

Evaluation of Stabilized Blood Cell Products as Candidate Preparations for Quality Assessment Programs for CD4 T-Cell Counting

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Background: Exceptionally robust cell preparations are needed for quality assessment programs (QAPs) such as the International Program for Quality Assessment and Standardization for Immunological Measures (QASI) relevant to HIV/AIDS. A suitable product must withstand environmental stress related to transportation for a minimum of 6 days. The two objectives of this study are (1) to evaluate the performance of various commercial preparations with multicenter participation and (2) to evaluate the robustness of stabilized blood cell products. **Methods:** Phase 1: The performance of stabilized blood cell products was evaluated in a multicenter QAP utilizing various staining procedures and flow cytometers. Absolute cell enumeration was achieved using single-platform T-cell subset methodology. Phase 2: The robustness of stabilized blood cell products was evaluated by monitoring T-cell subset values from samples stored at 4°C, 22°C, and 37°C for up to 10 days. **Results:** The largest interlaboratory variation in both absolute and relative T-cell values was 16% in samples with CD4 levels ≥ 400 cells per microliter and 21% in samples with CD4 levels < 400 cells per microliter. Six preparations retained their phenotypic expression for 7 days at 4°C and 22°C. However, only two preparations remained stable for 4 days at 37°C. **Conclusion:** Some stabilized cell preparations are more robust and therefore more suitable for quality assessment purposes. *Cytometry (Clin. Cytometry)* 50: 86–91, 2002. © 2002 Wiley-Liss, Inc.

Key terms: stabilized cell preparation; quality assessment program; T-cell subset; flow cytometry; immunophenotyping; morphospectral attribute; robust cell preparation

Immunophenotyping T-cell subsets of human immunodeficiency virus (HIV)-infected individuals is performed routinely by flow cytometry (1,2). It remains the best surrogate marker for monitoring the immune status of an HIV-infected individual (3). Using traditional or homogeneous gating strategies for T-cell subset enumeration, specimens must be processed within 24 h to preserve their integrity and obtain reliable absolute CD4⁺ T-cell values (4). There are numerous quality assessment and assurance programs (QAPs) for T-cell subset immunophenotyping, but only two employ overnight couriers to ship fresh HIV-positive whole blood specimens (5). These two programs support major HIV antiretroviral drug clinical trials (6).

QASI (International Program for Quality Assessment and Standardization for Immunological Measures relevant to HIV/AIDS) is an international QAP for the evaluation of T-cell subsets which operates globally in resource-poor settings. Participation in QASI is free of charge and it

supports over 120 laboratories in 46 countries. The distribution of fresh blood specimens for such a global program is impractical. In the past few years, a number of commercial stabilized preparations became available, most of which were developed to serve as batch-processed controls for general quality control schemes for leukocyte immunophenotyping (7). These preparations are designed to mimic the properties of whole blood; they can be lysed and generally can be processed as fresh whole blood specimens. The mimicry had only limited success with homogeneous gating protocols. When analyzing blood samples exposed to suboptimal conditions, heteroge-

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Table 1
*Multicenter Evaluation of Stabilized Blood Cell Products and Fresh Whole Blood for Absolute and Relative T-Cell Subset Determinations**

Material tested	CD4 level (cells/ μ L)	Absolute measurements (CV, %)				Relative measurements (CV, %)			
		N	CD3+4+	CD3+8+	CD3+	N	CD3+4+	CD3+8+	CD3+
CD-Chex low	181	39	12	9	9	na	na	na	na
CD-Chex normal	1,049	39	9	11	8	na	na	na	na
FluoroTrol low	211	40	21	10	13	40	15	9	10
FluoroTrol mid	441	40	11	10	11	40	7	11	6
FluoroTrol normal	857	40	7	10	7	40	9	9	4
Immuno-Trol cells	640	75	9	10	8	50	5	6	4
Ortho AbsoluteControl	722	40	11	15	10	38	6	8	4
StatusFlow ^{Lo} low	101	42	18	16	15	37	15	12	10
StatusFlow mid	400	42	13	16	13	37	7	10	6
Fresh whole blood, HIV+	135	29	11	14	11	28	9	6	4
Fresh whole blood, HIV+	403	29	14	10	10	28	6	6	5
Fresh whole blood, HIV-	875	29	9	11	9	22	4	5	4

*N, number of participating laboratories; na, not available.

neous gating is the most reliable option (8). Homogeneous lymphocyte gating is defined here as a two-parameter approach based on dual light scatter from selected leukocyte subpopulations. In this case, both side scatter (SSC) and forward scatter (FSC) parameters define morphological attributes. The heterogeneous gate is also based on a two-parameter approach. This mixed or combination gate includes FSC and CD45 lineage-specific fluorescence attributes. As this gate utilizes one morphological, intrinsic, and one spectral, extrinsic, parameter it is also referred to as a gate with morphospectral attributes. These commercial products remain stable for weeks when stored at room temperature and may be considered as potential candidates for use in QAPs such as QASI.

Phase 1 of this study was designed to assess the performance characteristics of several of the available preparations in a near-ideal multicenter environment. Phase 2 focused on the evaluation of some of the selected stabilized blood cell products under suboptimal environmental conditions.

PHASE 1

The objective of phase 1 was to establish the relative performance characteristics of various available commercial preparations in a multicenter environment where there was a significant diversity in use of flow cytometers, monoclonal antibodies (mAbs), and lysing reagents. In most cases, phase 1 preparations were expedited within 24 h to all participating laboratories in containers that provided protection from excessive heat exposure.

Materials

Nine commercial preparations from five manufacturers were evaluated to determine their relative performance in a multicenter environment. The preparations included Ortho AbsoluteControl (Ortho Diagnostics Systems, Raritan, NJ), StatusFlow mid and StatusFlow^{Lo} low (R&D Systems, Minneapolis, MN), FluoroTrol-CD4 trilevel low, mid, and normal (BioErgonomics, St. Paul, MN); CD-Chex PLUS low and normal CD4 (Streck Laboratories, Omaha, NE),

and Immuno-Trol cells (Beckman Coulter, Miami, FL). The evaluation included three fresh whole blood specimens with EDTA as anticoagulant, two from HIV-positive donors and one from an HIV-negative donor.

Methods

Six multicenter surveys took place between 1997 and 1999. Five shipments included members of the Canadian HIV Clinical Trials Network and one included the QASI participating sites. All preparations were analyzed using the laboratory routine whole blood immunophenotyping procedure. T-cell subsets were reported as lymphocyte percentages, absolute counts, or both. The absolute counts were performed using a single-platform method (9–11).

Statistics

The interlaboratory variation in T-cell value was established for each survey. Means and SDs were calculated. Values falling outside of 2 SDs were considered outliers and were excluded from the database for reassessment of the mean (aggregate mean) and SD.

Results

The coefficients of variation (CV) obtained for all six multicenter surveys are given in Table 1. The commercial stabilized blood cell products with CD4 T-cell levels below 250 cells per microliter had the largest CVs for relative and absolute CD3+4+ determinations. The aggregate means and SDs are shown in Table 2 along with the expected values and ranges provided by the respective manufacturers. The aggregate means fell within the expected ranges supplied by the manufacturers except for CD3+ with CD-Chex low. To various degrees, each stabilized cell preparation deviated from the relative fluorescence intensity levels observed with fresh whole blood. Table 3 shows the relative mean fluorescence intensities and ratio for both CD3+4+ and CD3+4- clusters observed with CD45-fluorescein isothiocyanate (FITC)/CD3-

Table 2
Multicenter Evaluation of Stabilized Blood Cell Products for Absolute and Relative T-Cell Subset Determinations*

Material tested	Absolute measurements mean \pm SD (cells/ μ L)			Relative measurements mean \pm SD (%)		
	CD3+4+	CD3+8+	CD3+	CD3+4+	CD3+8+	CD3+
CD-Chex low	181 \pm 21 (190 \pm 30)	710 \pm 62 (600 \pm 70)	1,005 \pm 93 (790 \pm 100)	na	na	na
CD-Chex normal	1,049 \pm 94 (na)	643 \pm 70 (na)	1,783 \pm 145 (na)	na	na	na
FluoroTrol low	211 \pm 44 (183 \pm 10)	518 \pm 65 (549 \pm 28)	855 \pm 113 (865 \pm 15)	13 \pm 2 (11.6 \pm 2)	33 \pm 3 (34.7 \pm 3)	55 \pm 55 (54.7 \pm 4)
FluoroTrol mid	441 \pm 50 (396 \pm 10)	357 \pm 36 (378 \pm 13)	895 \pm 98 (857 \pm 10)	30 \pm 2 (28.4 \pm 3)	24 \pm 3 (27.1 \pm 3)	61 \pm 4 (61.5 \pm 4)
FluoroTrol normal	857 \pm 64 (794 \pm 64)	480 \pm 47 (496 \pm 20)	1,456 \pm 101 (1,363 \pm 45)	41 \pm 4 (41.2 \pm 3)	23 \pm 2 (25.8 \pm 3)	69 \pm 3 (70.9 \pm 4)
Immuno-Trol	696 \pm 56 (704 \pm 165)	327 \pm 86 (353 \pm 118)	1,038 \pm 193 (1,075 \pm 260)	49.4 \pm 2 (51 \pm 9)	24.8 \pm 2 (26 \pm 6)	76.8 \pm 3 (80 \pm 9)
Ortho AbsoluteControl	722 \pm 80 (790 \pm 173)	314 \pm 47 (353 \pm 88)	1,093 \pm 108 (1,217 \pm 237)	48 \pm 3 (48.8 \pm 11)	21 \pm 2 (21.8 \pm 5)	73 \pm 3 (75.1 \pm 9)
StatusFlow ^{Lo} low	101 \pm 18 (94)	246 \pm 39 (244)	383 \pm 56 (373)	16 \pm 2 (17)	40 \pm 5 (43)	64 \pm 6 (66.4)
StatusFlow mid	400 \pm 50 (417)	151 \pm 25 (156)	578 \pm 77 (597)	48 \pm 3 (49.1)	18 \pm 2 (18.4)	69 \pm 4 (70.2)

*Manufacturer's values are given in parentheses, na, not available.

PC5/CD4-RD1/CD8-ECD staining. A low value of the ratio of CD3+4+ to CD3+4- corresponds to poor resolution of positive cells, leading to difficulty in setting proper quadrant cursors to define the T-helper cell cluster. This problem with resolution varied between types of preparation and between various CD4 levels. The lowest relative fluorescence ratios were observed with preparations having low CD4 levels.

PHASE 2

All stabilized blood products for evaluation in phase 2 had to be compatible with the FACSCount flow cytometer (BD Biosciences, San Jose, CA), a dedicated flow cytometer for CD3+, CD3+4+, and CD3+8+ absolute enumeration. This is an essential requirement because a large number of the laboratories enrolled in the QASI program use this instrument. Preparations that were not compatible with the FACSCount were eliminated from further consideration. Three stabilized preparations from phase 1 and TransFix (UK National External Quality Assessment Schemes [NEQAS], Sheffield, UK), a stabilizing reagent (kindly provided by Dr. D. Barnett, UK NEQAS), were evaluated for their robustness under suboptimal environmental conditions. The TransFix preparation was not available during phase 1 and therefore was only introduced for phase 2. The Ortho AbsoluteControl product was discontinued and therefore was not included in phase 2.

The objective of phase 2 was to assess the robustness of the remaining preparations to help with the selection of the best stabilized material for QASI. This preparation must have the capacity to withstand global transit for a minimum of up to 6 days and retain phenotypic integrity under suboptimal thermal conditions. The study was constructed to simulate shipping conditions to sub-Saharan Africa and to South America. Aliquots were stored at 4°C, 22°C, and 37°C and monitored for up to 10 days.

Materials

Seven stabilized whole blood preparations were evaluated in phase 2: FluoroTrol-CD4 trilevel (low, mid, and

Table 3
Comparison of CD3+4+ and CD3+4- Mean Fluorescence Intensity Observed With Testing Material When Stained With CD45-FITC/CD3-PC5/CD4-RD1/CD8-ECD

Material tested	Mean fluorescence intensity		Ratio of CD3+4+ to CD3+4-
	CD3+4-	CD3+4+	
Whole blood	0.149	39.5	265
StatusFlow ^{Lo} low	0.282	27.8	99
StatusFlow mid	0.255	35.9	141
Immuno-Trol	0.274	42.9	157
FluoroTrol low	0.223	19.7	88
FluoroTrol mid	0.196	35.1	179
FluoroTrol normal	0.167	35.6	213
CD-Chex low	0.174	9.6	55
CD-Chex normal	0.182	9.9	54
Ortho AbsoluteControl	0.220	40.9	186
TransFixed blood	0.105	15.7	150

normal), Status Flow mid and Status Flow^{Lo} low, ImmunoTrol cells, and TransFix, a whole blood stabilizing reagent. When TransFix is added to fresh blood, the sample retains morphology and preserves cell surface expression for an extended period. Based on previous studies (12,13), the optimal TransFix concentration was established at 1:10 (1 part TransFix to 10 parts blood).

On an Epics XL (Beckman Coulter) flow cytometer, a four-color immunophenotyping protocol was used to determine absolute counts. The mAb cocktail was CYTO-STAT tetraCHROME CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 (Beckman Coulter) and Flow-Count fluorospheres (Beckman Coulter) as volume calibrators were included as part of the single-platform absolute counting procedure.

Methods

All stabilized blood cell preparations were dispensed into aliquots and stored at 4°C, 22°C, and 37°C. The TransFix material was mixed with fresh HIV-positive and HIV-negative whole blood and then dispensed. All preparations were analyzed in triplicates on day 0 (fresh) to establish baseline T-cell subset values. Subsequent monitoring took place on days 2, 4, 7, and 10. Both percent and absolute cell counts were obtained.

Immunophenotyping

Each aliquot was processed for analysis on both the FACSCount and the Epics XL flow cytometers. With the FACSCount, 50 µL of stabilized cell preparation was added to a CD3/CD4 and CD3/CD8 reagent tube pair (accurate pipetting is essential). The mixture was incubated for 1 h in the dark at room temperature. After the addition of 50 µL of fixative, the samples were analyzed. With the Epics XL, 100 µL of sample is introduced into a 12-mm × 75-mm polystyrene tube (accurate pipetting is essential). Then, 10 µL of a four-color mAb combination CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 was added and the resulting mixture incubated for 10 min at room temperature. Samples were processed through the Coulter TQ-Prep (Beckman Coulter) workstation for automated and sequential distribution of the ImmunoPrep (Beckman Coulter) reagents for lysing, stabilizing, and fixing. After fixing, an additional 1 mL of 2% formaldehyde solution was added. Prior to immunophenotyping acquisition, 100 µL of Flow-Count fluorospheres was added to each preparation.

Flow Cytometric Analysis

Sample analysis was performed on an Epics XL using a universal template (6) for obtaining both single-platform absolute counts and percentages of CD4, CD8, and CD3 T

Table 4
Monitoring of Absolute T-Cell Subsets From FluoroTrol (mid) Stored at 37°C

No. of days stored at 37°C	CD3+4+ (cells/µL)	CD3+ (cells/µL)
0	285	558
2	240	464
4	196	378

cells. The universal template based on a double anchor gate strategy was used for the analysis of each processed aliquot.

The FACSCount single-platform flow cytometer is a dedicated instrument for the absolute enumeration of CD3+, CD3+4+, and CD3+8+ cells. The analysis is fully automated and the reagents are provided in a premeasured kit. A heterogeneous T gate is applied to a two-color, two-tube panel containing CD3/CD4 and CD3/CD8 reagents. Fluorospheres are included in the tubes and are used for single-platform absolute count calculations.

Statistics

Absolute and percent T-cell subset values from each time point were averaged and SDs and CVs were calculated. The definition of acceptable performance for a given preparation was based on the SD for relative measurements and on the CVs for absolute counts. According to the 1997 revised CDC guidelines (14), acceptable ranges for replicates on percent values were defined as ±2%, which is the variability determined from preparing and analyzing replicates. For absolute count determinations, the maximum acceptable variation was defined as 10%. Schnizlein-Bick et al. (9) and Reinman et al. (10) each reported results from a five-laboratory study using the single-platform method with TruCount and Flow-Count fluoromicrospheres, respectively. The mean of interlaboratory and intralaboratory variation for both studies was 10%. In this study, the 10% value was used as the acceptable limit for between-day variation on absolute counts.

Stability limits were assessed according to the CV and SD set limits produced for absolute and relative measures, respectively. When %CV and SD were above the acceptable limit, the most discordant entry (the last data point) was excluded and both CVs and SDs were recalculated. This process was repeated until CVs and SDs fell within the acceptable limit. An example of this type of elimination process is provided in Tables 4 and 5. In Table 4, the raw absolute count data for CD3+ and CD3+4+ from the

Table 5
Applied Statistical Analysis to Establish Temperature-Dependent Stability

No. of days stored at 37°C	Mean ± SD (cells/µL)		%CV (acceptable limit ≤10%)			
	CD3+4+	CD3+	CD3+4+	Status	CD3+	Status
4	240 ± 36	467 ± 74	15	Fail	16	Fail
2	263 ± 23	511 ± 47	9	Pass	9	Pass

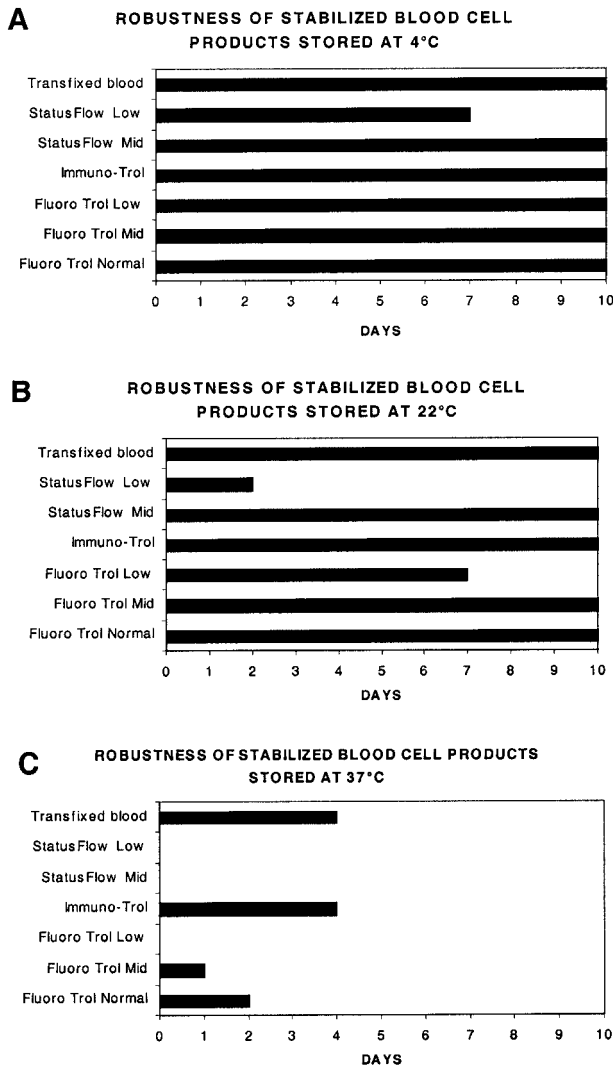


FIG. 1. Bar charts showing the stability (in days) of various blood cell products when stored at 4°C (A), 22°C (B), and 37°C (C).

FluoroTrol midrange preparation stored at 37°C were tabulated. The analysis was performed on a FACSCount instrument. Table 5 shows how the statistical cutoff points were established for temperature-dependent stability. After 4 days of exposure to 37°C, the CV values were above 10%. By removing the results obtained at day 4, the CVs were reduced to less than 10%, the acceptable limit. Therefore, the product had a stability of 2 days at 37°C.

Results

All preparations stored at 4°C were stable for 10 days except StatusFlow^{Lo} low, which retained its phenotyping integrity for 7 days. At room temperature, five preparations were stable for 10 days. FluoroTrol low and StatusFlow^{Lo} low were stable for 7 and 2 days, respectively. At 37°C, Immuno-Trol cells and the specimens treated with TransFix retained their integrity for 4 days. FluoroTrol

normal and mid remained stable for 1 and 2 days, respectively. The stability of each product is shown in Figure 1.

Some preparations collapsed before day 10, particularly those stored at 37°C. When stored at 22°C or 4°C, some preparations could be analyzed up to several days, although the lymphocyte cluster was difficult to identify (Fig. 2) and the T-cell subset values decreased over time.

DISCUSSION

A number of commercially available stabilized whole blood preparations were evaluated to determine which mimics most faithfully the morphospectral properties of fresh blood and yet remains robust for days while exposed to temperature variations including heat exposure up to 37°C. In the past, relying on the traditional combined light scattering patterns of stabilized preparations was often disappointing. The emphasis has shifted to a heterogeneous gating protocol such as CD45 gating (15). The combination of lineage-specific fluorescence and SSC has opened new possibilities for preserved blood preparations. This evaluation was based on the more stable morphospectral attributes of commercially available preserved blood preparations. With single-platform absolute counting protocols, there is an opportunity to consider these preparations as substitutes for fresh whole blood. Some rigorous requirements still remain, including preservation of orthogonal scatter, conservation of fluorescence intensity for the lineage-specific marker(s) (CD45 and/or CD3), dose-specific lysing properties, and robustness with respect to heat and duration of heat exposure.

Although stable blood preparations do not replace fresh whole blood, they offer a practical solution for quality assessment challenges in resource-poor settings where antiretroviral drug distribution is ongoing or will soon take place. Some of the tested products are good candidates for wide-scale QAPs. The multi-CD4 level preparations present a lot of interest to HIV clinical trial assessment programs. However, our results indicate that the preparations with low CD4 levels are more heat sensitive. Nevertheless, the preparations with low CD4 levels are improving and have been used successfully as quality assessment material for some international programs (data not shown).

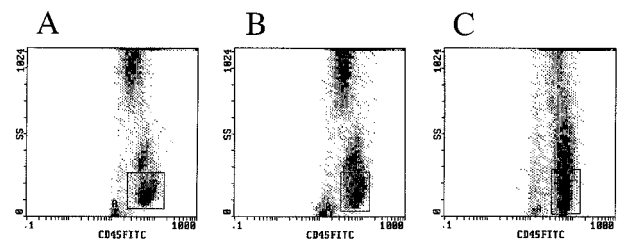


FIG. 2. The heterogeneous dot plots (A-C) show the leukocyte distribution of a stabilized blood cell product stored for various periods of time at different temperatures. A: Analysis of the product stored for 2 days at 4°C. B: Analysis of the same product stored for 10 days at 22°C. C: Analysis of the same product stored for 2 days at 37°C.

Immuno-Trol preparations and specimens treated with TransFix displayed the best resistance to temperature extremes. The TransFix solution is currently being tested for its potential to inactivate HIV and other viral agents. This is an important consideration in the face of rigorous regulations involved with international transportation of infectious goods. In conclusion, we have selected a commercially available product that is noninfectious and stable for at least 3 days at 37°C. Stabilized seronegative blood cell products could be shipped without the infectious labeling, therefore increasing the likelihood that they will reach their destination quickly and in a cost-effective manner.

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