# Affordable CD4 T-Cell Enumeration for Resource-Limited Regions: A Status Report for 2008

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Background: The global struggle with human immunodeficiency virus (HIV) and the battle to develop affordable CD4 T-cell counting technology are both unfulfilled goals in 2008. The need for such instrumentation is more critical now as implementation of antiretroviral therapy (ART) is in progress in many resource limited regions. Major scaling-up efforts in rural situations are difficult to implement without laboratory infrastructure. CD4 T-cell counting is especially critical when trying to reach individuals with HIV to have them enrolled in ART as soon as they qualify for treatment based on CD4 count.

Method: This review covers both the chronological evolution and the scientific milestones of technological development of affordable immunophenotyping. It is more focused on flow cytometry but does consider the potential contribution by digital image cytometry.

Results: Thus far flow cytometry offered only modest progress toward affordable immunophenotyping. A list with desirable features is offered for side by side comparison. Digital image cytometry has yet to show its enormous affordable market potential.

Conclusions: It is possible to develop truly affordable, portable flow cytometry but it is not here yet. There are some hopeful signs as there are innovative and practical technical components appearing at regular intervals. However, so far the technical breakthroughs have been fragmented efforts without any attempts to consider intercorporate collaboration to optimize critical mass and synergy. The smaller players in the industry have made some progress toward meeting the monumental needs in Africa and Asia. Digital image cytometry may well be the ultimate winner in the affordable technology race. © 2008 Clinical Cytometry Society

Key terms: human immunodeficiency virus-1; antiretroviral therapy; flow cytometry; resource-restricted conditions; CD4+; T cell enumeration

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In 2003, the World Health Organization (WHO) responded to the plight of millions of people living with HIV and introduced a strategy called the "3 by 5." The objective was to treat 3 million HIV-infected individuals by the end of 2005. The target was not achieved, but the movement had significant impact and pioneered a bold but only partial fulfillment of a commitment. The door to the concept of global implementation of antiretroviral therapy (ART) as the universal standard treatment for people who were going to perish with HIV has

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been opened (1). The dramatically accelerated implementation of ART combined with persistent global political pressure has resulted in a two-log cost reduction of first-line ART for adults and eventually for children. However, to support ART implementation with essential monitoring tests, significant cost reductions must also be achieved for essential clinical assays. In the long run, delivery of ART is impossible without sustainable critical monitoring tests. Without cost effective CD4 T-cell measurement services, reliable patient management is in serious jeopardy in resource-limited regions. It is important to mention that in situations where CD4 T-cell counting facilities do not exist, this should not hinder the introduction of ART to patients with clinical AIDS symptoms (1). However, for quality patient management the critical laboratory tools include reliable HIV diagnostics such as measurements of CD4+ T cells and HIV viral load (VL). To ensure continuation of scaling-up of ART in areas with limited resources two strategic targets must be achieved: (i) to reduce the cost of the diagnostic/monitoring assays; and (ii) to bring testing capacity to areas where the HIV-infected individuals live, often in remote rural areas amidst devastating poverty.

In resource-rich countries, HIV-related laboratory technologies have improved over time. Thirty years ago, CD4 T-cell counting was performed manually with an epifluorescent microscope. Today, multicolor flow cytometers with automated software are part of routine HIV immunophenotyping in most clinical immunology laboratories. Also in the past decade HIV VL assay sensitivity has increased dramatically, thanks to improved methods and introduction of costly automation. However, when the monitoring tests are compared with antiretroviral medication in resource limited countries, neither of these critically needed assays has undergone a dramatic cost reduction. This situation is especially troubling as CD4 T-cell counting has been around longer than AIDS has (2). In this review some of the technical issues that govern affordability are addressed as they are related to CD4- and cellular immunological assays. Most discussions are focused on recent developments of the affordable and sustainable immunophenotyping and provide some insight regarding the possible directions for future alternative assay developments. There are at least two options, one is to reduce the cost of dedicated CD4 immunophenotyping machines as for example Cytometry for Life (C4L) group is attempting. This is a Purdue University based consortium focused on changing lives through low-cost diagnostics. The second option is to develop robust flow cytometers designed as flexible diagnostic platforms capable of multitasking and delivering much more beyond T-cell subtype reports. In such cases, the goal is to try to amortize the higher up-front operational cost with reduced daily labor costs. This is the strategy PointCare Technology with PointCare NOW has introduced to penetrate this underserviced rural and urban market.

With the introduction of ART, there is a golden opportunity to assess the minimum need for infrastructure in resource poor locations in order to increase both the quality and the capacity of basic laboratory services in most of the disease-burdened rural locations. This kind off orward thinking can go a long way to introduce sustainable infrastructure building without compromising any precious resources committed to HIV. It is becoming evident, especially in Africa, that effective treatment of HIV includes the monitoring of tuberculosis (TB) and malaria. To provide an effective front to fight, these three diseases diagnostic activities need to be integrated. With any public health infrastructure enhancement, most HIV-infected individuals will benefit dramatically. TB will often present itself as a coinfection returning with HIV infection. The demand for low cost, easy-to-use, and reliable CD4 T-cell monitoring devices is enormous but so is a rapid test that is reliable for acute malaria.

WHO recommendations call for published scientific evidence independent of manufacturers to facilitate the effective and objective evaluation of newly introduced affordable CD4 measurement technologies. Paradoxically, the most acute need for such evidence-based evaluations is in resource poor countries that have the most limited capacity to conduct expensive method studies. CD4 T-cell numbers in peripheral blood was considered to be a reliable test to confirm and monitor immune suppression even before the arrival of AIDS (2–5). The accurate enumeration of the absolute numbers of CD4 T-lymphocytes remains the best indicator of HIV disease progression in adults. For a brief period, in the wake of the enthusiasm for the then novel quantitative VL assays, the clinical significance of CD4 T-cell enumeration as the best monitoring tool for immune status was challenged (6,7). Currently, HIV serology, T-cell subset enumeration, and HIV VL are considered to be essential complementary tests. VL is the best measure of drug efficacy during ART (8,9). Absolute CD4 T-cell counts, combined with VL remain the best predictor of clinical patient outcome (4,10), including those treated with ART (11,12). CD4 T-cell count is the marker to monitor HIV disease progression, level ofi mmune suppression, and overall assessment ofi mmune restoration of patients receiving ART (10,13). CD4 T-cell counts remain essential in deciding when to start ART (13,14) and when to consider medical interventions to prevent opportunistic infections (15) whereas increase in VL and synchronous reduction in CD4 count are indication to consider altering therapy. CD4 T-cell number trends in specific group of patients are also used in epidemiological studies to forecast the potential cost burden a specific HIV outbreak will have on the public health system (16). At this time, drug resistance assays are unavailable for direct patient management in rural resource poor regions.

The challenge facing the cell-based diagnostics industry is to create a CD4 T-cell counter that is simple to operate in rural settings while being very robust so it is compatible with the tropical environment that is known to be extremely hostile to equipment with sensitive electronics and optics. The assay must also be reproducible at a dramatically low cost. In 2005, the price for ART in many parts of Africa and Asia underwent a 50-to 100-fold reduc-



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tion (from $15,000 to $150 per year) albeit this low price comes with some qualifying conditions such as fixed formulation, specifications with large confirmed orders, only for point-to-point supplies, advance payments, etc. Should a cost-reduction model be applied to immunophenotyping similar to the one that was implemented to ART delivery, the price of CD4 testing would drop from the current $4 to $50 to under one dollar per test. However, such a low price level may not be sustainable especially in rural, low volume resource poor regions. The current consensus on CD4 T-cell counting is to aim for a $2 assay for remote rural locations in resource poor regions. Many believe that this challenge is an achievable and sustainable target without compromising assay quality. The question comes to mind, is flow cytometry necessary for CD4 counting? Do we really need a flow cytometer or will a low cost digital imaging device suffice. Howard Shapiro very elegantly and convincingly argues that flow cytometry is an over kill when all we need to know is how many dual-labeled cells (CD4 and CD3) are in a unit volume of whole blood (17).

## NO REVOLUTION JUST GRADUAL EVOLUTION TOWARD MORE ROBUST CD4 T-CELL IMMUNOPHENOTYPING

It is important to remember that there were no antiretroviral drugs available to combat AIDS in the 1980s. When they did emerge, it was clear that because of prohibitive cost, they would not be available in resource limited regions. In Africa, treatment was restricted to late stage secondary opportunistic infections where the monitoring of CD4 T-cell count is less important. Hence, the immunophenotyping assay development focused on the lucrative industrialized world market. By the late 1990s, clinical flow cytometry had steadily evolved from research-only applications to complex routine immunophenotyping protocols including polychromatic reagents. The assay procedures were complex to the point that they required guidelines and detailed protocols to render them reproducible for large multicentric clinical drug trials conducted and still ongoing, such as the NIH/ACTG in the USA (18).

One significant protocol switch occurred in some of the leading immunology laboratories in the 1990s. It was the elimination of the overt dependency on the dual light scatter strategy (forward vs. side scatter) as the initial step in the immunophenotyping gating protocol during the execution of any standard CD4 T-cell assay (reviewed in Ref. 5). The traditional dual light-scatter protocol is also referred to as a homogenous gating strategy. As long as the CD4 T-cell counts are reported as a lymphocyte % (percentage of total lymphocytes), the use of homogeneous dual-light scatter did not pose a significant problem with reproducibility. Nevertheless, such morphologic discriminator proved to be incompatible with ageing whole blood specimens, once the objective was to obtain an absolute CD4 T-cell count. However to report an absolute count with this traditional method, both the total white blood cell (WBC) count and the lymphocyte differential count (LD) had to be obtained from a second instrument, a hematology workstation

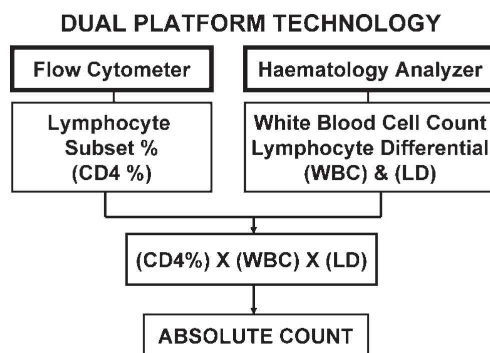


FIG. 1. Dual Platform Technology (DPT) for CD4 T-cell absolute counting. Clinical flow cytometers are designed as open flow systems. Traditionally they deliver relative numbers or the percentages of all the cells analyzed. For example, if 10,000 lymphocytes were analyzed and there were 5000 CD4 T-cells, the flow cytometer will report CD4 T-cells as 50% ofl ymphocytes. Once the demand switched to absolute count, the CD4 T-cell percentage ofl ymphocytes had to be converted. This requires multiplication with two additional parameters from a hematology analyzer. To obtain the absolute CD4 T-cell count, the white blood cell (WBC) count and the lymphocyte differential (LD) are taken from the hematology instrument to be multiplied through with the flow cytometry derived CD4 that is a percentage ofl ymphocytes. This method is the dual platform technology for absolute cell counting. There is a variation on the above described conventional DPT. In the case of the original panleucogating protocol only the WBC count is used from the hematology analyzer the two other numbers are obtained from the flow cytometer (see Fig. 10).

(Fig. 1). This dual assay combination is referred to as a double-platform technology (DPT). In general, the hematology instrument results had to be from a blood sample < 6 h old. If not, the CD4 T-cell absolute count would be unreliable. The mixing of one intrinsic and one extrinsic cell attribute for gating purposes is referred to as a heterogeneous gating strategy. As the traditional approach was replaced by more robust heterogeneous protocols, the absolute count problem was only half solved. The use of a combination of fluorescent and side scatter as the primary trigger (CD45/SS), enable a reliable lymphocyte count determination. However, the total WBC still remained problematic. To deal with this deficiency, some instrument manufacturers added another reagent, a volume-metering bead product, to enable absolute count to be incorporated into CD4 T-cell immunophenotyping process. With such an additional reagent, it was possible to compute cell concentration per unit volume of blood (panel B on Fig. 2). Known concentrations of microfluorospheres were introduced with each patient specimen; the absolute count challenge was solved and the process is known as single-platform technology (SPT). Several new gating combinations were introduced in the early 1990s (reviewed in ref. 19): these included CD45 (20), CD3 (21), and CD4 (22,23) with one light scatter such as orthogonal side scatter. The combination of bright CD45, side scatter, and counting beads is referred to as CD45-gating with SPT for absolute counting.

US companies manufacturing clinical flow cytometers introduced FDA approved reagent kits for CD4 T-cell assays to reduce the complexity of both protocol and reagent selection. In HIV clinics, a steady demand



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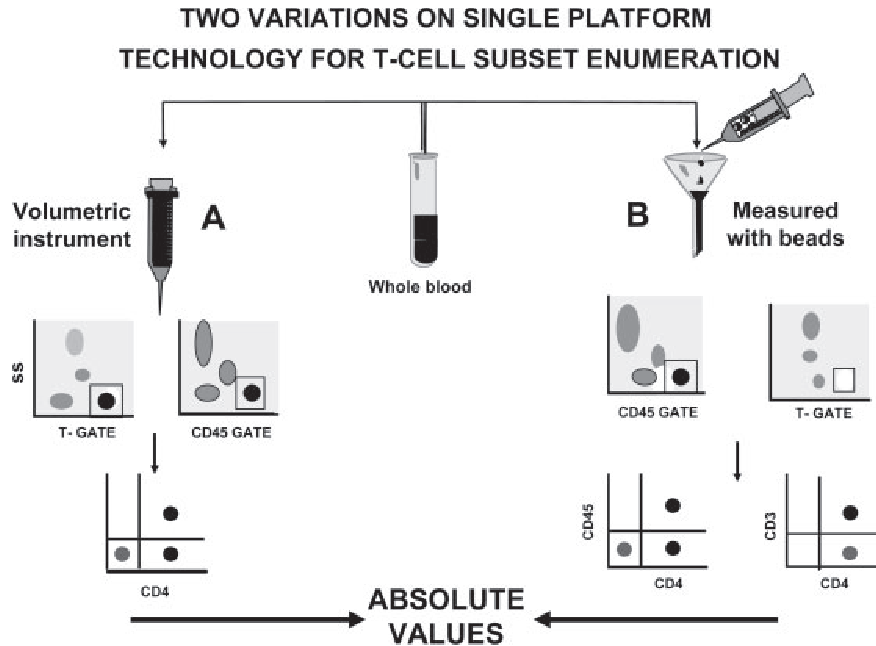


FIG. 2. Two Variations on Single Platform Technology (SPT) for T-cell subset enumeration. As demand increased for absolute counts on a single instrument, the SPT, two options became available: (i) The cytometer equipped with built-in syringe system in order to deliver a unit volume of blood for analysis. This is depicted on panel A on the left hand side. This intrinsic volumetric method does not require additional reagents to obtain absolu te cell counts. (ii) A known volume and concentration of microfluorospheres (or reference beads) are added to a clinical flow cytometer with each specimen, as illustrated on panel B. The additional reference reagents (beads) added to every specimen can considerably increase the per-test costs.

remained for "enumerating CD4 T-cells" with these expensive but relatively simple-to-use kits. With the rapid spread of newly introduced lymphoma and leukemia diagnostics services, the more frequent clinical use of flow cytometry shifted away from HIV to cancer diagnostics. Therefore, the driving force behind further development ofi mmunophenotyping protocols in resource rich countries switched to the new flow cytometry based diagnostics of onco/hematological disorders. Where a detailed "lymphocyte subset-analysis" had to be carried out for defining T cell subsets, B-lymphocytes, monocytes, and NK-cell populations these assays were driving the market (18). The complexity of the new assays for whole blood pathology based oncology, left CD4 T-cell counting in comparison a relatively simple-to-perform assay. Therefore throughout the next decade, the pursuit ofl ess expensive alternative instrumentation for CD4 T-cells was not a high priority for the well established flow cytometry industry. Equally clear was that selecting protocols requiring fewer reagents served neither the priorities ofl aboratory managers who were equipped with polychromatic instrumentation nor the reagent marketers and manufacturers of the profit oriented flow-cytometry industry.

## HISTORICAL DEVELOPMENT OF AFFORDABLE FLOW CYTOMETRY

### Instruments

With the onset ofi mplementation of 3 3 5 in Africa, the goal was to install instruments as fast as possible in high throughput laboratories representing the largest African consumer groups, which were easy to reach and needed affordable technology. Over the years, international agencies have learned that such technology installations require the implementation of both internal and external quality control programs for CD4 T-cell enumeration and technical training on continuous bases in order to achieve sustainable immunophenotyping, where instruments must survive with nominal infrastructure and extreme environmental conditions. Other considerations might include intermittent electric power delivery, with frequent fluctuations (including spikes that can damage instruments instantly), ambient room temperature can exceed 40 8C by midday, and refrigerator operating temperatures which may reach above 15 8C. Indeed, the US Army, in collaboration with the Los Alamos National Laboratory, developed a robust instrument to detect airborne lethal biological weapons in the battlefield. They produced a compact flow cytometer capable of operating under rugged desert conditions. A commercial version of this originally Los Alamos/BioRad instrument, Apogee A40 analyzer from UK/Italy, was introduced to the clinical market for CD4 T-cell counting around 2004 without much success (24). The first dedicated T-cell subset enumerating instrument, the FACSCount, was launched by BDIS about 15 years ago (25). It represented a remarkable achievement at a time when the other authoritative guidelines still called for 12 monoclonal antibodies (MAbs) in 6 tubes using complex dual-color protocols (18). The FACSCount is portable and still sells world-wide including in Sub-Saharan Africa,

delivering reliable CD4 T-cell counts to medium-sized rural health centers. This instrument was the first to count CD4 T-cells using whole blood, no-lyse, with a SPT for absolute cell count. A single tube is required containing two MAbs (CD3 and CD4) and microfluorospheres for counting absolute CD4 T-cells. Currently, the CD3/CD8 T-cell assay is an optional kit (25). However, FACSCount has been expensive to operate, selling initially with a reagent cost above $20 per test. For over a decade, it was the only flow cytometry option available for resource poor regions. In the past few years, the cost per test has been reduced significantly in selected resource-poor countries.

Another approach to affordability was developed by Guava Technologies. They redesigned the flow cell, the heart of the hydrodynamic fluidic system of a flow cytometer. This innovative alteration eliminated the need for sheath fluid that is traditionally essential to achieve cell alignment in the flow cell (26). The elimination of sheath fluid with microcapillary flow was an important breakthrough for regions such as Sub-Saharan Africa where clean water is a precious commodity. Conventional flow technology guzzles up liters of expensive filtered-water. Further advantage of this microcapillary system is the significant reduction in reagent-volume per test. The corresponding reduction of specimen volume helps with minimizing biohazard discharge volume, and it is also a welcome innovation for pediatric applications. The Guava gating strategy is similar to the FACScount, both technologies use a variation on T-gating protocol (Figs. 3A and 3B). Currently, the Guava Easy CD4 technology provides the lowest reagent cost per test, but the Guava PCA instrument cost is higher compared to the FACSCount. The integration ofi ntrinsic volumetric solution in flow cytometry is a significant engineering advancement in the direction of affordable absolute CD4 T-cell count. It eliminates the use of costly fluorescent microspheres with each specimen. Previously, Ortho Diagnostics with the Cytoron-absolute had already introduced a motorized syringe system for volumetric delivery for clinical application (22,27) but this instrument was not developed for resource-poor settings and it did not survive. Variants ofi ntrinsic volumetric technology were first incorporated into affordable systems by Partec GmbH (28) and later by Guava Technology (26); see panel A on Figure 5. The Guava PCA uses a syringe system whereas in the case of Partec, the volume measurement is achieved with a dual linear sequential air/liquid sensing device integrated with a motorized syringe feedback drive system. The least expensive Partec CD4 counting instrument, the CyFlow Counter (28), has this volumetric feature packaged together with "no-lyse" technology. The most recent robust simple-to-use arrival on the market, PointCare NOW, from PointCare Technology, also uses a syringe system for absolute count (29).

The next major innovation was to switch to freeze-dried and low-heat dried reagents to eliminate the excruciating cost burden of cold-chain, transportation/delivery, and on-site refrigerated storage. This strategy was

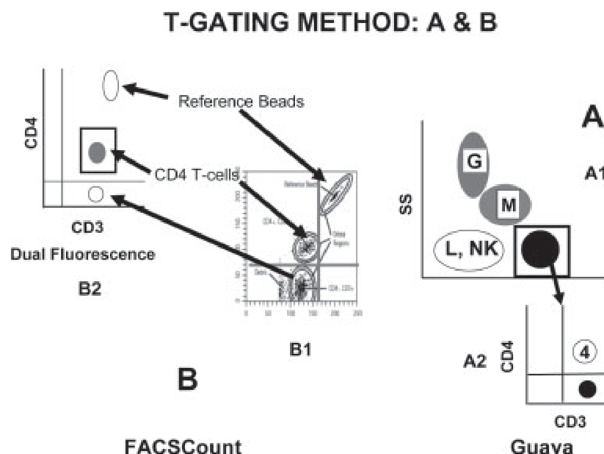


FIG. 3. T-Gating methods A and B. Method A: The Guava Easy CD4 protocol is represented on the right side in Panels A1 and A2. On Panel A1, a manually gated heterogeneous histogram (SS/CD3) represents T-cells as they are separated from monocytes (M), other lymphocytes (L), NK cells (NK) and granulocytes (G). The gated CD3 1 T-lymphocytes are all in the open square. Panel A2 illustrates the second manually gated homogeneous (CD3/CD4) bivariate histogram where the double labeled T-lymphocytes (CD3 1 /CD41 ) are in an open circle ˆ . Method B: The FACSCount protocol is represented on Panel B1 and B2 on the left side. Panel B1 illustrates the software controlled homogeneous dual fluorescent histogram (CD3/CD4) in the histogram only the dual labeled cells are in the gate. Panel B2 is a cartoon to illustrate how the automated gating software can be compared to the traditional bivariate histogram. CD4 T-cells are a subset identified as CD31 /CD41 they are in the open square. The reference beads which are used to assist with SPT absolute counting are identified in both Panels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

recently introduced by PointCare Technology (29) and ReaMetrix of India (30), respectively. PointCare NOW is an automated flow cytometer that operates with colloidal gold-labeled reagents. This approach eliminates the complexity of using expensive vibration resistant mounts for fluorescence signal detection filters and most of the potential optical alignment problems. It detects scattered light at four different angles all at once. Such a compact optical bench design is quite energy-efficient, thus a significant inroad toward affordability has been achieved with the LED powered optics (Fig. 4). With the PointCare NOW system, sample handling is fully automated with all manual pipetting eliminated. This is a great advantage since pipettes in Africa and Asia are rarely calibrated. Once the vacutainer tube containing the specimen is placed in the designated compartment, the complete analysis cycle is carried out without any further manual intervention. This approach comes a long way to fulfill the ideal low-throughput rural requirement. The current lack of compatibility with available international external quality control schemes is a problem from laboratory quality management point of view. This instrument is modestly priced, but the reagent costs per test are relatively high. The higher cost is in part is justified as the instrument provides a four-part hematology differential count, a hemoglobin count and both an absolute and a lymphocyte percent CD4 T-cell enumeration for about



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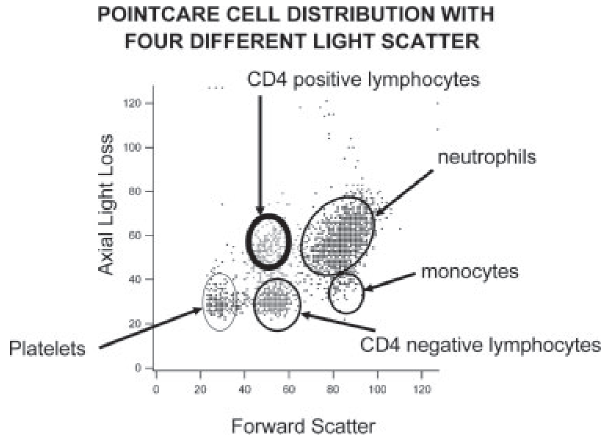


Fig. 4. PointCare cell distribution with four different light scatter. Colloidal gold labeling technique to count CD4 T cells withPointCare NOW. This gating technique is based on identifying leukocytes including monocytes, neutrophils and lymphocytes on a histogram with forward scatter (FS) on the " x" axis and Axial Light Loss (ALL) on the "y" axis. The lymphoid scatter on T-helper cells is altered by colloid gold labeled CD4 antibodies. They bind to CD4 1 T cells (see heavy black-rimmed circle). The CD4 negative lymphocytes include B, NK and CD8 T-cells. The colloidal gold tagged CD4 1 T-cell are pulled away from the monocytes. With this bivariate histograms both platelets and neutrophils are visualized. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

$10 in under 8 min. It will take some time before the market place will be able to assess the cost-effectiveness of providing such a comprehensive clinical data set while the patient is still with the health-care giver (i.e., at the point of care).

There are three combinations where flow cytometry functions are mixed with other technologies (Fig. 6). We already discussed the situation (panel B in Fig. 6) when a flow cytometry and hematology analysis are combined as is the case with PointCare NOW. A hematology analyzer can be modified to count stained cell nuclei such as the Symex PocH-100i (panel A in Fig. 5). It can read

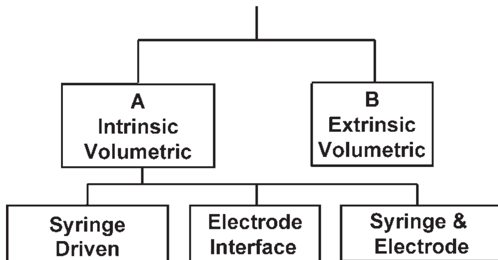


Fig. 5. Intrinsic Single Platforms on Some Affordable Flow Cytometers. Several instrument manufactures concluded that it is possible to offer volumetric solution with lower costs per test. This solution is available as a built-in syringe, electrode interface or a combination of both. The syringe driven option is utilized by Guava, PointCare and all the hybrid instruments. Partec CyFlow Counter uses the dual, syringe and electrode, volumetric option.

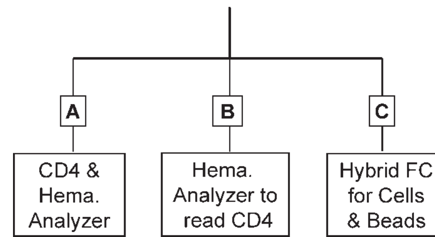


Fig. 6. Mixed Flow Cytometry. Currently there are three ways to combine flow cytometry with other technologies to obtain CD4 T-cell measurements: (A) Two models of bench-top Sysmex hematology analyzer can be used to read stained cell nuclei to support the Dynal manual CD4 T-cell enumerating kit; (B) The PointCare NOW instrument, a flow cytometer designed to operate both as a hematology analyzer and CD4 T-cell counter simultaneously; and (C) Hybrid flow cytometers designed with various capacities to perform automated analysis with both cell- and bead-based assays.

the final results from a Dynal manual kit for CD4 counting. Yet another approach is a hybrid- or bioanalyzer flow cytometry (panel C in Fig. 6). Such instruments are capable of analyzing both cell- and bead-based assays. With hybrid flow cytometers, it is important to think in terms of amortization ofi nstrument cost over several years. They are a relatively new class of cytometers on the market. There are three instruments of this type: the Luminex 100 (Luminex Corp., the Guava 96 PCA (Guava Technology), and the FACSArray (BDIS). All are equipped with built-in microtiter plate sample-handling to process 96 assays nonstop. These hybrid flow cytometers can also be referred to as hybrid flow application platforms (HyFAP). The capacity of these platforms can be enhanced to perform a variety ofi mmunoassays and molecular probe assays (31–34). In the future, it is probably the speed of software development that will determine the extent of success that this technology will have. It is possible that the broad application potential will justify the relatively high cost of such platform. With HyFAP, cells can be processed to provide CD4 T-cell counts (23), and bead-bound immune-based assays (replacement assays for various ELISA) simultaneously (31,34). A feasibility study on the use of HyFAP for HIV serology testing has been reported for the detection of circulating antibodies from fresh and dried blood spots (DBS) (33). Others have used this multitasking platform for the detection of vaccine-preventable infectious diseases in children (32). ReaMetrix is also developing a HyFAP platform system which will reach the Indian domestic market in 2008. The multitasking concept is the same; they will provide an instrument that will offer a whole panel of diagnostic assays that are required. To summarize, new alternative flow cytometers have arrived. They include instruments using microcapillaries and the nonfluorescent reagents. A flow chart (Fig. 7) places these various key features where they appear related to flow signal detection and cell propulsion methods available on affordable cytometers.



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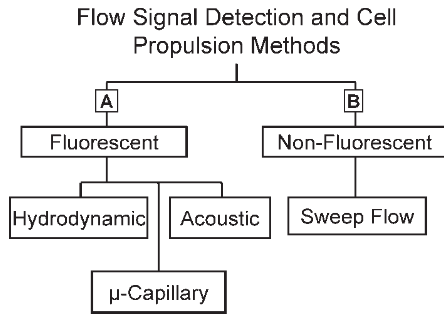


FIG. 7. Flow Signal Detection and Cell Propulsion Methods. This flow chart illustrates some of the most promising evolutionary developments in flow cytometry technology which may have impact on accelerating affordability of CD4 T-cell measurements in resource limited regions. Fluorescence is no longer the only option available for immunophenotyping as indicated in Panel 7B. The use of nonfluorescent tagging method gives the option to eliminate cold-chain, refrigeration and light sensitivity issues. The other innovation with potentially significant impact is the alternative cell propulsion technologies listed in both 7A and 7B. The utility of acoustic focusing to deliver affordable instruments is unknown at this point. However there are two options available to replace hydrodynamic focusing. Guava technologies has introduced l -capillary system and on Panel A. The sweep flow technology introduced by PointCare on Pane 7B also utilized less water compared to hydrodynamic focusing.

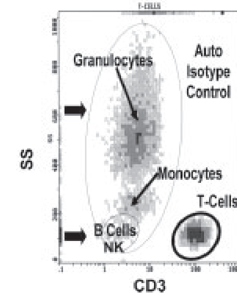


FIG. 8. A Built-in Advantage with Heterogeneous Gating Technology. Using a lineage marker as part of a heterogeneous gating (SS/CD3) strategy has some additional benefits. When implementing a T-gating protocol such as one used by Guava, the desired cluster ofl abeled T-cells pull away from the cells that are not CD3 positive. The non-T-cell population serves as a built-in automatic isotype control. It is known that it is unnecessary to add a reagent to CD4 T-cell immunophenotyping, however it is freely available. In some of the most hostile rural destinations this protocol provides an assurance that the reagents and specimen survived the trip to the laboratory in good condition. In the cartoon a strong separation between T-cell gate and other cells is visible. Monocytes, B cells ands NK-cells exhibit some nonspecific binding reaction as they are some distance from the " y" axis, see the dark horizontal arrows. However this low level staining does not jeopardize the rapid identification of the positive T-cell population.

## REAGENT AND NOVEL GATING STRATEGY DEVELOPMENT

One can also consider the innovative diversity that is evolving in terms of gating strategies, hence reagent selection. Efficient reagent selection can go a long way toward affordability (35). Various novel gating strategies have led to the cost effective gating strategies through the reduced sizes and volumes of the reagent panels. One international organization, the Clinton Foundation, looked at the various new affordable options and has accepted some of them. The Clinton Foundation negotiated CD4 T-cell counting assay prices around $4 per test (35), eliminating CD8 T-cell counting with the FACSCount and other systems.

### CD3 T-cell Gating

The extrinsic attribute of T-cells, the expression of CD3 molecules as a T-cell linage specific marker, was introduced in combination with CD4, a function specific marker (21). This T-gating combination was first implemented on the FACSCount (25). In this case, it is a combination of green and red colors that define the location of the orange colored CD4 T-cell (Fig. 3A). The red CD3 and the green CD4 MAbs provide a mixed orange color for the dual stained CD4 T-cell population. This CD4 T-cell count strategy requires the total of three reagents, two MAbs (CD3 and CD4 MAbs) and counting beads for each specimen. It also includes a free auto-isotype control feature (Fig. 8). With this method, there is no need to lyse the red cells as only fluorescent cells are observed by the optical system; hence there is a further reduction in reagents.

### Primary CD4 Gating

By 1999, "progress" dictated a six-tube, 12 MAb protocol for CD4 T-cell counting. Most guidelines recommended processing specimen within 6 h of phlebotomy (18). Sherman et al. introduced the concept of simplifying CD4 T-cell counting (36). First, the primary CD4 gating using a single-reagent, CD4 MAb, was developed (22,23). By combining a function specific marker CD4 with side scatter, CD4 T-cells could be resolved (Fig. 9).

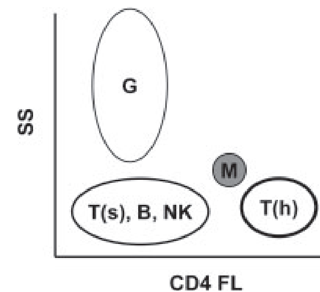


FIG. 9. Primary CD4 gating. The simplest immunophenotyping protocol for CD4 T-cell counts utilizes a single CD4 antibody with heterogeneous histogram: CD4 intensity (CD4 FL) on the " x" axis and side scatter (SS) on " y" axis. This combination discriminates between strongly CD41 lymphocytes T(h), CD4-negative lymphocytes (T(s), B, NK), monocytes (M) larger cells with lower fluorescent CD4 intensity and granulocytes (G). Primary CD4 gating works optimally in adults when volumetric flow cytometers directly count the numbers of CD4 positive lymphocytes present in a unit volume off resh blood. The side scatter (SS) is essential to resolve CD4 brightly fluorescing lymphocytes from the CD4 dimly fluorescing monocytes that are not always much larger then a lymphocyte.



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This simple gating strategy was also introduced on the Partec CyFlow SL (37,38) and later on the Partec CyFlow Counter but here without side scatter (28). More recently, a second parameter was again added to the CyFlow Counter so it gates much the same way as the primary CD4 gating protocol. In adults, the absolute CD4 T-cell counts obtained by primary CD4 gating on volumetric cytometers are generally reliable without using additional reagents (22,23,37,38). However, these methods rely on clear identification and separation of CD4 T-cells from monocyte population which in some situations can be problematic unless both the CD4 and side scatter parameters are used.

### CD45 Fluorescence-Based Panleucogating

Next, the "pan-leuco-" gating strategy was developed as a DPT for CD4 T-cell measurement using conventional flow cytometers in high throughput hospitals where there is a hematology analyzer near by. This simplified protocol designed to work on any full-featured clinical flow cytometer (20), eliminated the need for counting beads without compromising CD4 T-cell count quality (39,40) using only two MAbs, CD45 and CD4 (39). The original panleucogating method is a DPT where not one but two of the three numeric elements required to generate the absolute count (Fig. 1) are acquired with a flow cytometer. The components generated by flow cytometry on a heterogeneous histogram (SS/CD45) are the lymphocyte differential (LD) and CD4 T-lymphocyte percentage. The operator draws a large gate (Gate A) that includes all the CD45-tagged leucocytes (Fig. 10A). The second gate (Gate B) also in Figure 10A includes only the bright CD45 stained cells, the lymphocytes. It is the content of Gate A that is sent to the second heterogeneous histogram (SS/CD4) to determine the actual CD4 positive events (Fig. 10B). With this strategy only the total WBC count is obtained from a hematology analyzer. The CD4 T-cell absolute count is generated by multiplying the two elements from the flow cytometer with the one from the hematology analyzer. This strategy works well on samples that have been drawn up to 3–5 days earlier (39,41). A version of CD45-assisted gating strategy is now modified for use on clinical flow cytometers with SPT and included in most international guidelines published in the past 5 years (41–44). The above panleucogating method was further modified by Glencross et al. to incorporate inexpensive counting beads (45). There is also a multilaboratory study reported from a North American trial in this issue (46).

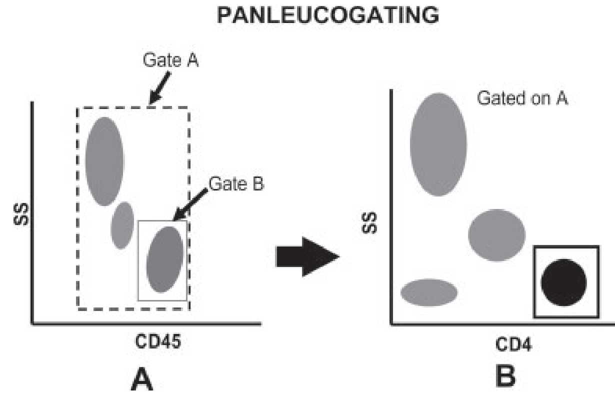


FIG. 10. Panleucogating Method. This robust method is based on initial gating with CD45 antibody - in combinations with CD4 antibody. The first step is to gate on all CD45 leucocytes (Gate A on Panel 10A), a large gate that includes all positive CD45 leucocytes including lympho-, mono- and granulocytes. The absolute counts for cells included in "Gate A" are quantified with a WBC count from a haematology analyser. Next, CD4 T-lymphocytes are counted among the leucocytes as shown in the smaller rectangular "Gate B" on Panel 10A. Finally, the bright CD45 lymphocytes "Gate B" are also identified to calculate the CD4 T-cells as percentage ofl ymphocytes as they are gated on Panel 10B in the open square.

## AFFORDABLE CD4 T-CELL COUNTING REMAINS AN ORPHAN TECHNOLOGICAL CHALLENGE

The blame for slow evolution cannot be placed just on myopic vision and greed associated with the healthcare industry of resource-rich countries. Companies with publicly traded shares must make profit and they are not in a position to become charitable organizations. There is, however, a general lack of vision and commitment on a global political level. Jeffrey Sachs, a leading global economist, published a book called "The End of Poverty." He makes a compelling case that elimination of global poverty is an economic possibility in our time.

There was historical evidence that pipeting was the major error source in absolute cell counting. This is why the entire hematology industry switched to hands-off cap piercing technology several decades ago. Such restrictions were not imposed on the flow cytometry industry when it became obvious that absolute CD4 T-cell counts were needed. Yet the cap piercing technology eliminates all manual pipeting errors and significantly reduces biohazard exposure associated with infectious specimens. Although it was recognized 20 years ago that it was important to provide an accurate absolute count, until recently, only three companies had developed an intrinsic volumetric solution (which also keeps this test more affordable). Most instruments to this day require the addition of costly beads to each specimen. While the pharmaceutical industry was developing powerful antiretroviral drugs, immunologist in the research laboratories gained considerable understanding regarding interaction between functional, linage, and activation markers. Cell-surface receptors such as the CD4 are functional markers. CD4 positive cells can belong to various lineages including the T-helper cell subset and monocytes. Other groups developed better understanding of how to preserve receptors or antigenic affinity while freezing cells and lyophilizing monoclonal antibodies. Powerful water-cooled lasers have given way to air-cooled units with more modest but still significant energy requirements. The latter development was followed by expensive but compact solid-phase laser modules, and then very low power consuming and affordable LEDs. We have learned that the two most critical compo-

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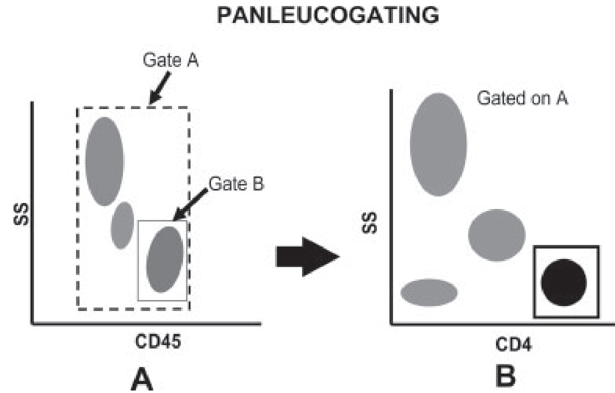


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Table 1
Guide for Selecting an Appropriate CD4 1  T-cell Counting System [a]

| Categories | Apogee A40 | FACSCount BDIS | Guava PCA | Partec CyFlow C. | PointCare NOW | Sysmex Poch-100i |
|---|---|---|---|---|---|---|
| Environmental energy issues (E) | | | | | | |
| E-1 Use ofl ow energy laser or LED | | | | 1 | 1 | |
| E-2 Low water consumption | | | 1 | | 1 | 1 |
| E-3 No cold-chain & or need for refrigeration | | | | | 1 | |
| E-4 Impervious to day-light exposure | | | | | 1 | 1 |
| E-5 Nominal waste collection | | | 1 | | 1 | |
| E-6 Protection against power fluctuations | | | | | | |
| E-7 Operates without AC | | | | | | |
| E-8 Eliminated stand-alone computer | | | | | 1 | 1 |
| E-9 Battery/solar power free or an option | | | | 1 | 1 | |
| Cost issues (C) | | | | | | |
| C-1 Minimal antibody numbers used in small volumes | 1 | | 1 | 1 | | |
| C-2 Simultaneous CD4 absolute and CD4% | | 1 | 1 | 1 | 1 | |
| C-3 Method without lysing reagent | | 1 | | 1 | | |
| C-4 Method without counting beads | 1 | | 1 | 1 | 1 | 1 |
| C-5 Patient/control data space onboard | | 1 | | 1 | 1 | |
| C-6 Minimal daily quality control cost | | | 1 | | 1 | 1 |
| C-7 Cost/availability of preventive/service contract | | 1 | 1 | 1 | 1 | 1 |
| C-8 Internet/satellite/GMS compatibility | | | | | 1 | |
| C-9 Compatibility with EQAP (NEQAS,QASI) | 1 | 1 | 1 | 1 | | |
| C-10 Maximum daily sample throughput | | | | | | |
| Labor-related issues (L) | | | | | | |
| L-1 Elimination of manual gating | | 1 | | | 1 | |
| L-2 Manual pipetting is avoided | | | | | 1 | |
| L-3 Onboard automated data management | | | | | 1 | |
| L-4 Availability of continuous-training | | | | | | |
| L-5 Portability: is transport case available | 1 | | 1 | | 1 | |
| L-6 Small bench-top foot-print | | 1 | 1 | | 1 | |
| L-7 Barcode reader is available or an option | | | | | 1 | |
| L-8 High skill-level for operator is eliminated | | | | | 1 | |
| L-9: Additional capacity fo hematology | | | | | 1 | 1 |

[a]This supplement to Clinical Cytometry includes publications which describe how to run conventional clinical flow cytometers that have originally been designed for affluent countries in challenging economical settings (45,55). Both the protocols of primary CD4 gating (Fig. 9) and CD45/CD4 gating (Fig. 10) are well suited to run on 4-colour flow cytometers marketed by different companies [45,55,67]. In this Table, however, only the instruments that are potentially capable of running with inferior infrastructure in rural or semi-rural conditions are included. The criteria shown in this Table are complementary to the other parameters such as the cost of the instrument, precision, accuracy and reproducibility, critical issues that are often available from observations by External Quality Assessment protocols. Importantly, the cost of the instrument may not be the most significant factor when all other issues factored into the total package. A prudent manager will study the operational budget adjusted for the specific requirements of his/her laboratory as well as the costs per tests with the three areas of cost containment: (i) environmental and energy management issues (''E''- issues in Part 1 above), (ii) options available for containment of cost per test (''C''-issues in Part 2 above), and (iii) labor cost containment (''L''-issues in Part 3 above). The projected test volume per week/month/year will help to guide decision making about sustainable service costs and will serve well for negotiations. The plus signs assignments are incomplete as not all data has been available at the time this table was generated.

ment. The accuracy of the projected test volume per month will have a significant impact and probably will be a key factor to guide decision making. See Table 1 for the breakdown off eatures listed for various affordable systems. When looking at long-term cost, an example from each of the three categories ofi ssues can be selected for illustrative purposes. One can enquire about the savings that are associated with reagents that have a year long shelf-life without any refrigeration or cold-chain transportation requirements. Similarly, in the case of cost per test, the saving can be assessed if the system requires only one or two MAbs but without any additional demand for lysing solutions or for flow counting beads; in such a case, the per-test costs over the years will be dramatically lower. A final example is from the labor cost containment and quality management section. In a case where the manufacturer might be able to provide a suitable rugged transport container for a robust instrument in order to regularly circulate between three or four villages on a weekly basis, the amortization cost of the system drops to below 30% per village. Many specific features of service (Table 1), needs to be confirmed with local suppliers, keeping in mind that services may change with time. Fluctuation in price is also likely to occur for the prices ofi nstruments and reagents; therefore, they are not included in this comparison.

## ISSUES RELATED TO TECHNOLOGY TRANSFER AND SUSTAINABLE QUALITY LABORATORY MANAGEMENT

Biosafety issues are often neglected in resource limited locations. They must be considered when establishing laboratory space, equipment lists and operating budgets. Laboratory managers can be held legally responsible for not implementing, accommodating, and enforcing



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proper safety procedures in the laboratory. In most resource-rich countries, external quality assessment programs (EQAP) for CD4 T-cell enumeration are mandatory and are in place (51–54). These protocols involve shipping stressed challenge specimens at regular intervals. Internationals agencies such as WHO, Global AIDS Program (GAP), the Clinton Foundation, among others, have learned that technology transfer workshops can accelerate the effective installation and acceptance of external quality assessment programs (EQAP) for resource poor regions. These topics are discussed in more detail by Glencross in this issue (55).

## CONCLUSIONS

During the past 5 years, flow-based cellular tests have improved and are more robust but they are still too expensive. Kestens et al. validated a primary CD4 gating protocol in Cambodia (56). Looking forward, there are two major strategies that may bring about sustainability and affordability where it is needed the most. First, more hybrid instruments might be introduced either as point of care or at regional laboratory level that will lower overall cost for a large variety of assays beyond CD4 T-cell enumeration. A new truly portable and affordable dedicated user-friendly flow cytometer such as the Cytometry For Life (C4L) could take care of most of the ART related CD4 monitoring needs of rural nursing stations. Flow cytometers will also have to deliver, in the future, versatility through effective implementation of flexible software-driven hybrid instrument platforms. They must be capable of a wide range multiplexed assay panels covering both ELISA-type bead- and cell-based assays (31–34,57) including, for example, antiretroviral drug toxicity assays. The multiplexing platform approach of the future can promote the "one-stop-shopping" concept for the divergent serological, immunological, hematological test market. With the new generation of flow cytometry platforms that show considerable versatility, alternative approaches to PCR amplified plasma viral RNA as a surrogate VL tests could be introduced. Cell-associated markers such as CD38 expression levels on CD8 T-cells (58,59) have also been proposed as an affordable alternative for monitoring ART in HIV-infected patients. Cell-associated viral RNA (60,61) and more recently, related flow-cytometric changes in monocytes (62) are being studied as candidate assays for viral reservoir monitoring by flow cytometry. Isothermal amplification of viral RNA combined with bead-based flow detection assays (63) may also be exploited in the future as an affordable and simpler alternative to PCR amplified nucleic acid assays. HyFAP will probably be in demand particularly in resource poor regions because of the more economical operation and servicing of a limited number ofl aboratory instruments where the objective is to provide a wide spectrum of clinical assays, performed automatically using a single platform. Assay combinations are user selected as required. It is a bold cost effective approach to consolidate clinical laboratory testing requirements with relatively low volume in remote locations with stable patient populations. By contrast, in resource rich countries the multifunctional HyFAP can only be effectively considered at the time when the various dedicated instruments may become obsolete. Consequently, a situation might arise where the Northern hemisphere will follow the example of the Southern hemisphere —much the same way as North America is catching up to 82% of the world with the switch of Global System for Mobile (GSM) phone communication system. A question was posed in the introduction, whether a flow or nonflow cytometry system will take the lead to reach the badly neglected rural market in resource poor regions. At rural decentralized zones affordable technologies with minimal moving parts and no requirement for air-conditioning or sheath fluid, remain an almost irresistible option for counting CD4 T-cells. Greve, Tibbe, and Li, from the University of Twente, built several portable compact image analyzer cytometers that have not yet reached the market (64). Shapiro is working on an image analyzer where CD4 T-cells are recorded by a digital camera much like the light is captured by digital imaging telescopes from stars. The pixels offthe fluorescing cells are analyzed with a process he refers to as "cellular astronomy" (65). There is also a commercial instrument that has been in the pipeline for 6 years using similar concepts, which has yet to reach the market. Cheng et al. are developing a microfluidic nonflow device that does not require labeling of the CD4 antibody, however at this point it is not a robust technology (66). Based on what is currently available and how little success nonflow instrumentation has had so far, it is more likely that the flow-based technology will be the first to make significant impact on the rural markets of Africa and Asia—but the proponents of flow cytometry may not have the last word, say, in another 5 years.

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