by the CD4 aptamer assay. The values of CD4 percentage obtained from the CD4 aptamer assay were compared with those from the predicate clinical assay.

A scatterplot showing the correlation for the CD4 T cell percentages by both assays are shown in **Image 12**, demonstrating excellent correlation with  $R^2 = 0.9288$  ( $P < 0.001$ ) between the two assays over the range of CD4 T cell percentages tested (CD4% range: 8.59% to 49.26%). Mean CD4 T cell measurement in percentage was  $35.55 \pm 12.65$  by CD4 aptamer, and  $30.15 \pm 10.64$  by the predicate method.

In the Bland-Altman analysis, comparison of CD4 percentages either by the CD4 aptamer or the CD4-A594 antibody in the aptamer assay with those by the predicate assay showed slight disagreement, as shown in **Images 13(A)** and **(B)**. The mean difference of CD4 percentages between the CD4 aptamer and the predicate measurements is +5.40% with limits of agreement between -1.90 to 12.68. This indicates that the aptamer gives higher values than the predicate clinical assay. The consistent pattern of higher CD4 values was also observed in data obtained by CD4-A594 antibody, which served as an internal antibody control in the aptamer assay, with a mean difference of +5.03 and limits of agreement between -2.69 to 12.76.

On the other hand, the performance of aptamer in CD4 percentage measurement was also compared directly to the CD4-A594 antibody, which was added in the aptamer panel as an internal control for CD4 T cell quantitation. The results in **Image 12(C)** show that the CD4 percentages obtained by the CD4 aptamer and CD4-A594 antibody were in excellent correlation, with  $R^2 = 0.9987$  ( $P < 0.001$ ). The Bland-Altman plot in **Image 13(C)** showed excellent agreement as well, with a mean difference of 0.37 and limits of agreement ranged from -0.65 to 1.38.

### **Evaluation of accuracy in absolute CD4 T cell numeration**

A scatterplot showing the correlation for the absolute CD4 T cell counts by both assays are shown in **Image 14**. A correlation of  $R^2 = 0.8643$  ( $P < 0.001$ ) was seen between two assays over the range of absolute CD4 T cell counts obtained from the 20 samples (150 cells/ $\mu\text{l}$  to 1342 cells/ $\mu\text{l}$ ). The Mean CD4 T cell count was  $788 \pm 405$  cells/ $\mu\text{l}$  by the CD4 aptamer and  $588.95 \pm 310$  cells/ $\mu\text{l}$  by the predicate assay.

In the Bland-Altman analysis of absolute CD4 counts between assays, as shown in **Image 15(A)**, paired data from the CD4 aptamer and the predicate method showed a mean difference of +198.7 cells/ $\mu\text{l}$  with limits of agreement between -122.2 cells/ $\mu\text{l}$  to +519.5 cells/ $\mu\text{l}$ . When analyzing the agreement between data from the CD4-A594 antibody and those from the predicate assay, as seen in **Image 15(B)**, the mean difference in CD4 T cell counts was +189.8 cells/ $\mu\text{l}$ , with limits of agreement between -137.3 cells/ $\mu\text{l}$  to +516.8 cells/ $\mu\text{l}$ .

Similarly to the finding in CD4 percentage measurements, the absolute CD4 counts obtained by the CD4 aptamer and CD4-A594 antibody showed excellent correlation in **Image 14(C)**, with  $R^2 = 0.9989$  ( $P < 0.001$ ). The Bland-Altman plot in **Image 15(C)** indicated that the paired measurements were in great agreement, with mean difference of +8.9 cells/ $\mu\text{l}$  and limits of agreement ranged from -18.4 cells/ $\mu\text{l}$  to

+36.2 cells/ $\mu$ l.

### **Evaluation of precision in CD4 T cell percentages**

The same 20 samples used in the accuracy study were also tested in duplicates for the repeatability study to evaluate the precision of CD4 aptamer testing. In the results, the duplicate testing by the CD4 aptamer showed that the coefficients of variation (CV %) were 1.97 for CD4 percentage and 2.14 for absolute CD4 count.

For the CD4-A594 antibody in repeatability study, the coefficients of variation (CV %) were 1.99 for CD4 percentage and 2.10 for absolute CD4 count.

## Discussion

As a promising alternative for antibody reagent in immunoassays, aptamers have previously been proven their utility in combination with antibodies for multicolor cell phenotyping.[16] In this study, we evaluated the clinical value of a CD4 aptamer in multicolor flow cytometric analysis for CD4 T-cell quantitation of patient specimens.

### Comparison of methods

In the methods comparison study, if a consistent bias is found between methods and the differences within mean  $\pm$  1.96 SD are not clinically important, it is possible to systematically adjust the results by subtracting the mean difference from the new method, and the two methods may be used interchangeably [17].

In the aptamer assay, higher values of CD4 measurement obtained by the CD4 aptamer were observed. The differences between the aptamer assay and the established assay in absolute CD4 counts are clinically significant (bias: +198.7 cells/ $\mu$ l; LOA: -122.2 to +519.5 cells/ $\mu$ l), since the recommended timing to initiate anti-retroviral treatment is set at 200 to 350 cells/ $\mu$ l, depending on which clinical stage the patient is in. Therefore, the CD4 aptamer assay is not acceptable to replace the established assay.

Interestingly, however, the discrepancies in CD4 T cell quantitation were also

observed between the CD4-A594 antibody control in the aptamer assay and the established method (bias: +189.8 cells/ $\mu$ l; LOA: -137.3 to +516.8 cells/ $\mu$ l). Moreover, when considering data from the CD4 aptamer and its corresponding internal CD4 antibody control in the aptamer assay, both CD4 proportions and absolute counts were in excellent agreement (bias: +8.9 cells/ $\mu$ l and LOA: -18.4 to +36.2 cells/ $\mu$ l). This finding indicates that the CD4 aptamer is highly comparable to the CD4 antibody which further suggests that variables other than the CD4 aptamer may be inherent in the aptamer assay.

Despite the differences seen between two methods, the CD4 aptamer showed good precision in the repeatability study, with less than 3% CV in both CD4 percentages and absolute counts.

### **Potential sources of bias**

A disagreement of absolute CD4 T cell counts was observed between two assays (aptamer-based versus antibody-based). This disagreement in absolute CD4 counts between assays is less likely caused by CD4 aptamer itself because CD4 counts obtained by the CD4 aptamer were in excellent correlation and agreement with the internal CD4 antibody control results. Factors that are likely to cause the variations

between assays are discussed as follows:

First, the use of two different approaches for cell enumeration is likely one of the causes leading to the lack of agreement in absolute CD4 counts between the aptamer assay and the established antibody-based one. This may be due to the fact that the total lymphocyte counts from the dual-platform aptamer method were obtained by a hematology analyzer, which has been shown to have more bias in determining absolute cell counts.[18]

Furthermore, erythrocyte lysing reagents have been reported to cause a significant loss of leukocytes due to cell membrane destruction.[19] Different erythrocyte lysis procedures also have been shown to affect cells with varying degrees. In addition, multiple washes and centrifugation in the aptamer assay may also lead to cell loss and changes in cell subset proportions, which would have a substantial impact on absolute cell enumeration and cell percentage measurement.

As shown in the preliminary studies, PBMC collection was also considered as a suitable approach for sample processing. It would be interesting to conduct a comparison study on sample preparation procedures by RBC lysis and PBMC collection. By evaluating and comparing the impact on CD4 T cell enumeration, we may be able to identify the potential variables that contribute to the bias in CD4 T cell enumeration.

Lastly, because only residual blood samples that were collected over the prior 24 hours were used in this study, cell viability may be affected over time and cause changes in cell quantitation and increased binding.

### **Strength and limitations**

As antiretroviral therapy is being successfully introduced to developing countries, a low-cost but accurate CD4 T cell quantitation assay must be implemented in the monitoring system to ensure optimal patient treatment. As compared to antibodies, aptamers can potentially be manufactured at a lower cost and be more cost- and energy-effective for storage and transportation due to their high stability in ambient temperature. These features particularly make them great candidate reagents for development of a cost-effective CD4 T-cell assay suitable for resource-limited countries where continued refrigeration may be problematic and affordable reagents are desperately in need.

CD4 percentage is the preferred measurement in children under five years old, according to WHO guidelines for CD4 T cells assessment in pediatric population. Even though the CD4 aptamer assay showed bias especially in absolute CD4 counts as compared to the predicate assay, this aptamer assay could nevertheless be used in

measuring CD4 percentage as an early monitoring assay or follow-up on effective ART to monitor the changes until the measured value approaches the clinical treatment threshold. At the time, sampling testing could then switch to the traditional antibody assay to ensure adequate measure of low CD4 values. This two-tiered approach will require clinical judgment of the local professionals whether the merits of a low-cost, but generally accurate aptamer assay substantially outweighs the limitations for the clinical use in poor resource settings.

### **Future studies**

In order to reduce the variation caused by the dual-platform method in T-cell subset quantitation, modifying the CD4 aptamer assay to a no-wash, single-platform method could increase the accuracy of an aptamer-based assay. A no-wash sample preparation will prevent the cell loss due to washing and centrifugation as well as reduce processing time by at least 50 minutes, which could be beneficial in urgent clinical situations.

One thing needs to be carefully considered when modifying the test is the erythrocyte lysis procedure in sample preparation. Low pH has been indicated to affect the three dimensional structure of aptamer, which would alter the structure of

aptamer binding site and substantially reduce its binding affinity to the target protein.[20] Moreover, monovalent ions like Na<sup>+</sup> have been reported to decrease sensitivity and affinity of aptamer to its target, probably due to the increased ionic strength and the shielding effect of Na<sup>+</sup> ion which leads to conformational changes of the aptamer binding site[21]. The findings in our preliminary study showed that the ammonium chloride-based RBC lysis buffer used in the lab somehow affected CD4 aptamer binding to CD4 T cells when it was added after the cell staining. However, it did not impact the aptamer-cell interaction when samples were first pre-lysed with the lysis buffer, and then re-suspended in wash buffer before the incubation. It would be valuable to further investigate the root cause of this effect in order to develop an enhanced RBC lysis procedure for the aptamer assay.

In the meantime, a more careful study to evaluate the context of the bias is needed and a thorough validation, including sensitivity, specificity, and reproducibility, etc., would be conducted to determine the performance of the modified CD4 aptamer assay. In addition, more data near cutoff of 200 and 350 CD4 T cells/ $\mu$ l could be pursued to determine the clinical agreement between the two assays, because reliability of the CD4 counts is particularly clinically important at these clinical treatment values.

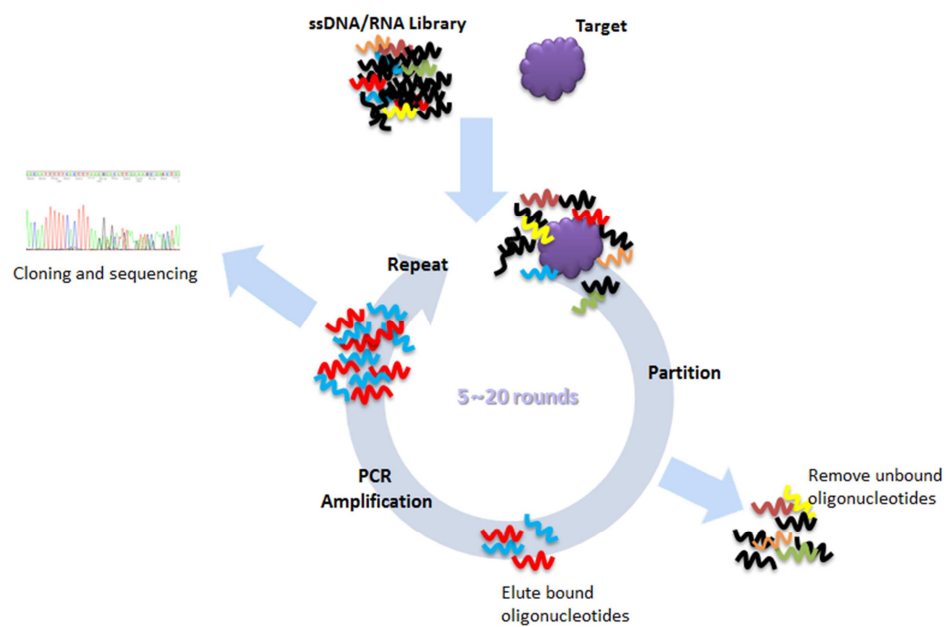
Ultimately, we would like to investigate the clinical utility of a multi-plexed

aptamer panel by combining multiple fluorochrome-conjugated aptamers in one test.

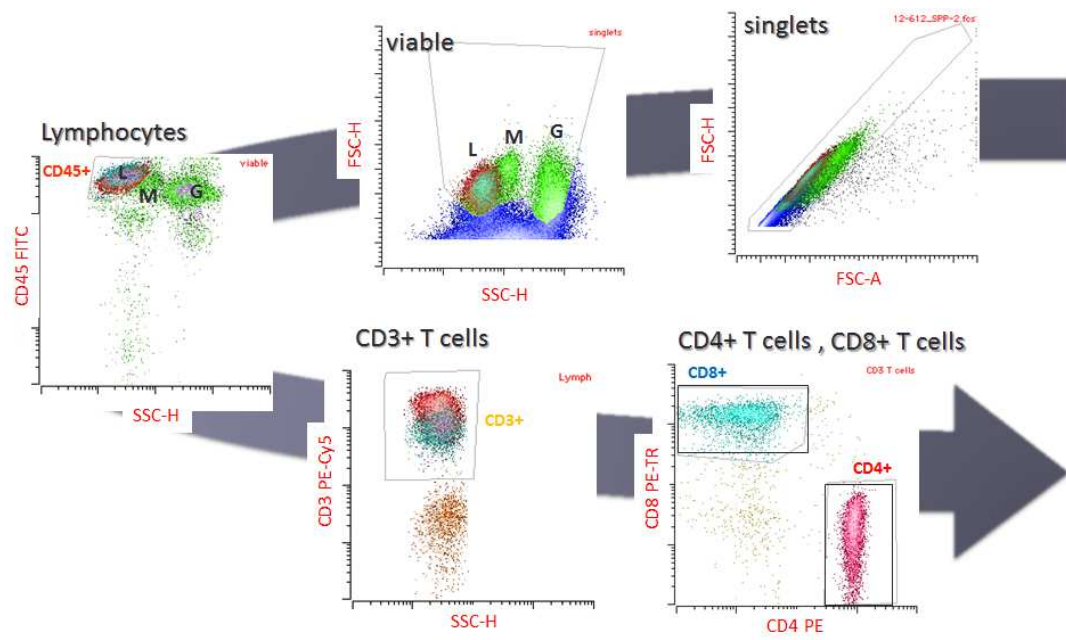
The goal would be to develop a model for cost-effective, multicolor aptamer flow cytometric assays, making the flow cytometry-based assays affordable and accessible to resource-limited settings. Discussions with the sponsoring company, SomaLogic, are underway to synthesize fluorochrome-conjugated aptamers targeting CD3, CD45, and CD8.

## **Conclusion**

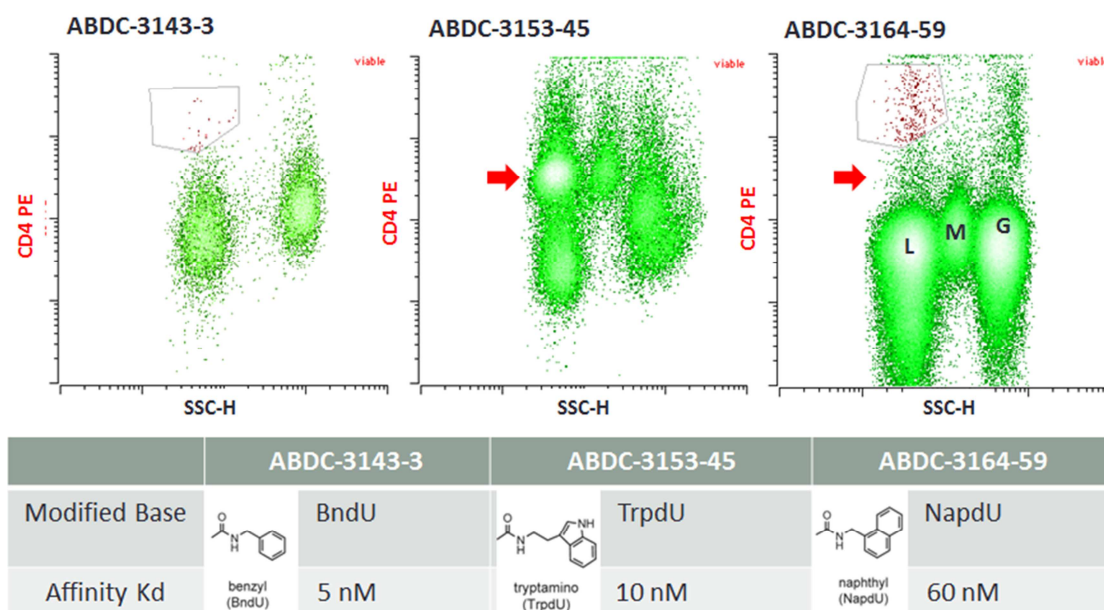
While the findings in the comparison study showed that the dual-platform aptamer assay is not yet sufficiently accurate to replace the established single-platform method, the CD4 aptamer did demonstrate a highly comparable performance in cell subset quantitation as compared to CD4 antibody. Therefore, the development of a single-platform CD4 aptamer assay would be desired to further validate its clinical value in parallel with the established assay before it can be implemented in the clinical setting.



**Image 1.** Scheme of systematic evolution of ligands by exponential enrichment (SELEX).

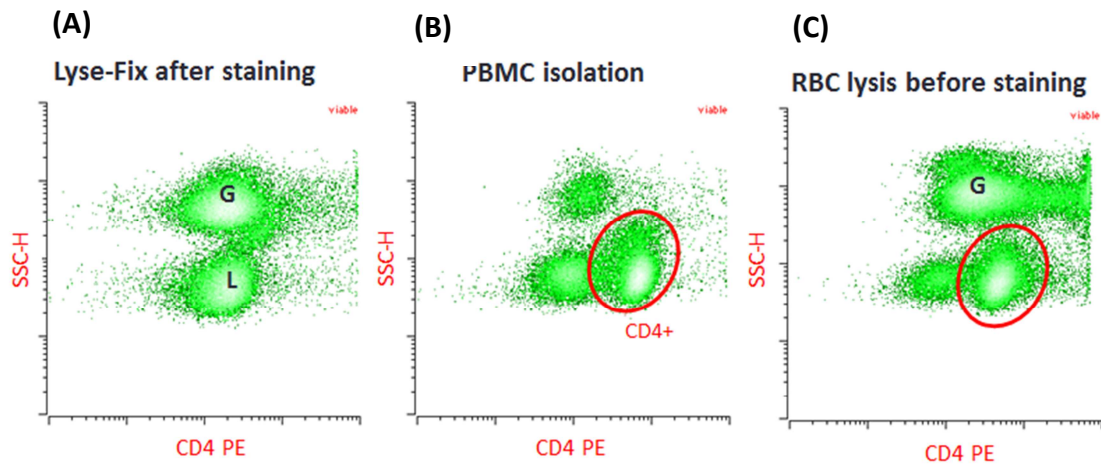


**Image 2.** Gating approach to identify CD4 T cells.



**Image 3.** Flow cytometric patterns of CD4 T cells by different CD4 aptamers.

Three CD4 aptamers provided by SomaLogic (Boulder, CO) with different modified bases were tested for CD4 T cell detection. Cells were simultaneously stained with CD3, CD8 antibodies, and CD4 aptamer. Viable cells distribution on the side scatter (SSC-H) and CD4 PE panel were shown. Cell populations are denoted as follows: L, lymphocytes; M, monocytes; G, granulocytes; Red arrow, CD4+ T cells.

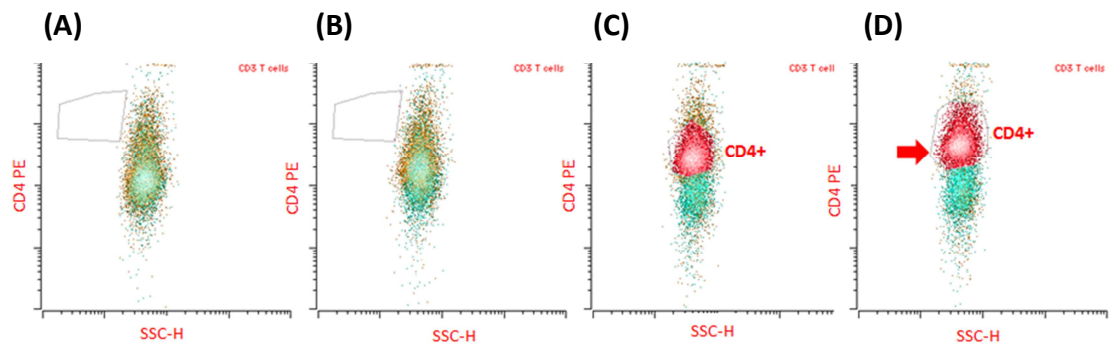


**Image 4.** Optimization of the sample processing for CD4 aptamer: CD4 T cell-binding patterns.

Cells were simultaneously stained with CD3, CD8 antibodies, and CD4 aptamer; and viable cells distribution on the side scatter (SSC-H) and CD4 PE panel were shown.

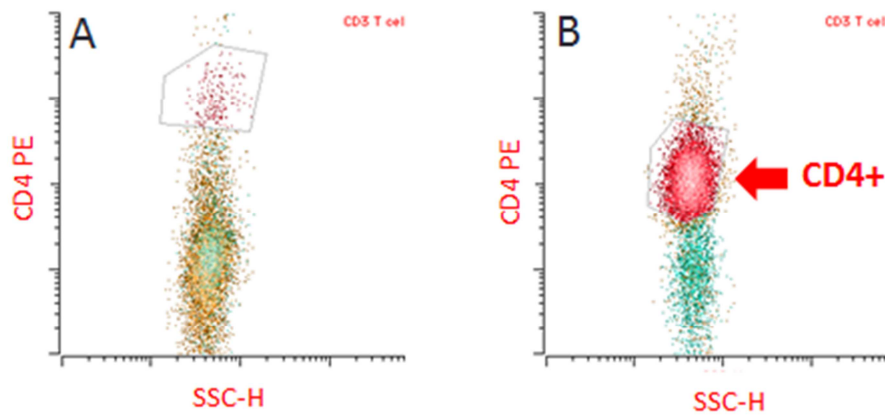
(A), Lyse-Fix buffer was added after whole blood sample was incubated with CD4 aptamer to remove red blood cells. (B), PBMC was isolated from whole blood and incubated with CD4 aptamer. (C), Whole blood was lysed and washed to remove red blood cells before aptamer incubation. Cell populations are denoted as follows:

G, granulocytes; L, lymphocytes; population highlighted in red, CD4+ T cells.



**Image 5.** Optimization of the concentration of CD4 aptamer.

Varying concentrations of CD4 aptamer reagents, 100 nM (A), 200 nM (B), 400 nM (C), 800 nM (D), were incubated with the same sample and assessed by flow cytometry. CD3 T cells distribution on the side scatter (SSC-H) and CD4 PE panel were shown. Cell subset in red, CD4+ T cells.



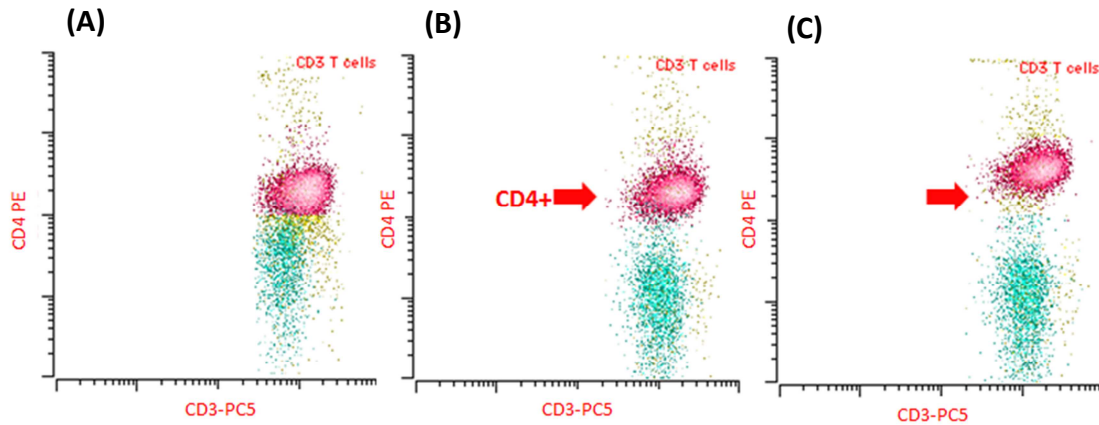
**Image 6.** Optimization of assay conditions with the CD4 aptamer.

(A), The CD4 aptamer reagent prepared in PBS+0.3%BSA was incubated with cells.

(B), The CD4 aptamer reagent prepared in SB18 solution was incubated with cells.

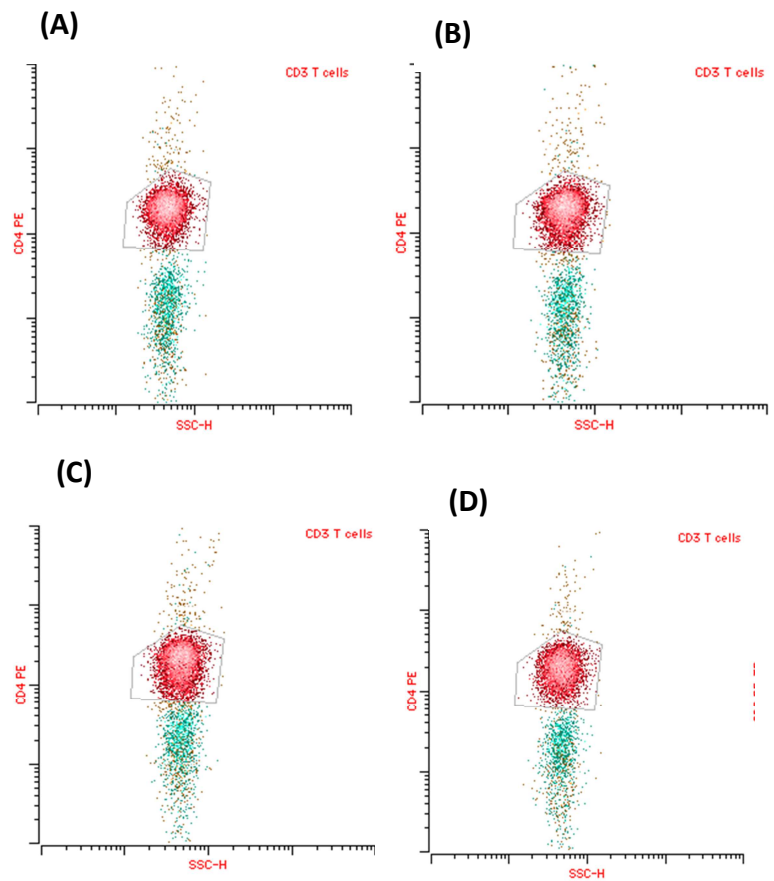
CD3 T cells distribution on the side scatter (SSC-H) and CD4 PE panel was shown.

Cell subset in red, CD4+ T cells; PE, Phycoerythrin.



**Image 7.** Optimization of incubation times for the CD4 aptamer

Multicolor panel using a combination of CD4 aptamer, CD3-PC5, and CD8-ECD was incubated with blood sample for 10 minutes (A), 20 minutes (B), and 60 minutes (C), respectively. CD3 T cells distribution on the CD3 PC5 and CD4 PE panel was shown. Cell subset in red, CD4 T cells; Cell subset in blue, CD8 T cells; PE, Phycoerythrin; PE-Cy5, Phycoerythrin Cyanin 5.1; FITC, Fluorescein isothiocyanate.

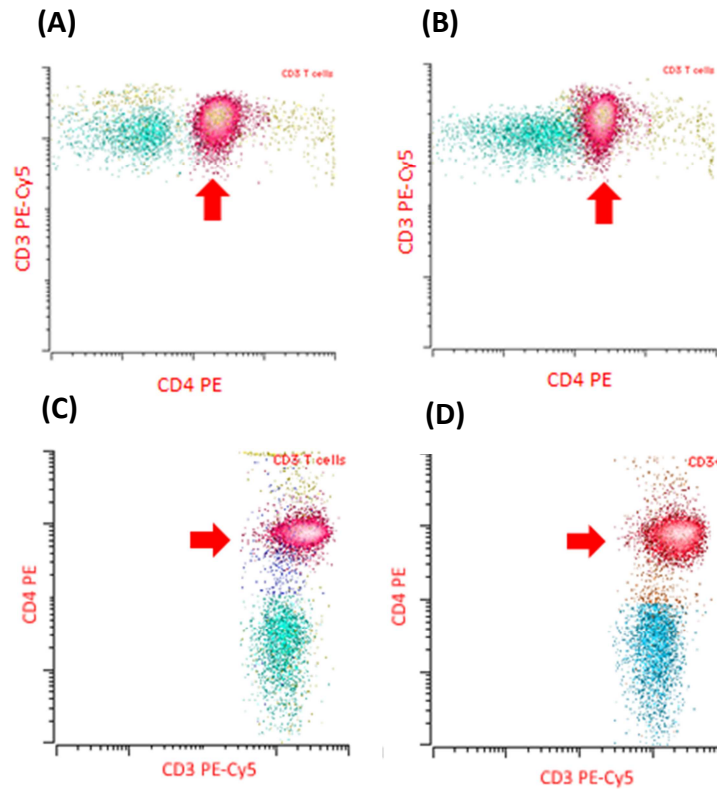


**Image 8.** Optimization of incubation times for Streptavidin-PE.

Stained samples were incubated with 20  $\mu$ l Streptavidin-PE for 5 minutes (**A**), 10 minutes (**B**), 15 minutes (**C**), and 20 minutes (**D**), respectively.

CD3 T cells distribution on the CD3 PC5 and CD4 PE panel was shown.

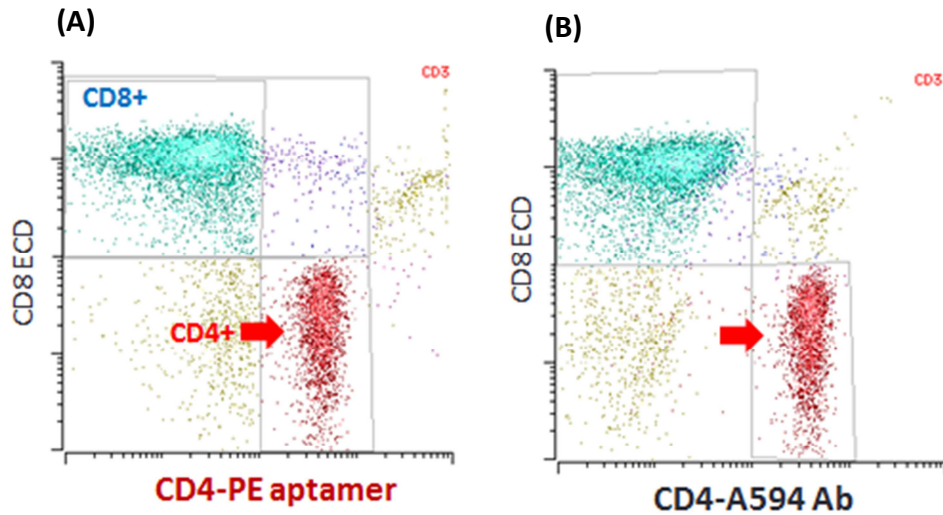
Cell subset in red, CD4 T cells; Cell subset in blue, CD8 T cells; PE, Phycoerythrin.



**Image 9.** CD45 antibodies multiplexing with multicolored aptamer and antibody panel.

(A) and (C), CD4-PE aptamer, CD3-PC5, and CD8-ECD antibodies were in combination used as control panel. (B), Cells were stained with the same set of aptamer and antibody panel, along with CD45-FITC (Clone J.33). (D), Cells were stained with the same set of aptamer and antibody panel, along with CD45-FITC (Clone HI30). CD3 T cells distribution on the CD3 PC5 and CD4 PE panel was shown. CD4 T cells, defined by CD4 positivity, were shown in red.

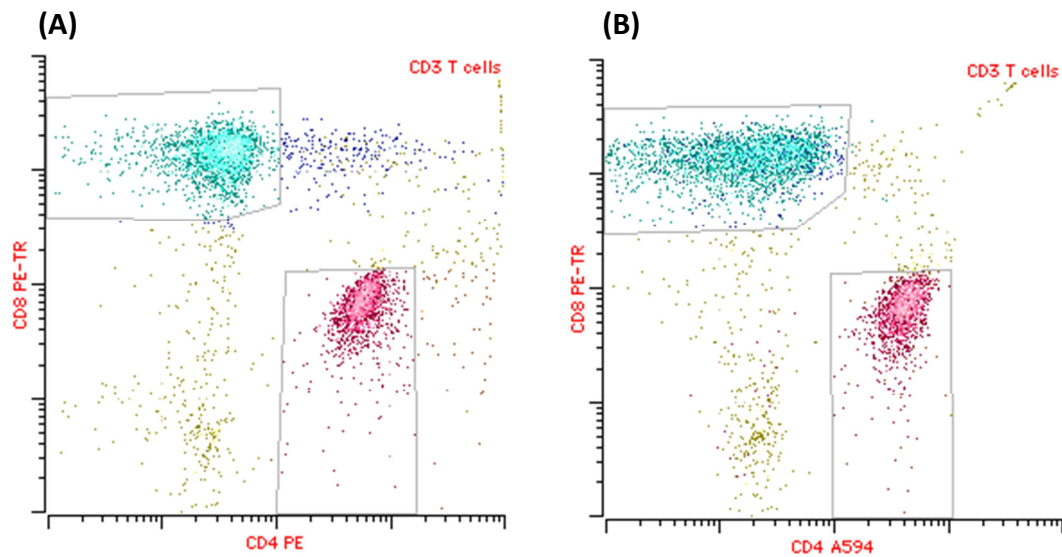
PE, Phycoerythrin; PE-Cy5, Phycoerythrin Cyanin 5.1; FITC, Fluorescein isothiocyanate.



**Image 10.** Sample simultaneously stained with CD4-PE aptamer and CD4-A594 antibody.

CD4-A594 antibody was simultaneously incubated with CD4 aptamer, CD3, CD8, and CD45 antibodies. CD3 T cells distribution on the CD8 ECD and CD4 PE panel was shown. (A), CD4 T cell population identified by CD4 aptamer. (B), CD4 T cell population identified by CD4 antibody.

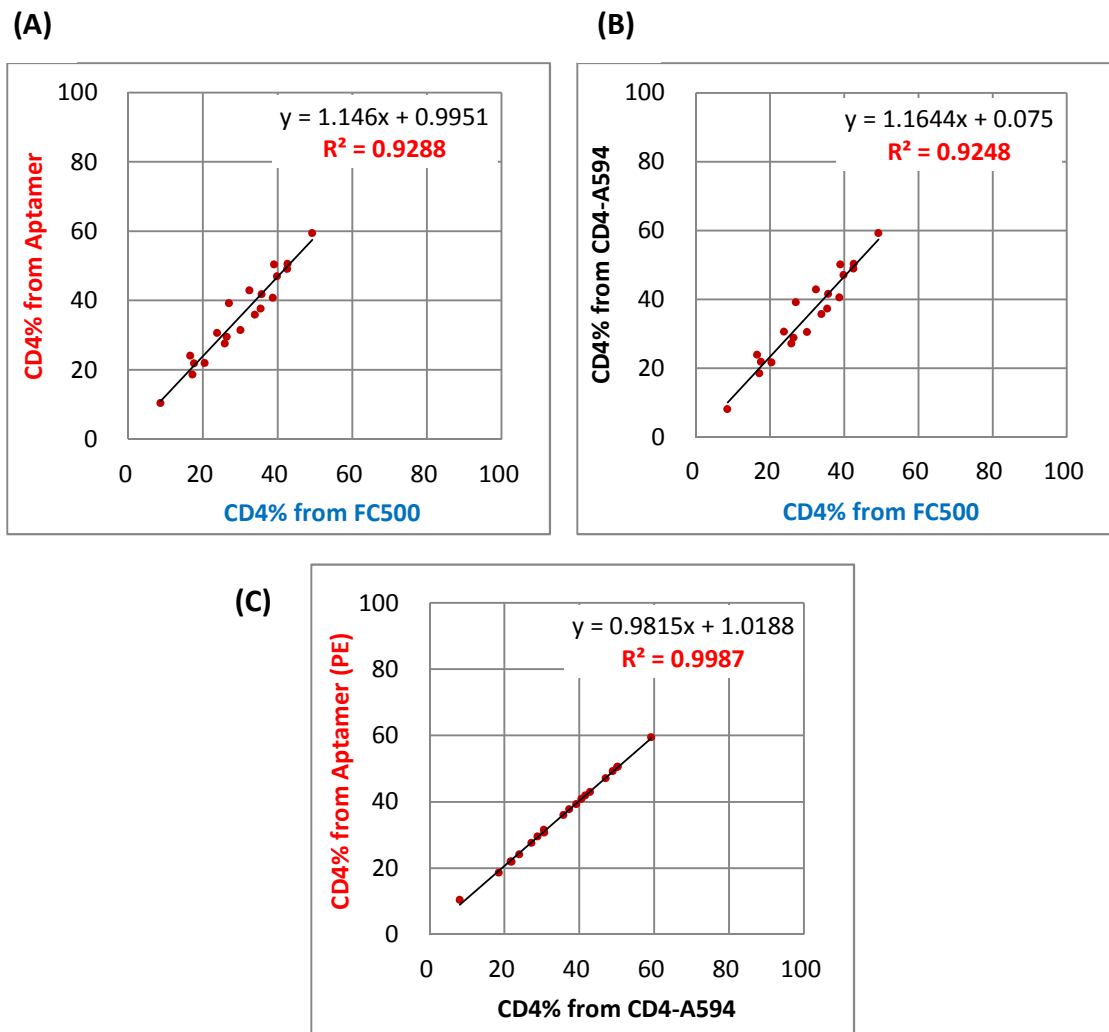
Cell subset in blue, CD8+ T cells; cell subset in red, CD4+ T cells; A594, Alexa Fluor 594; PE, Phycoerythrin; ECD, PE-TR, R-phycoerythrin(R-PE)-Texas Red.



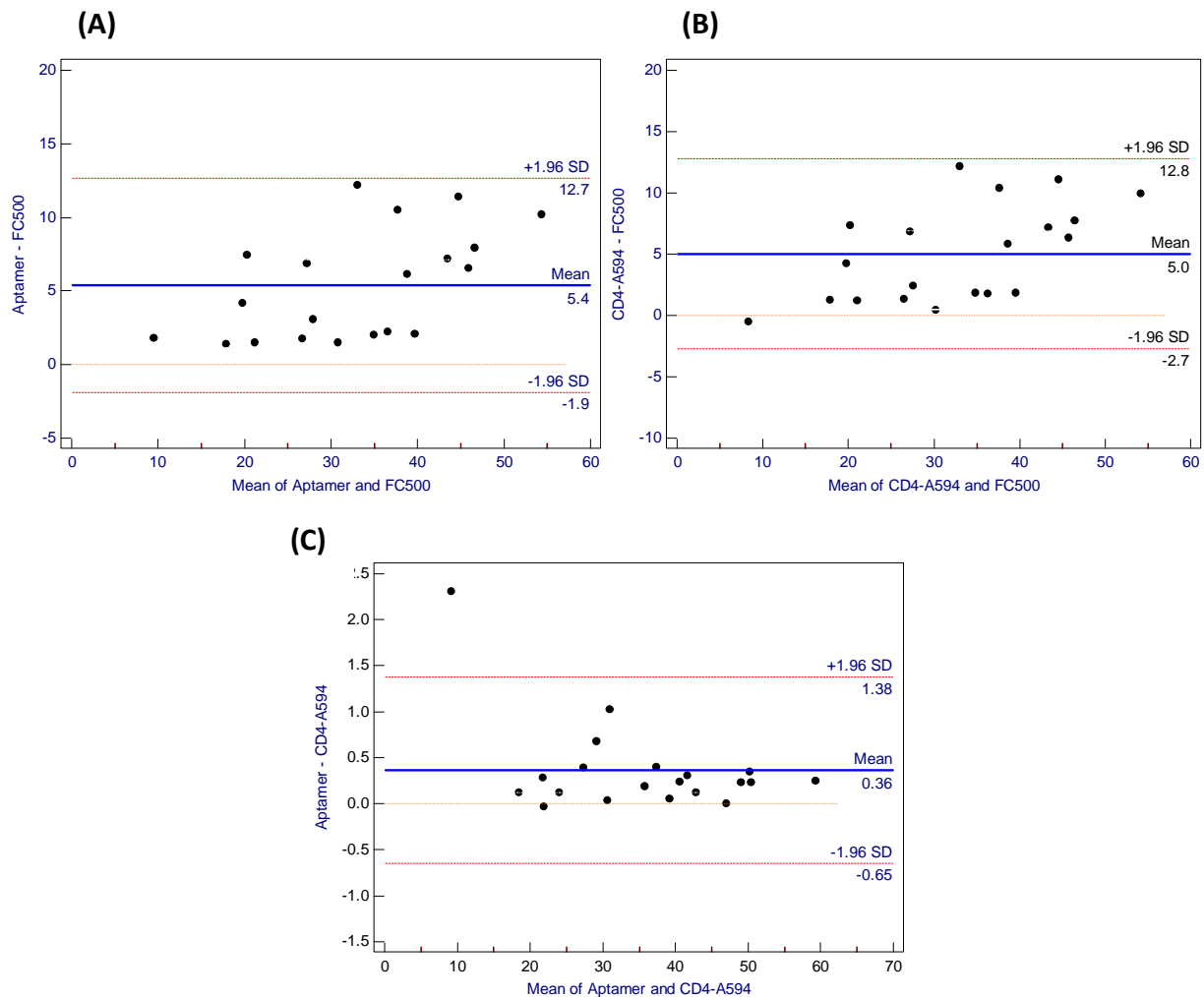
**Image 11.** Cell binding patterns of the CD4 aptamer assay.

The sample was simultaneously stained with CD4 aptamer panel and CD4-A594 antibody. CD3+ T cells were presented in the images. **(A)**, CD4 T cell binding pattern identified by CD4 aptamer. Non-specific binding of CD4 aptamer-PE to CD8 T cell subset was seen. **(B)**, CD4 T cell binding pattern identified by CD4 antibody.

Cell subset in blue, CD8+ T cells; cell subset in red, CD4+ T cells; A594, Alexa Fluor 594; PE, Phycoerythrin; PE-TR, R-phycoerythrin(R-PE)-Texas Red.

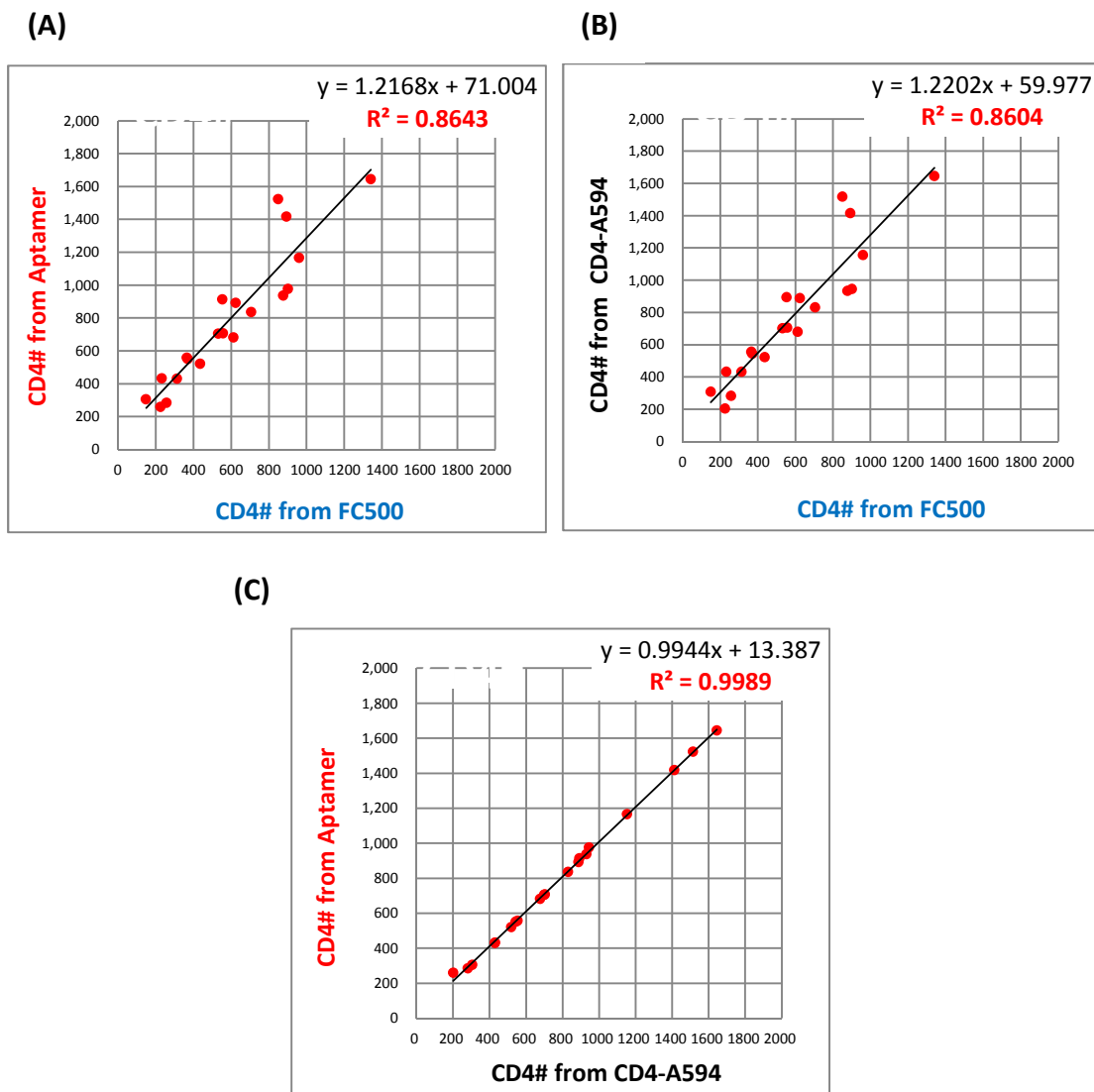


**Image 12.** Regression lines of CD4 percentages from the CD4 aptamer assay and the predicate assay. (A), Correlation between the CD4 T cell percentages obtained by the predicate method (FC-500) and the CD4 aptamer. (B), Correlation between the CD4 T cell percentages obtained by the predicate method and the internal CD4 antibody control from the aptamer method. (C). Correlation between the CD4 T cell percentages obtained by the CD4 aptamer and the corresponding internal CD4 antibody control.

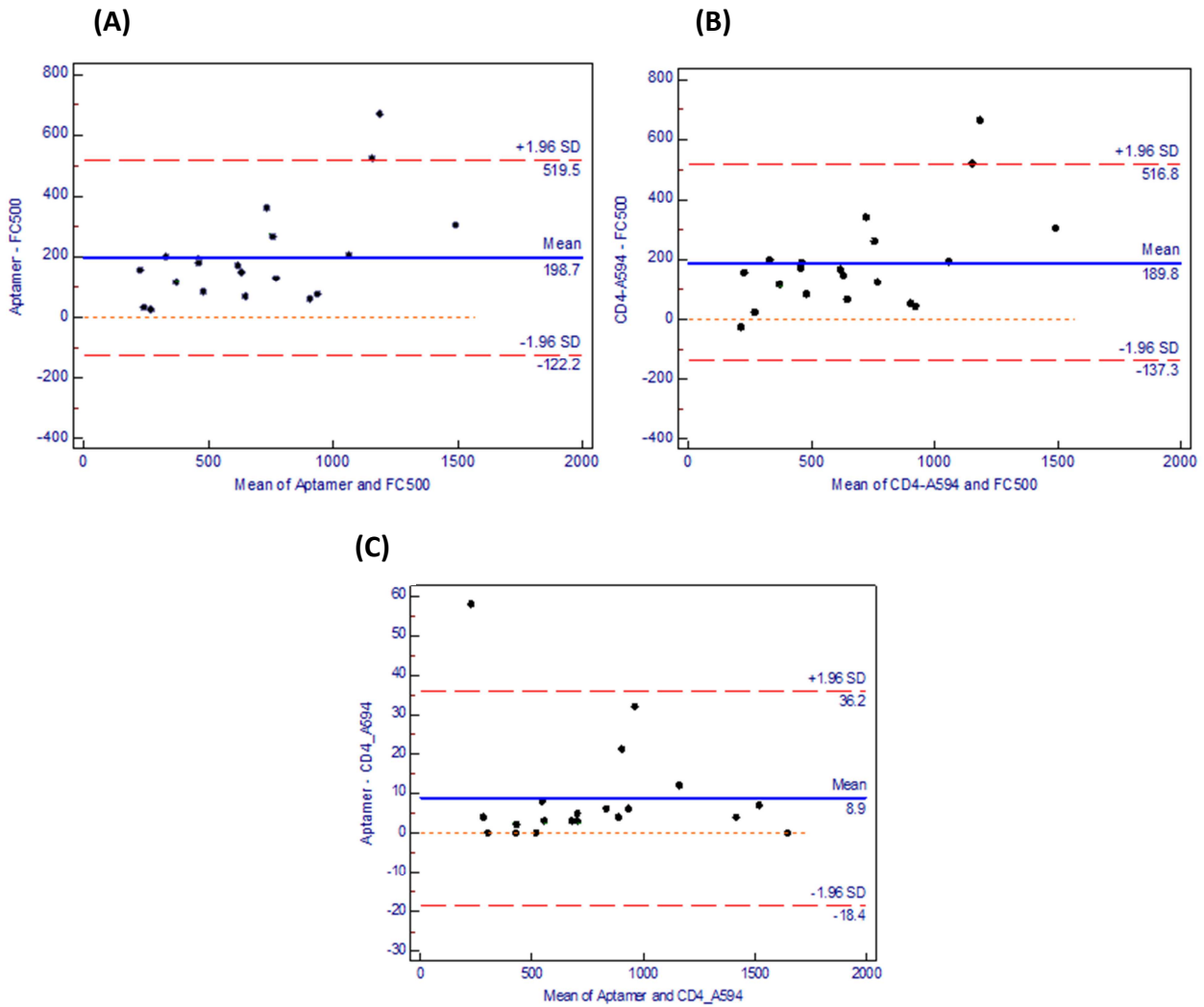


**Image 13.** Bland-Altman plots of CD4 percentages obtained by the CD4 aptamer assay and the predicate assay.

The plots depict the relative differences between the CD4 percentages obtained from the two methods which are plotted against the average CD4 percentages by the two methods in each subject. **(A)** The agreement between the predicate method and the CD4 aptamers. **(B)** The agreement between the predicate method and the CD4 antibody control from the aptamer method (CD4-A594). **(C)** The agreement between the CD4 aptamer and the corresponding internal CD4 antibody control (CD4-A594). Blue solid line, mean difference; red dotted line, limits of agreement.

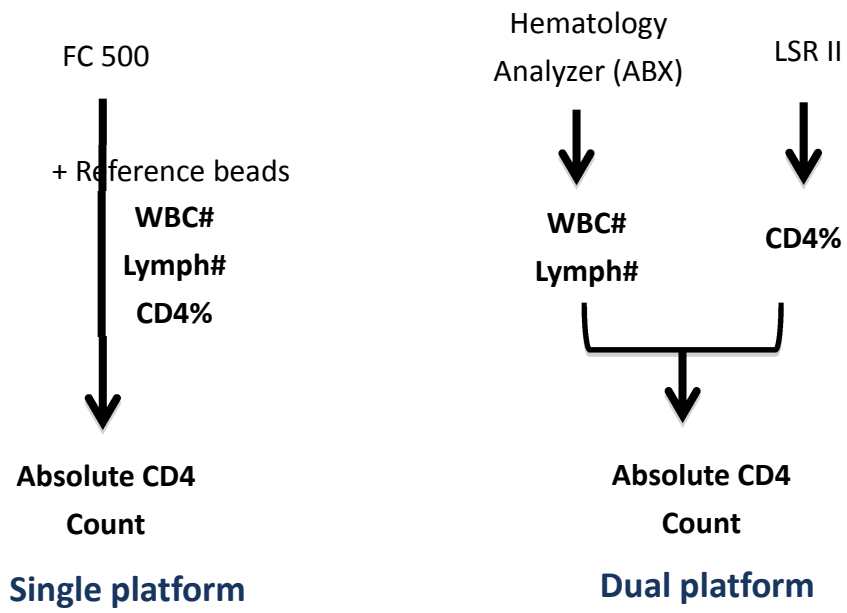


**Image 14.** Regression lines of absolute CD4 counts from the CD4 aptamer assay and the predicate assay. (A) Correlation between the CD4 T cell percentages obtained by the predicate method (FC-500) and the CD4 aptamer. (B) Correlation between the CD4 T cell percentages obtained by the predicate method and the internal CD4 antibody control from the aptamer method (CD4-A594). (C) Correlation between the CD4 T cell percentages obtained by the CD4 aptamer and the corresponding CD4 antibody control (CD4-A594).



**Image 15.** Bland-Altman plots of absolute CD4 counts from the CD4 aptamer assay and the standard assay. **(A)** The agreement between the reference method and the CD4 aptamers. **(B)** The agreement between the reference method and the CD4 antibody control from the aptamer method. **(C)** The agreement between the CD4 aptamer and the corresponding internal CD4 antibody control.

Blue solid line, mean difference; red dotted line, limits of agreement.



**Image 16.** Diagrams of single and dual platform approaches for absolute CD4 T cell counting.

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