# Enumeration of T, B and NK subpopulations in aged whole blood samples using AQUIOS CL instrument with Tetra application

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## Introduction

The use of flow cytometric analysis of peripheral whole blood to enumerate lymphocyte subsets is commonly used to assess the immunological status of patients in a wide variety of clinical conditions. The testing to enumerate lymphocyte subsets often occurs in specialized reference laboratories remote from the site of blood collection from the patient, often causing a delay between specimen collection and performance of the assay. In addition, once the lab has prepared the specimen for flow cytometry analysis, it is not uncommon for the lab to store this prepared sample prior to analysis or reanalysis.

Previous studies have shown that the scatter and fluorescence properties of various cell subsets of whole blood stored in EDTA change as a specimen or prepared sample ages. The general observations regarding specimen age have been that the relative proportion of T-cells increases, while the number of detectable B-cells decreases (Nicholson, et. al 1993, Ekong, et al 1993, Jalla, et. al. 2004).

The monocytes and neutrophils can lose their scatter characteristics which may lead to the contamination of the lymphocyte gate. Thus, it is essential for the lymphocyte subsets enumeration methods to provide evidence of integrity for the lymphocyte gating when the method is used, especially with the aged specimens.

In this study we demonstrate that the new AQUIOS-Tetra method developed for the enumeration of T, B and NK lymphocyte subsets provides accurate results for specimens stored in EDTA for up to 24 hours post venipuncture at room temperature. Monocyte/neutrophil contamination of Lymphocyte population in both fresh and aged specimens was assessed using CD13 and CD14 markers.

## Materials and Methods

The AQUIOS CL instrument with Tetra application is an integrated system of sample preparation and sample analysis; it analyzes samples within 3 min after the lysing reagent is added to the sample stained with antibodies. The software controls sample preparation and analysis to limit the overlysing impact. In this study, the whole blood samples were tested at 24 hours of post venipuncture and after 3min incubation time as prepared samples. Each specimen was tested in duplicate at each time point.

| Specimen Age (hours) | Prepared Sample Age | |
|---|---|---|
| | T0 | T3min |
| 0 | X (0-0) | |
| 24 | X (24-0) | X (24-3) |
| 32 | X (32-0) | |

**Table 1:** Time Points for each specimen

NOTE: The results from at least three time points testing were required to perform regression analysis

Seventy-three (73) specimens, including normal and clinical (HIV+) donors were tested for data analysis. The drift (change) in absolute count and percent positive for CD3+, CD3+/CD4+, CD3+/CD8+, CD3-/ CD19+ and CD3-/CD56+16+ lymphocyte subsets for all replicates were assessed between the reference point 0-0 and other time points (24-0, 24-3). A 95% confidence limit of the drift was also calculated.

AQUIOS drift analysis for Specimen Age

Data was modeled as a linear function of specimen using a mixed model including all the data collection at each time point (0-0, 24-0, and 32-0). Samples constituted the random component of the model while “age” was used as a linear regressoion fixed effect. The PROC MIXED routine of SAS 9.3 was used for data analysis.
Drift ($\delta_{Age}$) at a certain time point was calculated as the difference between the response at that time point and the response at time zero ($t_0$).

AQUIOS drift analysis for Prepared Sample

Drift of prepared samples (δPrepared) was calculated as the difference between the average response of samples aged for 24 hours and prepared for three minutes (24-3) and the average response of samples aged 24 hours tested immediately (24-0). $\delta_{Prepared} = X_{24hrs + 3\ min} - X_{24hrs}$

Where $X_{24hrs + 3\ min}$ and $X_{24hrs}$ were the respective averages. Standard error of this drift ($\sigma_{Prepared}$) was calculated as the standard error of the difference between these two time points.

AQUIOS total drift analysis

Drift at stability claim (24 hours aged and 3 minutes prepared) was calculated as the sum of the specimen age component and the prepared sample component as:

$$\delta_{Total} = \delta_{Age} + \delta_{Prepared}$$

The confidence limits of the drift were calculated based on the standard error of the drift and 95% confidence.

To evaluate the integrity of the gating and to verify the accurate recovery of samples after 24 hours of storage, the potential contamination of the lymphocyte gating from the monocyte and neutrophil cell populations was assessed. The AQUIOS algorithm employs a lymphocyte identification methodology that combines the lymphocytes identified in the CD45 vs SS and the EV vs SS plots to define the Lymphs All region which is intended to contain all the lymphocytes. To assess purity of the lymphocyte population, CD14-PC7 and CD13-PC7 were added to AQUIOS Tetra-1 and Tetra-2+ panels to evaluate presence of monocyte (CD14+) and neutrophil (CD13+) contamination in the total lymphocyte gate. The study was performed on fresh (<8 hours) and aged (≤24 hours) specimens.

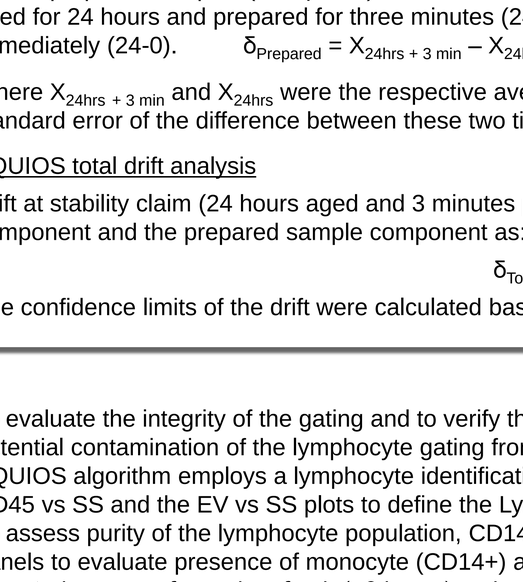
A total of 38 samples (24 clinical samples and 14 normal samples) were analyzed on three AQUIOS CL instruments. Each sample was tested at two time points as shown in Table 4.

**Table 4: Test reagents and test conditions**

| Test Reagents | Specimen – Prepared Sample Age | |
|---|---|---|
| | ≤8hrs (0) Standard (0) | ≤24±2hrs (24) Repeat (3 min) |
| Tetra-1 (T1) with CD14-PC7/CD13-PC7 | 0-0 | 24-3 |
| Tetra-2+ (T2+) with CD14-PC7/CD13-PC7 | 0-0 | 24-3 |

*Monocyte (CD14+) and Neutrophil (CD13+) Contamination analysis:*

**Fig 1: Gating Strategy**




All three populations are easily distinguished by the level of expression of the CD14/CD13 and CD56/16 markers.

Lymphocytes have 2 populations: CD14/CD13- and CD56/16- as well as: CD14/CD13- and CD56/16 medium.

Monocytes have 2 populations: CD14/CD13bright and CD56/16 - as well as CD14/CD13+ and CD56/16 medium. Neutrophils have CD14/CD13+ and CD56/16 bright population.

NOTE: The 5th PMT that is not available for the Tetra IVD application was used for evaluation of the CD13-PC7 and CD14-PC staining and the purity of the lymphocyte gating.

## Results

**Table 2: Mean values of the recovered drift at each time point**

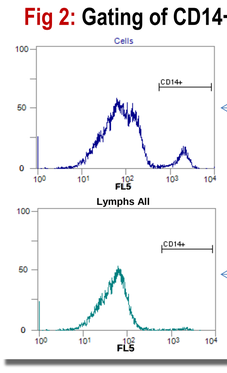
| Panel | Marker | Unit | 0-0 | 24-0 | 24-3 | 32-0 |
|---|---|---|---|---|---|---|
| AQUIOS Tetra-1 (CD45/4/8/3) | Total CD3+ | % | 73.66 | 74.00 | 74.19 | 73.75 |
| | CD3+/CD4+ | | 29.73 | 29.89 | 29.61 | 29.68 |
| | CD3+/CD8+ | | 41.43 | 41.60 | 42.07 | 41.59 |
| | CD45+ Low SS | | 35.41 | 35.72 | 36.18 | 36.32 |
| | Total CD3+ | cells/µL | 1327.20 | 1320.19 | 1310.22 | 1287.28 |
| | CD3+/CD4+ | | 530.02 | 527.13 | 517.85 | 518.14 |
| | CD3+/CD8+ | | 755.06 | 751.49 | 750.80 | 729.38 |
| | CD45+ | | 5380.35 | 5266.47 | 5161.18 | 5097.48 |
| | CD45+ Low SS | | 1811.50 | 1795.67 | 1778.31 | 1756.16 |
| AQUIOS Tetra-2+ (CD45/56+16/19/3) | Total CD3+ | % | 73.82 | 73.95 | 74.12 | 73.88 |
| | CD3-/CD19+ | | 13.57 | 13.29 | 13.33 | 13.42 |
| | CD3-/CD56+CD16+ | | 11.36 | 11.60 | 11.46 | 11.46 |
| | Total CD3+ | cells/µL | 1325.34 | 1332.01 | 1328.27 | 1306.19 |
| | CD3-/CD19+ | | 253.70 | 251.25 | 250.68 | 249.36 |
| | CD3-/CD56+CD16+ | | 202.70 | 207.37 | 203.77 | 201.65 |
| | CD45+ Low SS | | 1803.83 | 1811.39 | 1802.78 | 1778.75 |

**Table 3: Specimen and Prepared Sample Stability Results**

| Panel | Marker | Unit | Total Drift (includes 0-0 to 24-0 and 24-0 to 24-3) | 95% confidence limits | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| AQUIOS Tetra-1 (CD45/4/8/3) | Total CD3+ | % | 0.24 | 0.08 | 0.40 |
| | CD3+/CD4+ | | 0.02 | -0.09 | 0.13 |
| | CD3+/CD8+ | | 0.21 | 0.08 | 0.35 |
| | CD45+ Low SS | | 0.83 | 0.67 | 0.99 |
| | Total CD3+ | cells/µL | -35.82 | -43.79 | -27.85 |
| | CD3+/CD4+ | | -12.09 | -15.90 | -8.29 |
| | CD3+/CD8+ | | -22.50 | -27.67 | -17.33 |
| | CD45+ | | -275.04 | -308.92 | -241.16 |
| | CD45+ Low SS | | -52.88 | -63.20 | -42.55 |
| AQUIOS Tetra-2+ (CD45/56+16/19/3) | Total CD3+ | % | 0.14 | -0.02 | 0.30 |
| | CD3-/CD19+ | | -0.12 | -0.22 | -0.03 |
| | CD3-/CD56+CD16+ | | 0.08 | -0.03 | 0.19 |
| | Total CD3+ | cells/µL | -13.61 | -21.83 | -5.40 |
| | CD3-/CD19+ | | -3.64 | -5.89 | -1.39 |
| | CD3-/CD56+CD16+ | | -1.55 | -3.83 | 0.72 |
| | CD45+ Low SS | | -20.14 | -30.13 | -10.14 |

Since neutrophils have the potential of being incorrectly identified as NK Cells, the contamination of neutrophils (identified as CD13+) in the total lymphocytes gate (Lymphs All) was assessed in AQUIOS Tetra-2+ only.. The neutrophils are identified as cells expressing medium levels of CD13 and high levels of CD16 (CD16++). The CD16++ region was applied to the total lymphocyte gate (Lymphs All) to assess neutrophil contamination (Figure 3)

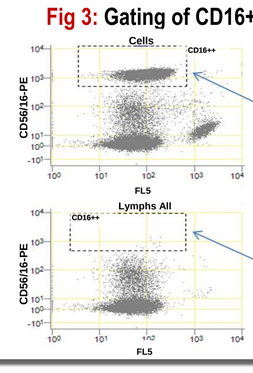
**Fig 2: Gating of CD14+ monocytes**




Gating of monocytes (CD14+) in ‘Cells’ gate (including lymphocytes, monocytes and granulocytes) showing clear separation of monocytes from other leukocyte populations.

Gating of monocytes (CD14+) in ‘Lymphs ALL’ gate to assess the monocytes contamination in total lymphocytes.

**Fig 3: Gating of CD16+ neutrophils**




Gating of Neutrophils (CD13+CD16++) in ‘Cells’ gate showing clear separation of neutrophils from lymphocytes.

Gating of neutrophils (CD13+CD16++) in ‘Lymphs ALL’ gate to assess the neutrophil contamination in total lymphocytes.

Based on the gating strategies of identifying monocytes and neutrophils, the results of monocyte and neutrophil contamination in the ‘Total Lymphocyte’ gate are summarized in the following two tables. Monocyte and neutrophil contamination are expressed as percent of total lymphocytes.

**Table 5: Monocyte contamination assessment (% of total lymphocyte)**

| Age/ Analysis | AQUIOS instrument | Tetra-1 | | Tetra-2+ | |
|---|---|---|---|---|---|
| | | MIN | MAX | MIN | MAX |
| Fresh/ Standard | CR21 | 0.15% | 1.54% | 0.04% | 1.47% |
| | CR22 | 0.13% | 0.85% | 0.14% | 0.85% |
| | CR27 | 0.27% | 1.66% | 0.20% | 1.31% |
| 24 hrs / Repeat | CR21 | 0.08% | 1.12% | 0.15% | 1.55% |
| | CR22 | 0.04% | 1.40% | 0.19% | 1.11% |
| | CR27 | 0.19% | 1.38% | 0.23% | 1.78% |

**Table 6: Neutrophil contamination assessment (% of total lymphocytes)**

| Age/ Analysis | AQUIOS instrument | Tetra-2+ | |
|---|---|---|---|
| | | MIN | MAX |
| Fresh/ Standard | CR21 | 0.00% | 0.22% |
| | CR22 | 0.00% | 0.37% |
| | CR27 | 0.00% | 0.27% |
| 24 hrs / Repeat | CR21 | 0.00% | 0.52% |
| | CR22 | 0.02% | 0.48% |
| | CR27 | 0.02% | 0.75% |

## Conclusions

- The AQUIOS CL instrument with Tetra application provides accurate results for recovery of the T, B and NK lymphocyte subsets in clinical and normal specimens collected into the EDTA K3 tubes when stored at room temperature for up to 24 hours.
- Monocyte and neutrophil contamination of total lymphocytes is less than 5%. No significant difference was observed within the tested conditions Fresh/standard vs. 24 Hrs/repeat and across different instruments.