FACSCount™

The most complete system for measuring absolute CD4, CD8, and CD3 counts

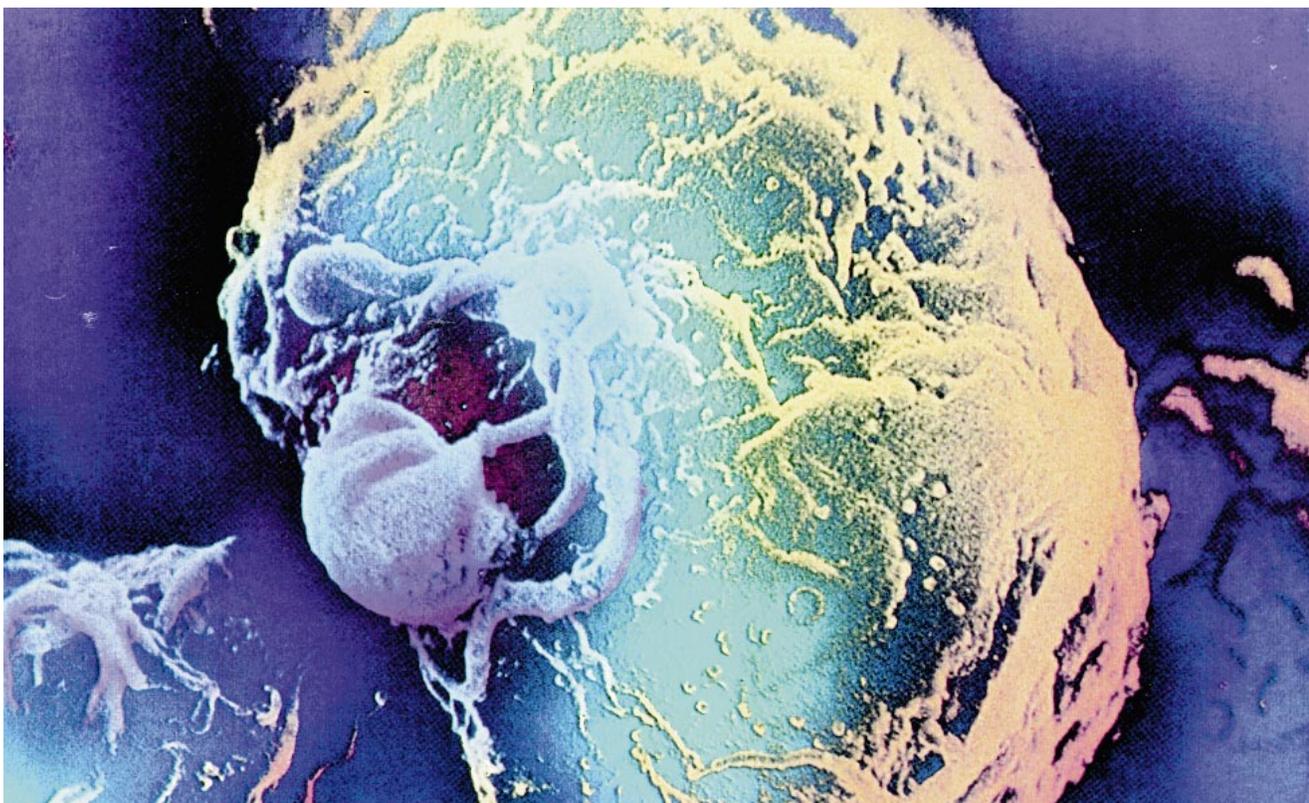*For in vitro diagnostic use*

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FACSCount White Paper • July 1994

AIDS virus budding from human lymphocyte

Clinical Significance of CD4

The World Health Organization estimates that by the year 2000, 30 to 40 million people worldwide will be infected with the human immunodeficiency virus (HIV).[1] The continued spread of the disease and the lack of a vaccine or cure, poses a tremendous challenge to worldwide health care. The average time it takes for a person to progress from seroconversion to AIDS is 8 to 11 years. The appropriate intervention and management over the course of the infection is the focus of many ongoing clinical studies.

50,000 +

500,000 +

50,000

1 million +

100,000

2.5 million

10 million

2 million

25,000
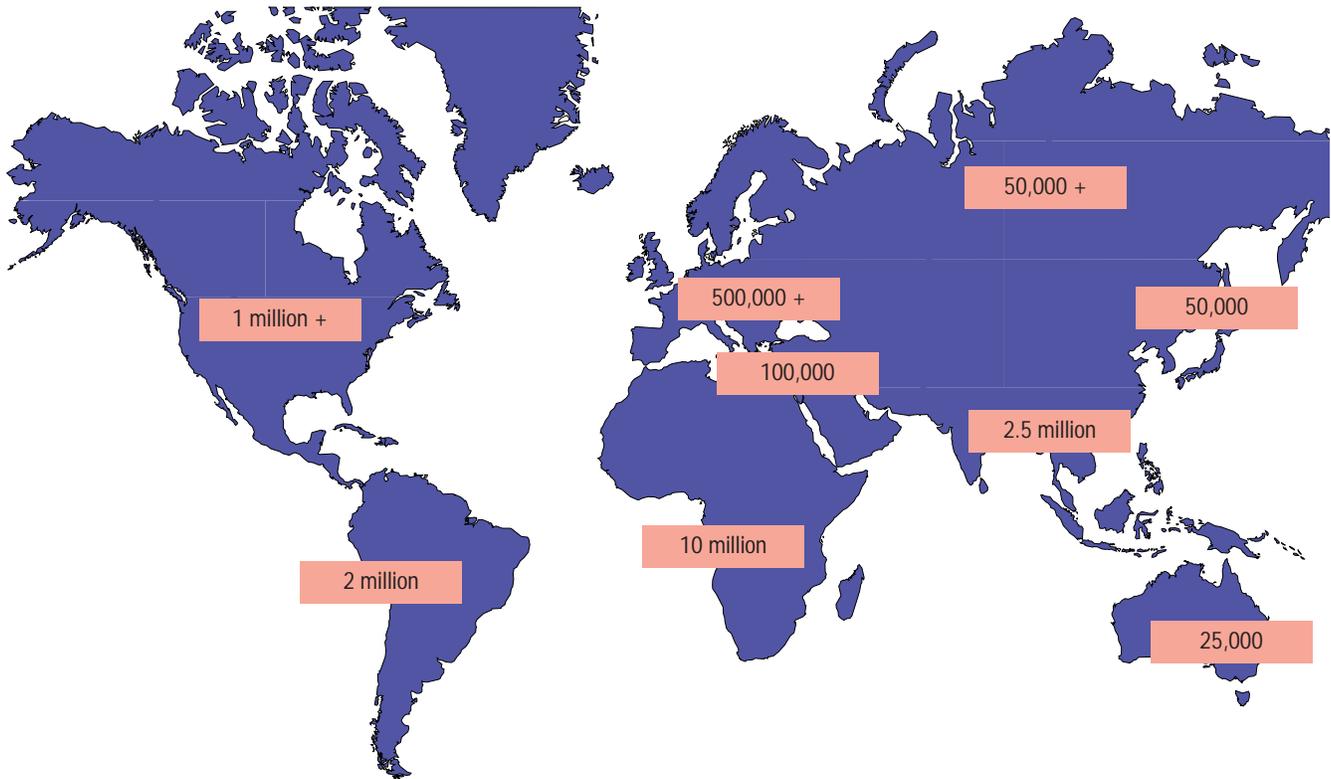
1993 Estimated Worldwide Distribution of Cumulative Adult HIV Infections
*Source: WHO Global Program on AIDS, late 1993.*

Immunophenotyping of T-lymphocyte subsets provides important information for a number of patient conditions. As the primary target of HIV infection, CD4 cells are preferentially infected and depleted with progression of disease. It is widely recognized that as the number of CD4+ T lymphocytes declines the risk and severity of clinical symptoms increases.[2] For persons infected with HIV, routine monitoring of absolute CD4 counts provides important information on their immune status. Serial measurements of absolute CD4 counts may be used to determine patient treatments, response to therapy, and disease progression. Many infected persons are acutely aware of changes over time in their CD4 counts. Physicians may also use target CD4 counts to guide them to the most probable clinical conditions and appropriate therapies at various stages of HIV disease.[3] The T lymphocytes expressing the CD8 marker are the suppressor/cytotoxic T lymphocytes. CD8 T-lymphocyte counts increase at the onset of HIV infection and continue to rise through the progression of the disease.[4]
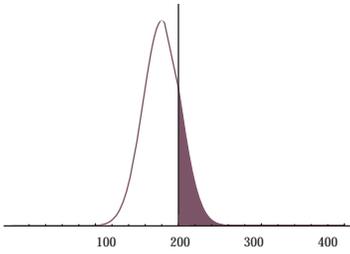
Patient's true CD4 count: 180

Probability of misclassification

by conventional technology: 13%
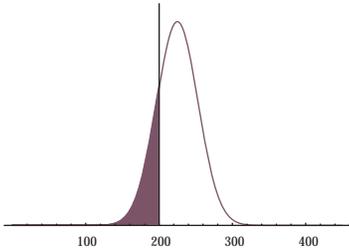
Patient's true CD4 count: 225

Probability of misclassification

by conventional technology: 19%

Therapeutic Decision Levels (3) *Absolute CD4 T-Lymphocytes*	
500 cells/µL	initiation of antiretroviral therapy
200 cells/µL	prophylaxis for Pneumocystis carinii pneumonia
100 cells/µL	increase in opportunistic infections
< 50 cells/µL	high occurrence of HIV-related deaths

The graphs immediately to the left illustrate the importance of precise measurements in the determination of patient therapy. Each curve describes the expected distribution surrounding a true CD4 count of 180 and 225. Conventional methodology uses absolute lymphocyte counts from a hematology analyzer multiplied by the %CD4+ cells from a flow cytometer to produce absolute counts. Based on reported variation with conventional methodology, the probability of misclassification above or below 200 cells/µL with a single site is determined by the area under the curve. The probability of misclassifying a person increases with multiple sites reporting CD4 counts with conventional technology.

Research into improving patient care continues and clinical studies are key to determining safety and efficacy. Absolute CD4 counts are an important parameter in many clinical studies to evaluate the efficacy of new and different regimens of therapy. As an example, the combination of zidovudine (AZT) and zalcitibine (ddC) for the treatment of advanced disease was based on data showing increases in CD4 counts.[5]

Clinical studies also use CD4 counts as a criterion for determining study groups and study endpoints. To enable consistent and comparable results across multi-center evaluations, improved standardization of absolute count determinations is critical.

In the United States, CD4+ T lymphocytes are also incorporated into the case definition of AIDS published by the Centers for Disease Control. The revised classification system, detailed in the December 1992 Morbidity and Mortality Weekly Report, integrates clinical categories with CD4+ T-lymphocyte counts. Precise and reproducible CD4 counts are important to the clear assessment of the HIV pandemic.

1993 revised classification system for HIV infection and expanded AIDS surveillance case definition for adolescents and adults*

Clinical Categories			
CD4+ Cell Categories	A Asymptomatic, acute (primary) HIV or PGL[†]	B Symptomatic, not A or C conditions	C AIDS-indicator conditions
1. ≥ 500/µL	A1	B1	C1
2. 200–499/µL	A2	B2	C2
3. < 200/µL AIDS-indicator T-cell count	A3	B3	C3

* The purple-shaded cells illustrate the expanded AIDS surveillance case definition. Persons with AIDS-indicator conditions (Category C) as well as those with CD4+ T-lymphocyte counts <200/µL (Categories A3 or B3) will be reportable as AIDS cases in the United States and Territories, effective January 1, 1993.
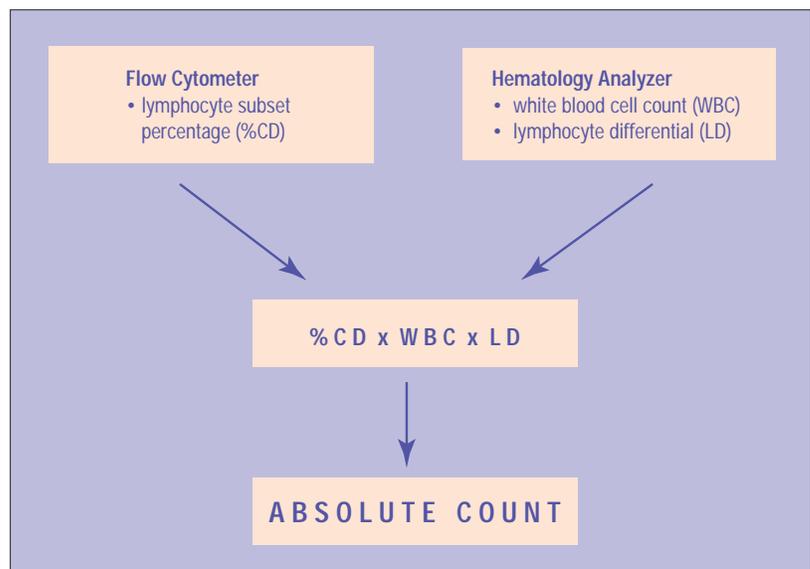
† PGL=persistent generalized lymphadenopathy. Clinical Category A includes acute (primary HIV) infection. MMWR, December 18, 1992.

Precise absolute CD4+ T-lymphocyte values have become an accepted and important surrogate marker in patient management of HIV infection.

- Physicians treating HIV-infected persons determine the appropriate staging and treatment in conjunction with absolute CD4 counts.

- Persons infected with HIV rely on CD4 counts for information on their immune status.

- Clinical investigators use CD4 counts as criteria for entry into drug trials and as a surrogate marker to measure drug activity and efficacy.

- A precise CD4 count is necessary to accurately describe the epidemiology of HIV and the direction for economic and healthcare policies.

Overview of Conventional Methodology for CD4 Count

The importance of absolute CD4 counts to persons infected with HIV has increased the use of subset immunophenotyping by flow cytometry. Absolute CD4 T-lymphocyte count determinations by the conventional flow cytometric methodology require a combination of results from a flow cytometer and a hematology analyzer.

Flow Cytometer
- lymphocyte subset percentage (%CD)

Hematology Analyzer
- white blood cell count (WBC)
- lymphocyte differential (LD)

% C D x W B C x L D

A B S O L U T E   C O U N T

Guidelines and recommendations have helped to standardize the practice, but the reliance on two instruments for an absolute count increases the variability of the result. Most of this error is due to the variability in the hematology analyzers.[6]

As the need for CD4 counts in the clinical laboratory increases, limitations of the conventional methodology may prohibit their accessibility. As an example, different instrument platforms have different restrictions on the time in which a sample may be processed. The technical skill required for flow cytometry operators may also be prohibitive to smaller laboratories.

An accurate and reproducible clinical system is required for improved standardization of absolute CD4 count determinations.

FACSCount™ System Overview

FACSCount is a complete system incorporating instrument, reagents, controls, and software. It employs a direct two-color immunofluorescence method for enumerating absolute lymphocyte counts (cells/µL whole blood) of the following mature human lymphocyte subsets: T lymphocytes (CD3+), T-helper/inducer lymphocytes (CD3+CD4+), and T-suppressor/cytotoxic lymphocytes (CD3+CD8+). In addition, the helper/suppressor T-lymphocyte (CD4+/CD8+) ratio is provided. These data can provide important information for staging and monitoring patients infected with HIV.

The FACSCount System consists of the following key components:

- FACSCount Instrument and System software
- Unit test reagent pairs
    —tube of CD4/CD3 reagents and reference beads
    —tube of CD8/CD3 reagents and reference beads
- Fixative solution (5% formaldehyde)
- Control beads solutions (four levels—zero, low, medium, and high)
- FACSCount automated pipette

FACSCount Reagents

The FACSCount Reagent Kit consists of 50 paired reagent sets containing a mixture of monoclonal antibody reagents conjugated to two fluorochromes and a known number of fluorochrome-integrated polystyrene beads. The first tube in each pair contains CD4 and CD3 antibodies while the second contains CD8 and CD3. The kit also contains two vials of formaldehyde fixative (fixative solution), sufficient to prepare 50 tube pairs.

FACSCount Controls

The FACSCount Control Kit consists of paired control bead sets containing fluorochrome-integrated (2-µm) polystyrene beads at four levels (zero, low [50 beads/µL], medium [250 beads/µL] and high [1000 beads/µL]). The control kit allows 25 control runs.

Basic Operation

Sample Preparation

FACSCount sample preparation consists of three easy steps with minimal hands-on time. This reduces the risk of exposure to biohazards and improves laboratory efficiency.

1 Pipette 50 µL whole blood into each tube, vortex and incubate.

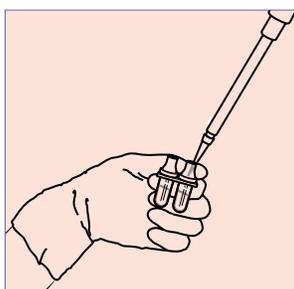**2** Pipette 50 µL of fixative solution into each tube and vortex.

3 Run on the FACSCount instrument.
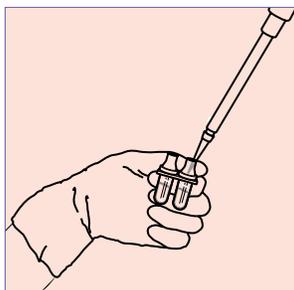
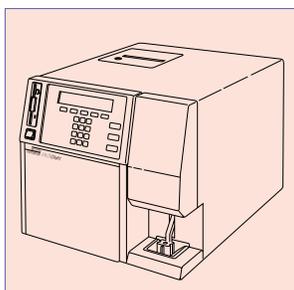Step 1a
Add 50 µL blood to each
of the two tubes.

Step 1b
Cap, vortex, and incubate
for 60 minutes.

Step 2a
Add 50 µL fixative to each tube.

Step 2b
Cap and vortex.

Step 3
Run the tubes on the FACSCount.

Control Preparation

1 Pipette 50 µL of normal whole blood into two pairs of FACSCount reagents and prepare as noted on previous page.

2 Before running on the instrument, pipette 50 µL of control beads into the corresponding reagent pairs.

3 Vortex and run on the FACSCount instrument.

Analysis

The FACSCount System software provides automated analysis, requiring no operator intervention.

The FACSCount System provides a report quantifying the CD4+, CD8+, and CD3+ T lymphocytes as absolute numbers of lymphocytes per µL (mm^3) of blood, and the CD4+/CD8+ T-lymphocyte ratio. The absolute CD3+ lymphocyte count is reported as the average of the total CD3+ values from the same blood sample stained with the two reagent tubes.

The reportable ranges generated by the FACSCount System for each lymphocyte subset are as follows:

CD4	50–2000 cells/µL
CD8	100–2000 cells/µL
CD3	100–3500 cells µL

Sample Printout

```
FACSCount SW  Version 1.0  4/93

Sample Results
Reagent Lot ID : 20052121
Reference Beat Counts – beads/ul
CD4 Tube : 1004
CD8 Tube : 2032

Date : 3/15/93  18:51

Last Control Run : PASSED
Control Run Date : 3/15/93  18:42
Control Run Reagent Lot ID : 20052121
Control Run Control Lot ID : 29031721

Accession #:5678


Absolute Counts – cells/ul
     CD4 :  838
     CD8 : 1107
Total CD3 Average : 2002
CD4/CD8 :  0.76
CD4/CD3 :  0.42
CD8/CD3 :  0.55

Signature:_____
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Comments:_____
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Control Printout

```
FACSCount SW  Version 1.0  4/93

Control Results
SMITH MEDICAL CENTER
Operator : PJT
Control Bead Lot ID : 29031721
Control Bead Counts – beads/ul
   low :   50  med : 247   high : 960
Reagent Lot ID : 20052121
Reference Bead Counts – beads/ul
CD4 Tube : 1004
CD8 Tube : 2032

Date : 3/15/93  18:42

Control Run : PASSED

Lab Normal ID:  12345678


Absolute Counts – cells/ul:
   CD4      zero tube  :   796
   Total CD3 zero tube  : 1927
   CD8      low tube   :  1113
   Total CD3 low tube   : 1993
   CD4      medium tube:   799
   Total CD3 medium tube: 1897
   CD8      high tube  :  1040
   Total CD3 high tube  : 1882
                      Mean      Range
                     Counts
                     ------    -----
   CD4 (2 tubes)     :  797        3
   CD8 (2 tubes)     : 1076       73
   Total CD3 (4 tubes) : 1924      111
Absolute Counts – beads/ul:
   Control Bead zero tube  :    0
   Control Bead low tube   :   56
   Control Bead medium tube :  231
   Control Bead high tube  :  964
Control Bead Results:
   R        :   0.9998
   Slope    :   1.00
   Intercept :  -3
Reference Bead Cluster Locations:
   zero tube     : 220,211
   low tube      : 220,212
   medium tube   : 221,213
   high tube     : 219,211

 Signature: _____
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 Comments: _____
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Principles of the Procedure

Staining

The FACSCount System is designed to use unlysed whole blood, collected in liquid EDTA. When whole blood is added to the tubes of a sample reagent pair, the fluorochrome-labeled antibodies bind specifically to antigens on the surface of lymphocytes. The FACSCount instrument detects two colors and measures relative cell size. The CD3 cells will fluoresce red and the CD4 and CD8 cells will fluoresce yellow when analyzed on the FACSCount instrument. A known number of reference beads is contained in each reagent tube and functions as a fluorescence and quantitation standard for calculating the absolute counts for the CD4+, CD8+, and CD3+ T lymphocytes. Fixative solution is added to the stained samples prior to analysis to preserve the integrity of the antibody binding. No lysing is necessary.
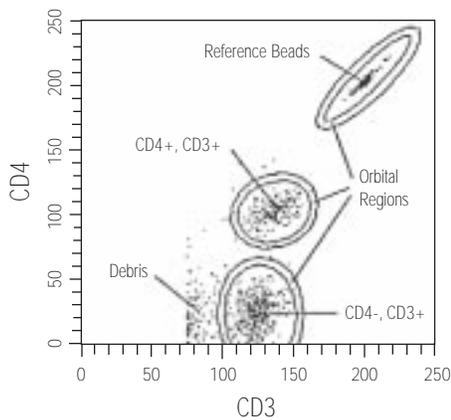
Graphic representation of the software algorithm

System Software and Analysis

When running the sample tubes, the system automatically sets the threshold and acquires a fixed number of events. The events are analyzed in two dimensions—red versus size—and most of the erythrocytes, platelets, monocytes, and granulocytes in the sample are excluded from the analysis by the placement of the threshold. The software evaluates the location and cluster integrity of the beads and each of the cell populations. This is done again in two dimensions: red versus yellow. The starting point for ellipse placement is predetermined from the control run. The ellipses optimize their position using the center of each population. After the initial placement of the ellipses, an orbital region is placed around each ellipse. The software will collect a sufficient number of events, up to 30,000 events per sample, so that the precision of the measurement is not limited by statistical sampling variation. The orbital region is used for data quality control. If the data meet the quality limits defined in the software, the software will proceed to report results. If the data do not meet the quality limits, the appropriate error messages are printed and sample results (cells/µL blood) are withheld.

In summary, there are five steps that occur for each sample: the threshold is positioned, the bead and cell populations are located, the ellipses are positioned, the orbital regions are placed, and the data are collected. The steps are performed for both the CD4 and the CD8 tubes. They are all done automatically by the system with no user intervention.

The data is then processed and reported. The absolute counts are determined by a simple ratio:

$$\frac{\text{observed counts from the population of interest}}{\text{observed reference bead counts}} \times \frac{\text{reference bead count}}{\text{µL whole blood}} = \text{absolute count (cells/µL) of population of interest}$$

The numbers are reported on the printout as CD4, CD8, and CD3. Since there is a CD3 count with each tube, the CD3 is reported as the average of the CD3 counts from each tube.
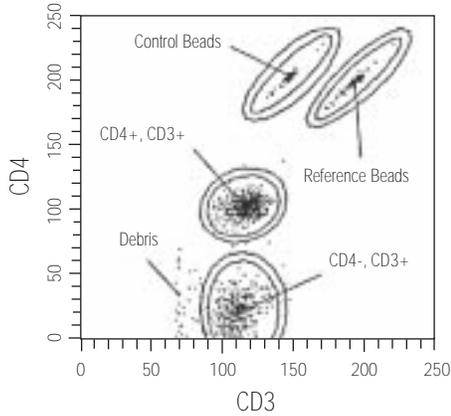
Graphic representation of the control software algorithm

Control Runs

The control run is handled similarly to the sample run, except the control beads are added to the prepared normal samples immediately before running on the instrument. Becton Dickinson recommends that controls are run at the beginning of each day. The control run determines the counts of four control bead suspensions. During a control run, the software will locate and count the control bead populations in order to verify the linearity of the system. The only difference from the sample run is that an additional ellipse is positioned around the control beads after locating the reference beads. The printout reports the correlation, slope, and intercept for the observed counts versus the expected counts. The control run also:

- confirms there is staining of the cells
- fails if any cell counts are less than the reportable range
- checks the ranges on the quadruplicate samples of CD3 and the duplicate samples of CD4 and CD8

The results (Pass or Fail) of the control run are reported on each subsequent sample. After running the controls on the system, the data can be recorded on a control chart for that instrument.

Quality Control

The FACSCount has quality control built into its operation so erroneous results are not reported. The highlights of the internal quality control are:

- automatic debris gating: the system finds an optimal gate to eliminate debris from the analysis.
- detection of population overlap: the orbital bands are used to ensure each population maintains its integrity and isn't affected by others.
- quality control limits in orbital regions: these regions are used to determine if abnormal distributions of data exist so erroneous information isn't provided.
- count limits on background events to prevent event distributions outside of the regions: the data space is defined with unique positions for each of the data clusters. This check looks for undefined data distributions and, if found, prevents reporting of any results.

FACSCount Evaluation Clinical Data

FACSCount System performance was evaluated in clinical studies in the United States, Belgium, and England. Additional evaluations are also ongoing in Spain and Poland, with increased emphasis on improved understanding of disease progression and correlation to other surrogate/cellular markers.

Accuracy

With the advent of new counting technologies, it is important to verify that counts are accurate relative to current technology. Evaluation of system accuracy was determined in comparison to the conventional methodology for immunophenotyping: a cytometer and hematology analyzer. Conventional methodology at all sites consisted of a Becton Dickinson FACScan flow cytometer along with their available hematology analyzer. The hematology instruments at the clinical trials sites were the Ortho ELT-1500, Coulter STKS, Sysmex NE 8000, and the Cell-Dyn 1600.

Each whole blood sample was prepared and analyzed in parallel with conventional methodology and the FACSCount System. Accuracy, for purposes of the studies, was defined as the correlation coefficient (r) between the measures obtained from the conventional methodology and the FACSCount System.

Whole Blood Sample

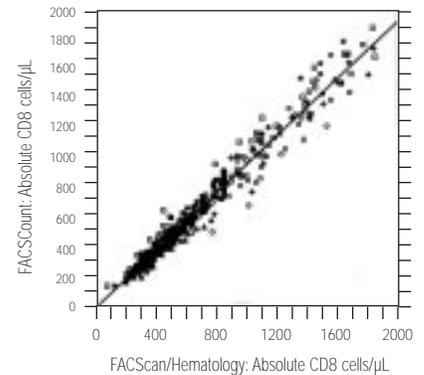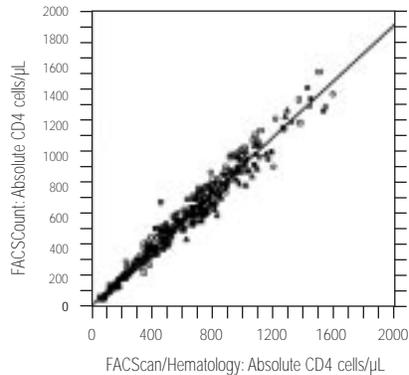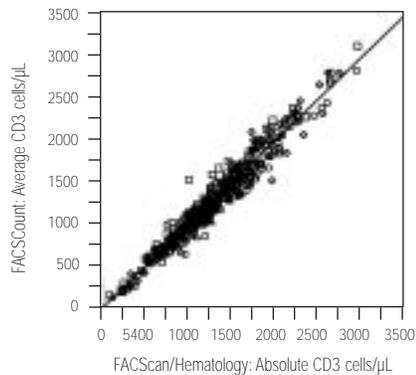**Conventional Methodology**　　**FACSCount System**
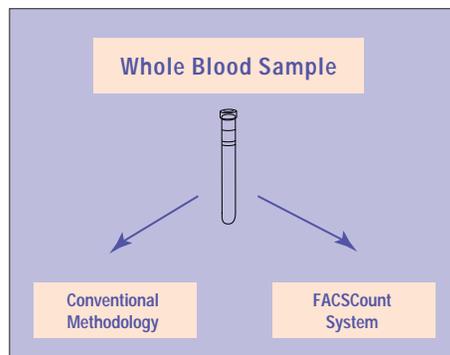
Accuracy Results from Clinical Trials

The FACSCount System produced equivalent results to the current standard for subset immunophenotyping for absolute CD4, CD8, and CD3 T-lymphocyte counts.

Regression statistics of comparison of absolute counts obtained with the FACSCount System versus results calculated by the conventional methodology

| Absolute CD4+ T Lymphocytes	Absolute CD8+ T Lymphocytes	Absolute CD3+ Lymphocytes
n = 351	n = 378	n = 383
R = 0.981	R = 0.983	R = 0.975
slope = 9,823	slope = 0.943	slope = 0.960
intercept = 15.161	intercept = −7.58	intercept = 18.895

Regression graphs for comparison of absolute counts obtained with the FACSCount System versus results calculated by the conventional methodology

Precision

The sequential monitoring of persons infected with HIV has emphasized the need for reproducible counts. An important aspect of FACSCount clinical trials has been to estimate the reproducibility of the system with replicate processing of individual samples.

To characterize tube-to-tube reproducibility, four FACSCount reagent pairs were stained separately for each sample and acquired on the system. The replicate data were used to calculate coefficients of variation across the reportable range of the system as well as at CD4 values surrounding therapeutic decision points.

The FACSCount System produced improved precision over the reported variability in conventional methodology.

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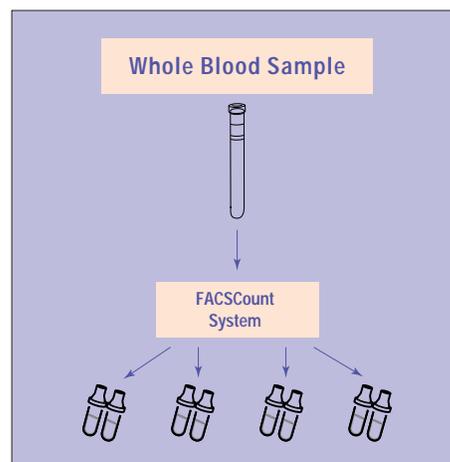
Coefficients of variation (CVs) determined across the reportable range of
FACSCount System parameters at two clinical sites. Additional abnormal samples
were collected to calculate CVs within the specific CD4 ranges.

	CVs at Site 1	CVs at Site 2
Absolute CD4+ T lymphocytes		
100–300 cells/µL	2.32%	3.38%
400–600 cells/µL	3.95%	4.59%
50–2000 cells/µL	3.75%	5.10%
Absolute CD8+ T lymphocytes		
100–2500 cells/µL	2.99%	4.05%
Absolute CD3+ Lymphocytes		
100–3500 cells/µL	2.51%	3.07%
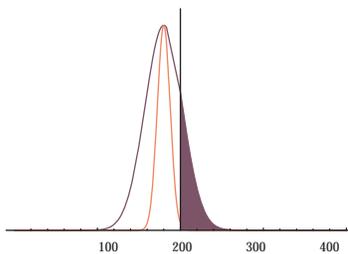
Patient's true CD4 count: 180

Probability of misclassification

by standard technology: 13%

by FACSCount: 0.06%
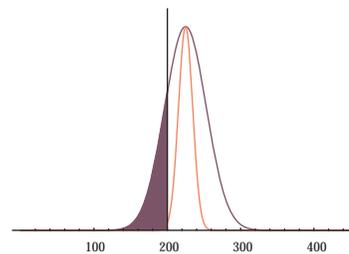
Patient's true CD4 count: 225

Probability of misclassification

by standard technology: 19%

by FACSCount: 0.5%

Interlaboratory Reproducibility

As a criterion for patient enrollment in clinical studies, specific levels of absolute CD4 are often used to define study groups. Consistent and comparable reporting of absolute counts becomes critical to the results and the conclusions of the study. As a dedicated system for providing absolute CD4, CD8, and CD3 cell counts, the FACSCount System produces comparable results across different laboratories.

Comparability and reproducibility were accomplished by preparation and analysis of whole blood samples in parallel at two different clinical sites. Regression analysis was performed to determine the comparability of absolute counts obtained using the FACSCount System at both sites. Tube-to-tube reproducibility was also estimated at both sites by four separately stained replicates of each sample. The replicates were used to calculate coefficients of variation across the reportable range of the system for normal controls and abnormal samples separately.

Regression statistics for comparison of absolute counts obtained from the same samples by two different sites

	n	R	slope	intercept
Absolute CD4+ T Lymphocytes	103	0.993	0.984	10.180
Absolute CD8+ T Lymphocytes	124	0.995	1.014	−0.347
Absolute CD3+ Lymphocytes	126	0.995	0.996	21.177

Absolute CD4+ T Lymphocytes

| Site	n	mean	df	SD*	% CV
Controls					
Site 1	34	786.39	49	23.50	2.99
Site 2	34	771.53	78	31.88	4.13
Abnormals					
Site 1	14	292.81	39	12.14	4.15
Site 2	14	288.18	48	14.92	5.18

* SD as an estimate of within-sample reproducibility

Stability

To increase flexibility in sample processing, comprehensive studies evaluated the performance of the FACSCount System for extended whole blood and stained sample storage times. Stability was determined by comparing the results from each time point to time zero. Time zero results were collected immediately following the recommended sample preparation procedure using whole blood less than six hours from draw. For these studies, stability was defined as changes equal to or less than 10% of the time zero value for 24-hour storage times and 15% of the time zero value for 48-hour storage times.

Storage of whole blood produced stable results up to 48 hours from draw when stained and analyzed with the FACSCount System.

Storage of stained samples produced stable results up to 48 hours following sample fixation with the FACSCount System.
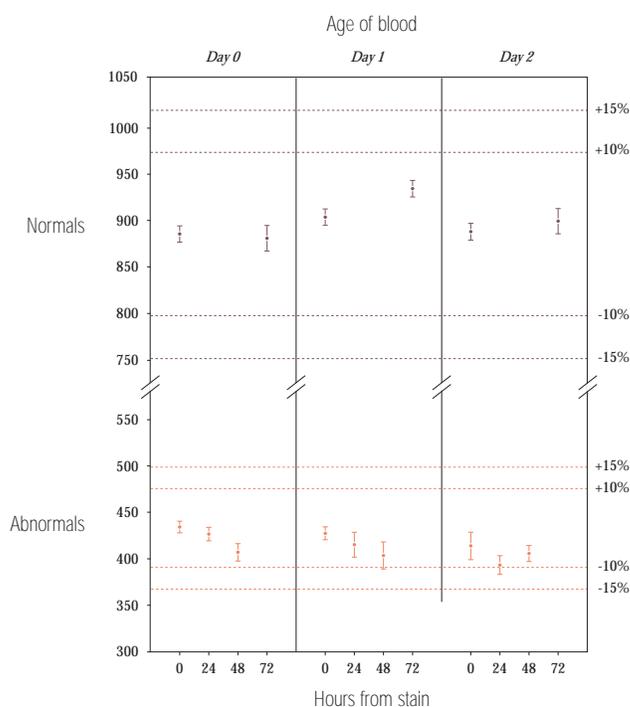
Absolute CD4+ T-lymphocyte counts across donors at each time point, including the 95% confidence interval (CI), and % CV

	Day 0				Day 1				Day 2			
	0 hr	24 hr	48 hr	72 hr	0 hr	24 hr	48 hr	72 hr	0 hr	24 hr	48 hr	72 hr
Normals mean (cells/µL)	885	–	–	881	993	–	–	934	887	–	–	898
95%	±8.72	–	–	±13.75	±8.92	–	–	±8.95	±9.13	–	–	±13.49
% CV	2.66	–	–	4.22	2.67	–	–	2.59	2.78	–	–	1.05
Abnormals mean (cells/µL)	434	426	407	–	427	425	403	–	413	393	405	–
95% CI	±6.25	±7.33	±4.92	–	±6.87	±13.34	±14.52	–	7.96	5.90	4.56	–
% CV	3.29	3.92	5.28	–	3.67	7.34	8.22	–	7.96	5.90	4.56	–

Normal Range

To establish reference ranges for FACSCount System parameters, data were collected from three geographically distinct sites on hematologically normal samples. The age criterion for donors in the study was 18 to 65 years. Differences in age, gender, and race was considered in the poolability of the data across sites. Age differences were observed for CD8+ T lymphocytes and ratio values; therefore two reference ranges are presented. Gender differences were observed for CD4+ T-lymphocyte values and two ranges are also presented. No differences ascribed to race were observed in the study.

FACSCount System parameter reference ranges

Parameter	Sex	Age	n	Mean cells/µL	95%* range cells/µL
CD4+ Lymphocytes	Male	18–65	92*	702	355–1213
	Female	18–65	57	798	470–1298
CD8+ T Lymphocytes	Both	18–40	92†	433	208–796
	Both	41–65	58	346	144–699
Average CD3+ T Lymphocytes	Both	18–65	151	1206	688–1955
CD4:CD8 Ratio	Both	18–40	92†	1.87	0.92–3.411
	Both	41–65	58	2.49	0.83–6.10

* The 95% range is obtained by fitting an appropriate distribution to the data and then calculating the central 95% area of the fitted distribution.
† Two did not have gender designation.

Conclusion

The FACSCount System is a unique and complete system of software analysis, unit dose reagents, and four levels of bead controls to provide absolute counts. Because it is a complete system it eliminates the need for an external hematology result. This change removes the variability found in absolute counts, which had been introduced by combining flow cytometry results with those from a variety of automated cell counters.[7] With conventional methodology, absolute count values for lymphocyte subsets may not always be comparable across laboratories. The FACSCount System reduces interlaboratory variability.

As a complete system, the FACSCount System precisely measures absolute numbers of CD4+ T lymphocytes, the cellular parameter most closely associated with HIV disease progression and therapy decisions. Even with low CD4 counts, the FACSCount System gives consistent, accurate results. In addition, the FACSCount System measures the absolute numbers of CD8+ and CD3+ T lymphocytes and the Helper:Suppressor ratio.

Today it is imperative that clinicians have cost-effective, reliable tools to monitor the immune status of patients, especially those infected with HIV. Clinicians utilize absolute CD4 T-lymphocyte counts in the staging and monitoring of disease progression and in making therapeutic decisions. The FACSCount System, which is dedicated to providing information used to monitor immune status, meets all these requirements and more.

1   World Health Organization. Global Program on AIDS. Current and future dimensions of
    the HIV/AIDS pandemic. A capsule summary. January 1992.

2   Amman AJ, Abrams D, Conant MA, et al. Acquired immune dysfunction in homosexual
    men: immunologic profiles. *Clin Immunol Immunopathol.* 1983;27:315-25.

3   Hollander H, Loveless MO, Montauk SL. The HIV-infected patient: early-stage care.
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