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ABSTRACT

BACKGROUND: Monitoring CD4⁺ and CD8⁺ T cell counts has become essential in research on blood from healthy individuals and immunodeficient patients such as those with HIV/AIDS. Most procedures for CD4⁺ and CD8⁺ T cell enumeration require the blood to be <48 hrs old for accurate results. This can often be a problem in resource-poor settings where samples need to be transported long distances for analysis due to a lack of instrumentation or operator availability. It has been reported that TransFix can stabilize blood for CD4⁺ T cell enumeration for up to 14 days. In this study, we demonstrate the compatibility of aged, TransFix-treated blood with the Guava EasyCD4[™] and EasyCD8[™] assays, which use low volumes of blood (10 µL/test) and have previously been found to provide accurate and precise results in multi-site studies compared to predicate methods.

METHODS: TransFix was mixed with EDTA-anti-coagulated whole blood (from HIV-seropositive, n=10 and -seronegative donors, n=5) at a 1:10 ratio and incubated at RT (20–25°C). TransFix-treated samples were analyzed using the EasyCD4 and EasyCD8 assays on Days 0, 2, 15 and 30. CD4⁺ and CD8⁺ T cell counts were also obtained from fresh blood on Day 0.

RESULTS: CD4⁺ and CD8⁺ T cell counts of TransFix-treated samples on all days showed good agreement with counts obtained from fresh blood on Day 0. Average CD4⁺ T cell counts of 0, 2 and 15 day old TransFix-treated blood, when compared to the counts obtained from fresh blood on Day 0, gave correlation coefficients of 0.979, 0.98 and 0.98. The average % differences were -1.3%, -0.3% and -2.0%, respectively. Similarly, the average % differences for CD8⁺ T cell counts for TransFix-treated blood on days 0, 2 and 15 were -4.5%, 6.4%, and 3.0%. The staining profiles of the TransFix-treated blood were essentially identical to those at Day 0 for fresh blood. With TransFix-treated HIV-seronegative donors, the CD4⁺ T cell counts were accurate even after 30 days.

CONCLUSIONS: TransFix-treatment and storage for at least 15 days is compatible with the Guava EasyCD4 and EasyCD8 assays to obtain accurate CD4⁺ and CD8⁺ T cell counts in HIV-seropositive and -seronegative specimens. EasyCD4 and EasyCD8 assays along with TransFix allow analysis of aged blood samples in a low volume, low cost format with minimal reagent consumption and provides an attractive solution for CD4⁺ and CD8⁺ T cell determinations in resource-limited settings.

INTRODUCTION

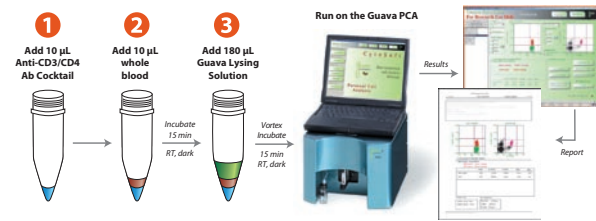
CD4 and CD8 T-cell monitoring has become an important component of biological research and clinical monitoring of several immunodeficiency diseases such as HIV. Most procedures for CD4⁺ and CD8⁺ T cell enumeration require blood samples to be <24-48 hrs old in order to obtain accurate T-cell counts. This can often be a problem in areas where CD4 counting technologies are not easily accessible and samples need to be transported long distances for analysis due to a lack of instrumentation or operator availability. This can also be a problem in situations where labs are overburdened and there is not enough time to get through T-cell analyses of the samples before they disintegrate.

TransFix is a whole-blood fixative developed at United Kingdom National External Quality Assessment Service (UK NEQAS) that when added to whole blood facilitates sample preservation, helps retain sample morphology and preserves cell surface expression of antigens over time. Several studies have evaluated the efficiency of TransFix in stabilizing blood samples and demonstrated that it can provide stable CD4 counts over a period of 10 to 15 days when used at a 1:10 ratio¹⁻⁴.

The Guava EasyCD4 and EasyCD8 assays are low cost assays for T-cell enumeration that use low volume of blood samples (10 µL) and utilize the Guava[®] PCA[™], a micro-capillary platform for analysis. The EasyCD4 and EasyCD8 assays have been previously demonstrated to provide comparable precision and accuracy to the clinically accepted BD Multi-Test method^{5,6}. In this study we evaluated TransFix-treated HIV seronegative and seropositive blood samples stored over a 30 day period for compatibility with the Guava EasyCD4 and EasyCD8 assays and determined the accuracy and precision of CD4 and CD8 T cell counts obtained from these samples over time compared to untreated control on day 0.

The results demonstrate good compatibility of TransFix reagents for use with the Guava EasyCD4 and EasyCD8 assays and demonstrate that accurate T-cell counts can be obtained on TransFix-treated aged blood samples.

BACKGROUND



The Guava EasyCD4 and Guava EasyCD8 assays as shown in the Figure above, are simple two-color assays that use antibody staining of 10 µL of blood, lysing of stained samples followed by analysis of samples on the Guava PCA a bench-top micro-cytometry platform to obtain accurate enumeration of CD4 T cells/µL blood and CD8 T cells/µL blood.

TransFix is a blood stabilizer developed by UK United Kingdom National External Quality Assessment Service (NEQAS) that is added to fresh whole blood to enable analysis of aged blood samples. In this study the performance of TransFix-treated blood with the Guava EasyCD4 and EasyCD8 assays was evaluated over a period of 30 days.

MATERIALS & METHODS

Materials

The EasyCD4 and EasyCD8 kits were from Guava Technologies, Hayward, CA. TransFix was a kind gift from Dr. David Barnett at UK NEQAS, Sheffield, UK.

Methods

Blood Sample Handling: 180 µL of EDTA-anti-coagulated blood (from HIV-seropositive, n=10 and seronegative donors, n=5) from purple-top lavender tubes were mixed with 20 µL of TransFix (a 1:10 ratio) and stored at room temperature (20–25°C) for a period of 0–4 hrs (Day 0), 2, 15, and 30 days after draw.

Easy CD4 Assay: The EasyCD4 assay was performed according to manufacturer's instructions. Briefly, 10 µL of whole blood from each sample was aliquoted to 1.5 mL microfuge tubes. A 10 µL cocktail of antibodies (CD4-PE with CD3-PECy5) was next added to the blood in the tube, vortexed and incubated for 15 min at room temperature in the dark. 180 µL of a Guava Lysing Solution was next added to lyse red blood cells, vortexed and incubated at room temperature in the dark for 15 minutes. Tubes were read within 15 minutes to 3 hrs of lysis using the Guava PCA.

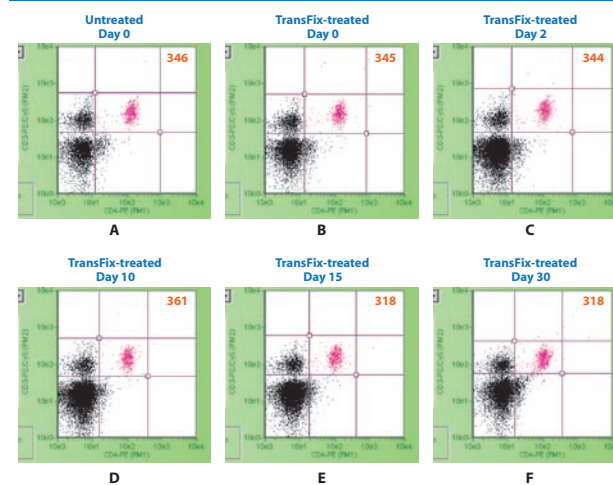
Easy CD8 Assay: The EasyCD8 assay was performed according to manufacturer's instructions. Briefly, 10 µL of whole blood from each sample was aliquoted to 1.5 mL microfuge tubes. A 10 µL cocktail of antibodies (CD8-PE with CD3PECy5) was next added to the blood in the tube, vortexed and incubated for 15 min at room temperature in the dark. 180 µL of a Guava Lysing Solution was next added to lyse red blood cells, vortexed and incubated at room temperature in the dark for 15 minutes. Tubes were read within 15 minutes to 3 hrs of lysis using the Guava PCA.

Data Acquisition and Analysis: Data was acquired on a Guava PCA instrument equipped with a 532 nm laser using the Guava EasyCD4 software. Data were exported to csv files and analyzed in Excel to obtain count and MFI results.

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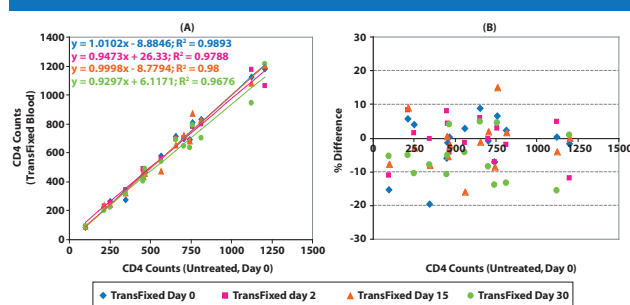
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Figure 1. EasyCD4 Staining Patterns of TransFix-treated Blood Samples Stored for Different Durations of Time



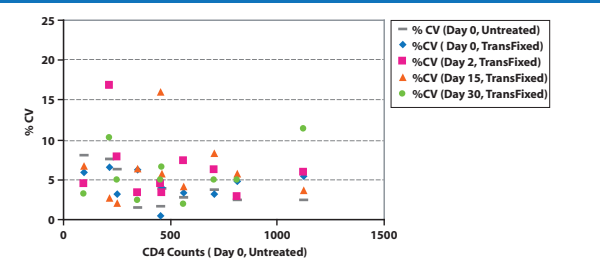
Freshly drawn blood samples were TransFixed on day of draw and stored at room temperature for 0, 2, 10, 15 and 30 days. The figure above is a compilation of the dot plots of the CD3-PECy5/CD4-PE staining of blood from a single donor untreated (A), TransFix-treated and stored for 0 days (B), 2 days (C), 10 days (D), 15 days (E) and 30 days (F). The CD4 counts are indicated in red. The figure clearly shows that the staining pattern of fresh, untreated blood is identical to the staining pattern of blood TransFixed and stored for 0, 2, 10 and 15 days. The TransFix-treated blood at 30 days (F) shows a distinct CD3⁺, CD4⁺ population but is beginning to show slightly diminished separation from the other populations and also exhibits some streaking of the monocyte population.

Figure 2. Accuracy of CD4 Counts from TransFix-treated Aged Blood Samples



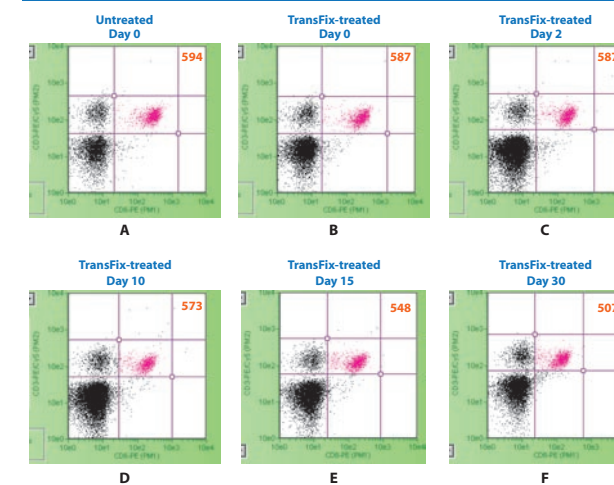
CD4 counts were obtained after performing the EasyCD4 assay on TransFix-treated blood stored for 0, 2, 15 and 30 days and fresh blood on day of draw. A linear correlation plot of CD4 counts of TransFix-treated blood for 0–30 days vs. CD4 counts from fresh untreated blood (Day 0) is shown in Panel A. The data show that excellent correlation of CD4 counts is obtained throughout the 30-day duration of TransFix-treated blood storage. This is further supported by the plot in Panel B, which depicts the percent difference of CD4 counts of TransFix blood versus the counts of untreated Day 0 blood. The results in Fig 2B clearly show that in most cases TransFix-treated blood stored at 0 days, 2, 15 and 30 days did not show significant variation from the CD4 counts compared to fresh blood on Day 0. The average % difference for Day 0 was -1.3%, Day 2 was -0.31%, Day 15 was -2.0% and for Day 30 was -5.3%. It was observed that the 30-day TransFix-treated samples showed an increased negative bias (average bias -5.3%) compared to the CD4 counts from samples TransFixed for 15 days or less. However in all cases CD4 counts within 20% of CD4 counts from Day 0 untreated sample could be obtained. The data thus suggests that it is possible to obtain accurate CD4 counts from normal and HIV blood TransFixed and stored for a period of up to 30 days when used with the Guava EasyCD4 assay.

Figure 3. Precision Data for EasyCD4 Assay on TransFix-treated Blood Samples over a Period of 30 Days



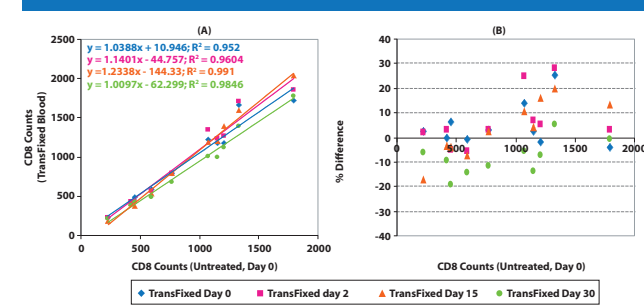
The %CVs of triplicates were calculated for the TransFix-treated and fresh blood samples and plotted as shown above. The average %CV for fresh untreated blood (Day 0), TransFix-treated blood for 0, 2, 15 and 30 days was 3.6, 4.1, 5.1, 5.1, 6.5 and 5.0%, respectively, supporting that good precision could be obtained on the TransFix-treated aged samples.

Figure 4. EasyCD8 Staining Patterns of Blood Samples TransFixed and Stored for Different Durations of Time



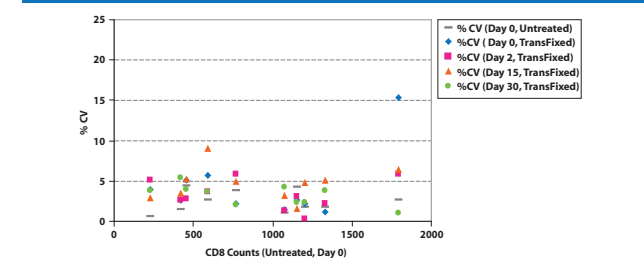
Freshly drawn blood samples were TransFixed on day of draw and stored at room temperature. The figure above is a compilation of the dot plots of the CD3-PECy5/CD8-PE staining of blood from a single donor untreated (A), TransFix-treated and stored for 0 days (B), 2 days (C), 10 days (D), 15 days (E) and 30 days (F). The CD8 counts are indicated in red. The figure clearly shows that the staining pattern of fresh, untreated blood is identical to the staining pattern of TransFix-treated blood and stored for 0, 2, 10 and 15 days. Again the blood at 30 days (F) shows a distinct CD3⁺, CD8⁺ population but is beginning to show much diminished separation between the other populations and indication of streaking from the double-negative population.

Figure 5. Accuracy of CD8 Counts from TransFix-treated Aged Blood Samples



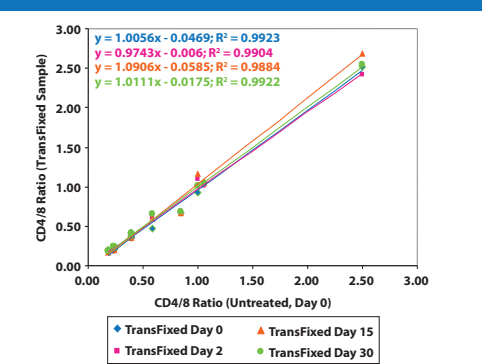
CD8 counts were obtained after performing the EasyCD8 assay on TransFix-treated blood stored for 0, 2, 15 and 30 days and fresh blood on day of draw. The linear correlation plot of CD8 counts of TransFix-treated blood for 0–30 days vs. CD8 counts from fresh untreated blood (Day 0) is shown in Panel A. The data show that excellent correlation of CD8 counts is obtained throughout the 30-day duration of TransFix-treated blood storage. This is further supported by the plot in Panel B, which depicts the percent difference of CD8 counts of TransFix blood versus the counts of untreated Day 0 blood. The results in Panel B clearly show that in most cases TransFix-treated blood stored at 0, 2, 15 and 30 days did not show significant variation from the CD8 counts compared to fresh blood on Day 0. The average % difference for Day 0 was -4.5%, Day 2 was 6.4%, Day 15 was 3.0% and for Day 30 was -8.4%. Again it was observed that the CD8 counts from 30-day TransFix-treated samples showed an increased negative bias (average bias of -8.4%) compared to the TransFix-treated samples from Day 15 or less. However in most cases CD8 counts within 20% of CD8 counts from Day 0 untreated sample could be obtained. The data suggest that it is possible to obtain accurate CD8 counts from blood TransFixed and stored for a period of up to 30 days when used with the Guava EasyCD8 assay.

Figure 6. Precision Data for CD8 Assay on TransFix-treated Samples Over a Period of 30 Days



The %CVs of triplicates were calculated for the TransFix-treated and fresh blood samples and plotted as shown above. The average %CV for fresh (Day 0) or TransFix-treated blood held for 0, 2, 15 and 30 days was 2.4, 4.2, 3.2, 4.7 and 3.2%, respectively, supporting that good precision could be obtained on the TransFix-treated aged samples.

Figure 7. Accuracy of CD4/CD8 Ratio for TransFixed Samples



The CD4/CD8 ratio for each TransFixed blood sample was plotted against the CD4/CD8 ratio of untreated sample on Day 0. The data shows that a good correlation ($R^2 = 0.99$, 0.99 , 0.98 and 0.99) of CD4/CD8 ratio is obtained on all days for TransFixed samples. Thus accurate CD4/CD8 ratios can be obtained with blood TransFixed for 30 days as shown in this study.

CONCLUSIONS

- ◆ Use of TransFix as a stabilizer at a 1:10 ratio when added to whole blood shows good compatibility for use with Guava EasyCD4 and EasyCD8 assays to obtain accurate and precise CD4 and CD8 counts.
- ◆ The staining pattern from TransFixed samples over 15 days is almost identical to the staining pattern at day 0. At 30 days the staining pattern is starting to show signs of deterioration, however accurate counts could still be obtained.
- ◆ Accurate CD4 and CD8 T-cell counts were shown to be obtained from TransFix-treated samples over a period of 0–15 days (within 10% of untreated control) when compared to fresh untreated blood on Day 0. The 30-day TransFixed-samples show an increased negative bias compared to samples treated for 15 days or less, however in most cases counts were within 20% of counts from untreated samples from Day 0. Accurate CD4/CD8 ratios could be obtained for the entire period of 30 days.
- ◆ Since low volumes of blood (10 µL) are needed for Guava assays, the amount of TransFix needed for stabilizing samples is also low (1 µL per assay). Use of TransFix along with Guava CD4/CD8 assays allows low volume and low cost assays on aged blood samples with minimal reagent consumption and is an attractive option in resource-poor settings.

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