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Center for Health Services
Research and Policy

Department of
Health Policy

School of Public Health
and Health Services

Forum for Collaborative HIV Research

Transfer of HIV Diagnostic and Monitoring Technologies Into Resource-Poor Settings

*A meeting organized by
the Forum for Collaborative HIV Research*

April 22, 2002 ■ Washington, DC

*Prepared by Mark Mascolini
Incorporating notes prepared by
Susan Fiscus, Alan Landay, and Laurence Weiss*

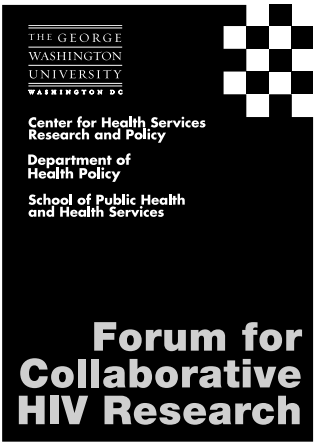
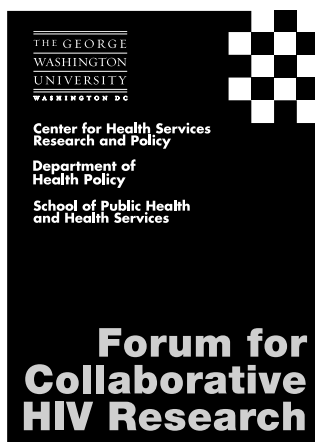


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Acknowledgements

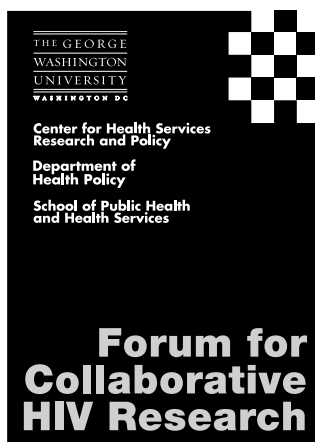
This project was a direct outcome of the workshop “Monitoring and Diagnostic Tools for the Management of Antiretroviral Therapy in Resource-Poor Settings” held on November 11-13, 2001 in Bethesda Maryland. The Forum would like to acknowledge and thank the organizers of that workshop, Ben Cheng and Gregg Gonsalves and their respective organizations, Project Inform and Gay Men’s Health Crisis for their insight and vision in convening that workshop which brought together the key players, highlighted new technologies in development and allowed critical discussion of many of the issues pertinent to the use of diagnostic and monitoring technologies in resource poor settings. The stage having thus been set, the Forum was able to convene a smaller workshop to specifically describe, discuss and facilitate the transfer process.

The Forum would like to express many thanks to the chairs for this project, Drs Susan Fiscus and Alan Landay for their dedicated and enthusiastic leadership and the steering committee for providing expertise and guidance in the planning of this project. The contribution of each and every one of the participants of the workshop was absolutely required for the success of the workshop and we thank all participants for the time invested in summarizing information, for their willingness to collaborate and share data, for bringing their expertise to the table. Two working groups were formed at the end of the April 22nd meeting, and they have logged in many hours since the day of the workshop to continue working on the “transfer of technology” steps outlined in this report, keeping the project alive. We thank the members of these working groups (listed on pages 4 and 5) and the chairs for the continued commitment.

We are grateful for the support of the diagnostic industry with direct contributions to the meeting (Beckman Coulter) and also highlight the pledges by all participating industries to support the further work required in the clinical validation and transfer process. We also gratefully acknowledge the support from the Rockefeller Foundation for this project. The Federal agencies (Centers for Disease Control and Prevention, National Institutes of Health) have committed to continued work with the research networks and the diagnostic industry to ensure that a transfer of technology will be possible. We thank them, as well as the WHO, for their commitment to keep working with us in this field.

We thank the Forum’s administrative staff: Paul Oh, Houtan Mova and Ipsita Das who through their skillful organizing of the logistics of this project, collecting and collating information, both before and after the meeting helped assure the success of this project.

Finally, we acknowledge Mark Mascolini’s excellent skills in preparing this report which will form the basis of future work in this area.



Summary

The Forum for Collaborative HIV Research organized a meeting of key international researchers, industry representatives, and others working on efficient and economical diagnostic and monitoring assays for people with HIV infection. Held on April 22, 2002, the meeting reviewed the development status of CD4 T cell and HIV quantitative assays and considered steps for further study with the ultimate goal of transferring validated technologies to resource-limited settings. The Rockefeller Foundation and Beckman Coulter provided funding for this workshop.

The meeting had five specific goals:

- Assess the current use and validation status of these assays.
- Assess the projected utilization of these assays in planned clinical research activities.
- Identify the requirements for clinical validation and assessment of these assays.
- Obtain commitment from participants to take the steps necessary for clinical validation.
- Develop recommendations to continue this process for newer technologies as they become ready to enter clinical trials.

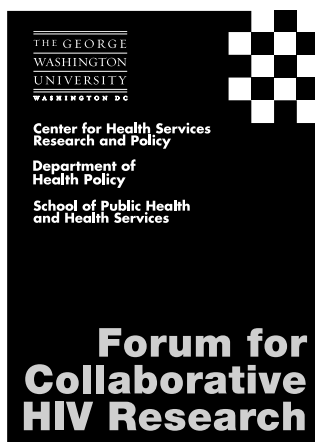
Two sets of assays were considered at the meeting:

- A heat-denatured HIV p24 assay (PerkinElmer Life Sciences) as an alternative to PCR- and bDNA-based viral load assays
- Cyto-Spheres (Beckman Coulter) and Dynabeads (DynaL Biotech) as alternatives to flow cytometric quantitation of CD4 T cells

The heat-denatured p24 assay has excellent sensitivity and specificity in diagnosing infection with HIV-1 subtype B. It has also performed well as a clinical monitoring tool and as a correlate of disease progression in people infected with subtype B virus. Results with non-B subtypes have been less consistent, perhaps because of inadequate training or experience in using the assay. Establishing the assay's value in people infected with non-B viruses is an urgent priority.

At least nine studies, several involving African populations, have correlated CD4 counts assessed by Cyto-Spheres or Dynabeads with counts measured by flow cytometry. In most (but not all) studies, the correlation has been excellent. Cyto-Spheres have already been approved by the US Food and Drug Administration. Both assays involve the physical counting of cells, which can be done under a light microscope.

Meeting cochairs Susan Fiscus (University of North Carolina, Chapel Hill) and Alan Landay (Rush Presbyterian-St. Luke's Medical Center, Chicago) organized two working groups that will take immediate steps to set priorities for further research on these assays and to create a network of test sites in poor and developing countries.



Virus Load Working Group

Chair: Susan Fiscus, University of North Carolina, Chapel Hill

Candidate members:

Gunnel Biberfeld, Swedish Institute for Infectious Disease Control

Bernard Branson, Centers for Disease Control and Prevention

Isabel Cabruja, PerkinElmer Life Sciences

Sharon Cassol, Africa Centre

Suzanne Crowe, MacFarlane Burnett Center for Medical Research

Robert Downing, Centers for Disease Control and Prevention

Mark Rayfield, Centers for Disease Control and Prevention

Richard Respass, Centers for Disease Control and Prevention

Tobias Rinke de Wit, PharmAccess International

Christine Rouzioux, Hôpital Necker (ANRS representative)

Jörg Schüpbach, University of Zurich

Wendy Stevens, University of Witwatersrand

Oliviero Varnier, University of Genoa

CD4 Assay Working Group

Chair: Alan Landay, Rush Presbyterian-St. Luke's Medical Center, Chicago

Candidate members:

Gunnel Biberfeld, Swedish Institute for Infectious Disease Control

Suzanne Crowe, MacFarlane Burnett Center for Medical Research

Serge Diabougoua, Centre Muraz

Berit Marie Eidal, Dynal Biotech

Debbie Glencross, National Health Laboratory Service

Daniella Livnat, National Institutes of Health

Steve McDougal, Centers for Disease Control and Prevention

Pat Roth, Beckman Coulter

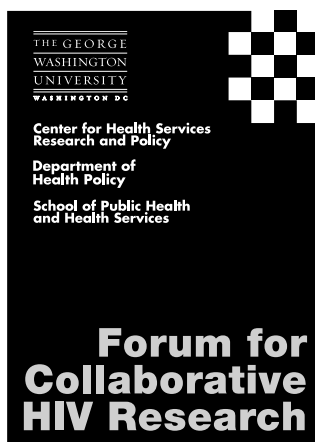
Gaby Vercauteren, World Health Organization

Kyle Webster, Becton Dickinson, Inc.

Laurence Weiss, Hôpital European Georges Pompidou (ANRS representative)

Forum director Veronica Miller agreed that the Forum would consider workshops or round table discussions to address:

1. Quality assurance and quality control of CD4 and p24 assays
2. Toxicity monitoring for trials in resource-limited settings

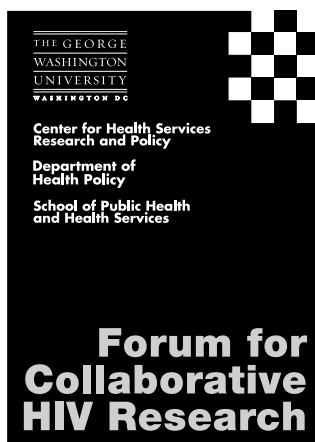


Attendees

Appendix A lists (1) technology transfer roles that can be played by institutions represented at the meeting, (2) their specific contributions, and (3) their specific needs.

Appendix B lists experience of institutions represented at the meeting with (1) heat-denatured p24, (2) Cyto-Spheres, (3) Dynabeads, (4) modified flow cytometry.

Gunnell Biberfeld Swedish Institute of Infectious Disease Control Solna, Sweden	Susan Fiscus University of North Carolina Chapel Hill, North Carolina, USA
Bernard Branson Centers for Disease Control and Prevention Atlanta, Georgia, USA	Charles Gilks World Health Organization Geneva, Switzerland
Isabel Cabruja PerkinElmer Life Sciences Varese, Italy	Gregg Gonsalves Gay Men's Health Crisis New York, New York, USA
Ada Cachafeiro University of North Carolina Chapel Hill, North Carolina, USA	Dennis M. Israelski San Mateo County Health Services Agency San Mateo, California, USA
Sharon Cassol Africa Centre Durban, South Africa	Jonathan Kaplan Centers for Disease Control and Prevention Atlanta, Georgia, USA
Ben Cheng Forum for Collaborative HIV Research Washington, DC, USA	Oliver Laeyendecker Johns Hopkins University Baltimore, Maryland, USA
Suzanne Crowe MacFarlane Burnett Center for Medical Research Fairfield, Victoria, Australia	Alan Landay Rush Presbyterian-St. Luke's Medical Center Chicago, Illinois, USA
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Serge Diabougoua Centre Muraz Bobo-Dioulasso, Burkina Faso	Steve McDougal Centers for Disease Control and Prevention Atlanta, Georgia, USA
Robert Downing Centers for Disease Control and Prevention Entebbe, Uganda	Veronica Miller Forum for Collaborative HIV Research Washington, DC, USA
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Mark Rayfield
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Tobias Rinke de Wit
PharmAccess International
The Hague, Netherlands

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Massachusetts General Hospital
Charlestown, Massachusetts, USA

Jörg Schüpbach
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Zurich, Switzerland

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Department of Health and Human Services
Washington, DC, USA

David Stanton
USAID
Washington, DC, USA

Timothy R. Sterling
Johns Hopkins University
Baltimore, Maryland, USA

Wendy Stevens
University of Witwatersrand
Johannesburg, South Africa

Milton Tam
Program for Appropriate Technology in Health
Seattle, Washington, USA

Gary Toedter
Beckman Coulter
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Michael Ussery
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Charles Van der Horst
University of North Carolina
Chapel Hill, North Carolina, USA

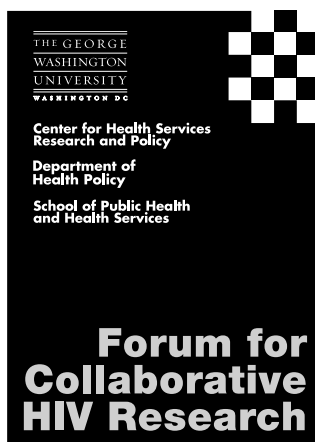
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Kyle Webster
Becton Dickinson
San Jose, California, USA

Laurence Weiss
Hôpital European Georges Pompidou
Paris, France



Background

On November 11-13, 2001, Gay Men's Health Crisis and Project Inform sponsored a workshop, "Monitoring and Diagnostic Tools for the Management of Antiretroviral Therapy in Resource-Poor Settings." Funded by the Rockefeller Foundation and the National Institutes of Health (USA), the workshop considered several low-cost diagnostic and monitoring technologies currently being used or adaptable for use in resource-poor settings.

At the conclusion of that workshop, the organizers approached the Forum for Collaborative HIV Research and requested that the Forum take on the next phase of this effort: overseeing the validation of such technologies in resource-poor settings with the ultimate goal of transferring appropriate technologies to those settings. The Forum is a public/private partnership funded through government agencies and the pharmaceutical industry and thus is ideally suited to coordinate and oversee this project.

A steering committee was formed to organize a 1-day workshop that would gather leaders in this field to focus on immediate hurdles to validation and transfer of these technologies and to organize working groups to address these issues.

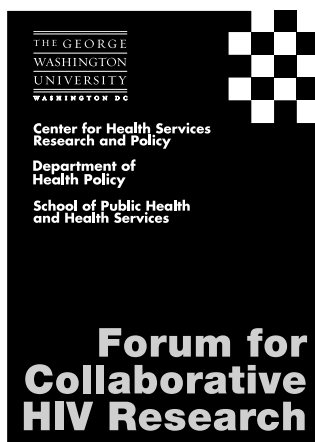
Steering Committee for "Transfer of Diagnostic and Monitoring Technologies Into Resource-Poor Settings"

Ben Cheng, Project Inform (current affiliation: Forum for Collaborative HIV Research)
 Susan Fiscus, University of North Carolina, Chapel Hill
 Gregg Gonsalves, Gay Men's Health Crisis
 Jonathan Kaplan, Centers for Disease Control and Prevention
 Alan Landay, Rush Presbyterian-St. Luke's Medical Center
 Veronica Miller, Forum for Collaborative HIV Research

The steering committee decided to concentrate on three assays that have been partially tested in various settings and are farthest along the developmental pathway but still require rigorous clinical validation in developing countries and standardization across laboratories. These technologies, referred to as "Set A," are Cyto-spheres and Dynabeads (CD4-cell assays) and the heat-denatured p24 assay (a viral assay).

The November 11-13 meeting also identified several other low-cost technologies at earlier stages of development, such as dipstick- and microchip-based assays. These "Set B" technologies can be added to the validation and transfer process once mechanisms for that process are defined with the Set A technologies.

Before the April 22 workshop, invited attendees submitted information on the current status of these assays by responding to surveys sent by the Forum. Responses to these surveys summarized work by attendees as well as other studies they knew about. Thus the workshop did not include individual presentations from attending investigators. Rather, the chairs of each session summarized that work.



Steering committee member Susan Fiscus outlined a set of steps to validation of viral diagnostic and monitoring technologies, and the committee adopted them as a guide to this project:

Steps to validation and technology transfer of HIV and CD4 assays for resource-poor settings

1. Performance characteristics
 - a. Sensitivity
 - b. Specificity
 - c. Accuracy
 - d. Precision
 - e. Linearity (dynamic range)

2. Clinical validation

For virologic assays:

 - a. Subtype B
 - i. Diagnosis
 - ii. Clinical monitoring
 - b. Non-B subtypes
 - i. Diagnosis
 - ii. Clinical monitoring

For CD4 assays:

 - a. Staging of disease
 - b. Monitoring of treatment

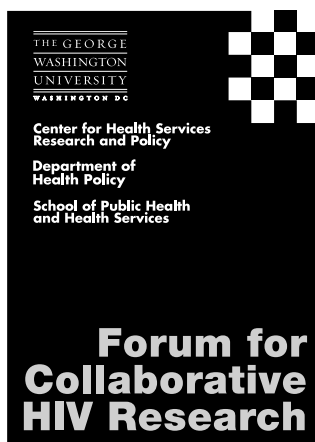
3. Logistics
 - a. Kit costs
 - b. Instrument costs
 - c. Shipping costs

4. Quality control/quality assurance

5. Technology transfer
 - a. Training checklist
 - b. Training and orientation program

6. Proficiency testing

7. Dissemination/acceptance
 - a. Technical reports
 - b. Peer-reviewed publications



Heat-Denatured p24 Assay

Among the less costly viral assays that may be appropriate for resource-poor countries, the workshop steering committee selected the heat-denatured p24 assay for consideration at this meeting because of its advanced stage of development. The Forum recognizes that other technologies, such as reverse transcriptase-based assays, may also become good candidates for validation in developing countries and that these technologies should be studied more thoroughly.

Susan Fiscus (University of North Carolina) overviewed the current status of research on the heat-denatured p24 assay. Developed by Jörg Schüpbach (University of Zurich), this assay for HIV p24 antigen is more sensitive than the original p24 assay used as a viral marker in early clinical trials of HIV therapies.

The assay uses heat denaturation in a conventional p24 antigen ELISA assay. Boiling for 5 minutes destroys antibody and preserves antigen. A commercially available kit is then used for signal amplification.

Appendix C lists the steps necessary for heat denaturation and ELISA as well as principles of the procedure from product literature.

PerkinElmer “Lab-in-a-Box”

PerkinElmer has developed this technology, now called the HIV-1 p24 Ultra Ag Assay. It will be packaged as a “lab-in-a-box” kit that includes all components necessary to use the assay except plate wash and electricity. Plans call for two versions of the assay:

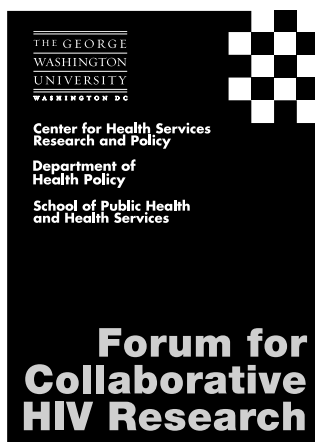
- “Ultradetect” for diagnosis
- “Ultraquant” for clinical monitoring, which will include an ELISA reader and Quanti-Kin software

Precision, Reproducibility, and Linearity

A single operator in Dr. Fiscus’s lab assessed the assay’s precision by running two specimens in duplicate on 10 different assays on 10 different days. Coefficients of variation were 6.1% and 7.9% for the two specimens.

Nadal and colleagues (1999) assessed diurnal variation in 5 HIV-infected children by testing samples from four time points on 2 days for a total eight specimens per child. The standard deviation for eight samples from a single patient ranged from 0.07 to 0.15 log₁₀ for the Roche RNA assay and from 0.02 to 0.11 log₁₀ for the heat-denatured p24 assay. Dr. Fiscus and coworkers (Pascual, 2002) tested six specimens in triplicate or quadruplicate in both assays. The standard deviation ranged from 0.07 to 0.20 log₁₀ for the Roche RNA assay and from 0.03 to 0.12 log₁₀ for the heat-denatured p24 assay.

Dr. Fiscus and colleagues (Pascual, 2002) determined the linear dynamic range of the assay to be at least 4 x 10⁶ fg/mL to 4 x 10³ fg/mL. Others have set the lower limit of detection at 200 to 500 fg/mL (Böni, 1997; Nadal, 1999). The PerkinElmer package insert will claim a dynamic range from 500 to 6,250,000 fg/mL. Values below that will be in a “gray zone.”

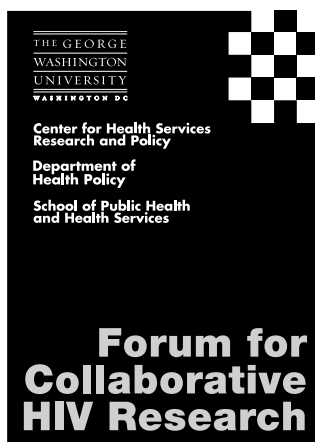


Sensitivity and Specificity

Dr. Fiscus reviewed several studies in which the specificity of the heat-denatured p24 assay was 99% or 100% in Malawian, Tanzanian, and European populations (Table 1). Sensitivity was more variable, ranging from 46% in Africans and Thais infected with subtypes A, B, C, D, E, F, G, O, or recombinants to 99% in Tanzanian infants with the high viral load characteristic of neonates (Table 1).

Table 1. Sensitivity and specificity of the heat-denatured p24 (HDp24) assay

Author, site	Population	Sensitivity	Specificity
Schüpbach, 1996, Switzerland	182 adult patients at various stages of chronic HIV-1 infection	187/182 (97.8%) 112/117 (95.7% by Roche 1.0)	0/107 (100%)
Böni, 1997, Switzerland	127 samples from 23 HIV+ adults (on ART, in Switzerland)	92/125 + by Roche 1.5 (74%) 96/127 + by HD p24 (76%)	No data
Bürgisser, 2000, Switzerland (African and Thai patients)	83 samples from 83 HIV+ African and Thai patients from Swiss Cohort Subtypes A, B, C, D, E, F, G, recombinants, O	55/83 + by Roche 1.5 (66%) 38/83+ by HDp24 (46%)	No data
Schüpbach and Ledergerber (unpublished), Switzerland	Serum samples Swiss cohort from 1989/90, batch-tested 10 years later (frozen at -70° C); 6 narrowly defined CD4 strata (median ± 10%) (RNA mostly degraded; was weakly positive in only 2 of 11 samples, all being positive for p24)	Median CD4; n +/- tested (%): 50; 114/115 (99.1%) 100; 82/84 (97.6%) 200; 87/88 (98.8%) 350; 96/103 (93.2%) 500; 87/102 (85.3%) 750; 40/55 (72.7%) Overall: 506/547 (92.5%)	No data
Pascual, 2002, mostly USA	130 samples from 130 adults (112 US; 18 Malawian, subtype C)	US 94/112 + by Roche 1.0 (84%) Malawi 18/18+ (100%) by NucliSens US 92/112 + by HD-p24 (82%) Malawi 18/18 + by HDp24 (100%)	0/25 (100%)
Fiscus and CDC, unpublished, USA	34 newly diagnosed HIV+ and 5 seronegative controls	32/34 + (94%) Roche 1.0 29/34 + (85%) HDp24	0/5 + (100%)

**Table 1. Sensitivity and specificity of the heat-denatured p24 (HDp24) assay, continued**

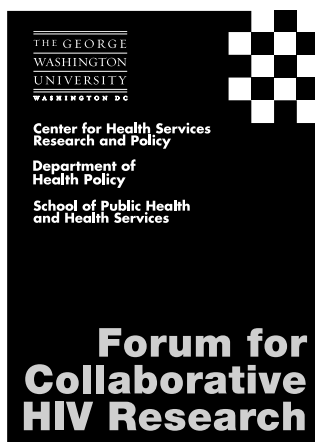
Author, site	Population	Sensitivity	Specificity
Nadal, 1999, Switzerland	232 samples from 61 HIV-infected infants	226/232 + by HD p24 (97%); only ones missed were <10 days old	
	230 samples from 25 HIV-infected infants	197/230 + by Roche (86%) 201/230 + by HDp24 (87%)	
	643 samples from 246 HIV-uninfected infants born to HIV-positive mothers (most samples positive for HIV antibodies)		5/643 + (99%) 40 initially weakly +, 5 still + after neutralization
Lyamuya, 1996, Tanzania	125 samples from 76 HIV+ infants	123/125 (99%)	
	116 samples from 116 HIV+ mothers	21/116 (18%)	
	106 samples from 101 HIV- infants		0/106 + (100%) 15 weakly +, but 0 neutralized
	160 samples from 160 HIV- mothers		0/160 + (100%) 2 weakly +, but 0 neutralized

- Böni J, Opravil M, Tomasik Z, Rothen M, Bisset L, Grob PJ, Lüthy R, Schüpbach J. Simple monitoring of antiretroviral therapy with a signal-amplification-boosted HIV-1 p24 antigen assay with heat-denatured plasma. *AIDS* 1997;11:F47-F52.
- Bürgisser P, Vernazza P, Flepp M, Boni J, Tomasik Z, Hummel U, Pantaleo G, Schüpbach J, and the Swiss HIV Cohort Study. Performance of five different assays for the quantification of viral load in persons infected with various subtypes of HIV-1. *AIDS* 2000;23:138-144.
- Nadal D, Böni J, Kind C, Varnier OE, Steiner F, Tomasik Z, Schüpbach J. Prospective evaluation of amplification-boosted ELISA for heat-denatured p24 antigen for diagnosis and monitoring of pediatric human immunodeficiency virus type 1 infection. *J Infect Dis* 1999;180:1089-1095.
- Lyamuya E, Bredberg-Raden U, Massawe A, et al. Performance of a modified HIV-1 p24 antigen assay for early diagnosis of HIV-1 infection in infants and prediction of mother-to-infant transmission of HIV-1 in Dar es Salaam, Tanzania. *AIDS* 1996;12:421-426.
- Pascual A, Cachafeiro A, Funk ML, Fiscus SA. Comparison of heat-dissociated "boosted" p24 antigen with the Roche Monitor Human Immunodeficiency Virus (HIV) RNA assay. *J Clin Microbiol* 2002. In press.
- Schüpbach J, Flepp M, Pontelli D, Tomasik Z, Lüthy R, Böni J. Heat-mediated immune complex dissociation and ELISA signal amplification render antigen p24 detection in plasma as sensitive as HIV-1 RNA detection by polymerase chain reaction. *AIDS* 1996;10:1085-1090.

Appendix D includes all references for Dr. Fiscus's presentation.

Clinical Validation With Subtype B Virus

For diagnosis of HIV infection in a cohort of subtype B-infected Swiss infants, the heat-denatured p24 assay had a sensitivity of 97% and a specificity of 99% (Nadal, 1999, Table 1). In a US study the assay correctly classified 47 of 55 samples from HIV-1 subtype B-infected infants (for a sensitivity of 86%) and all samples from 32 uninfected infants in the study (for a specificity of 100%) (Fiscus and CDC, Table 1).



Five studies reviewed by Dr. Fiscus appraised the accuracy of the heat-denatured p24 assay for clinical monitoring of people infected with subtype B virus (Böni, 1997; Nadal, 1999; Pascual, 2002; Schüpbach, 2001; Schüpbach and Ledergerber, unpublished). These studies confirm two points:

- HIV p24 measured by the heat-denatured assay decreases in parallel with HIV RNA in successfully treated patients.
- Levels of p24 measured by the assay correlate inversely and significantly with CD4 changes in virally suppressed patients.

For patients in whom therapy is failing, Dr. Fiscus added, more frequent monitoring would be possible with this assay than with standard viral load testing because it is less expensive. She also noted that residual viral production and viral blips observed during therapy are insufficient to maintain concentrations of p24 over time. Thus CD4 changes may be more directly linked with viral protein levels than with levels of RNA.

In a study of 169 HIV-infected people at all stages of infection, the heat-denatured p24 assay proved a consistently better predictor of CD4 declines than HIV RNA and a better predictor of survival (Ledergerber, 2000). But RNA was a better predictor of progression to clinical AIDS. In an unpublished study of 494 first-visit samples from drug addicts, Timothy Sterling and coworkers (Johns Hopkins University) found that heat-denatured p24, CD4 count, and HIV RNA were all highly predictive of progression to AIDS in 5 years. Of the three, heat-denatured p24 had the highest Wald Chi square for disease progression.

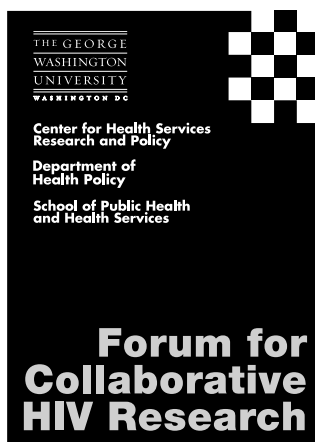
Dr. Fiscus proposed that one research goal should be to establish p24 strata that correlate with rates of disease progression, as John Mellors did in his seminal study of HIV RNA in the Multicenter AIDS Cohort Study (MACS) population.

Clinical Validation With Non-Subtype B Virus

The heat-denatured p24 assay had 99% sensitivity in diagnosing HIV infection in Tanzanian infants (Lyamuya, 1996, Table 1). Subtypes A and D prevail in Tanzania.

As a clinical monitoring tool, the assay correlated strongly with HIV RNA ($r = 0.72$, $P < 0.001$) in a study involving three samples from 46 patients in Côte d'Ivoire (Téhé, unpublished). One sample was collected before treatment, and two during treatment. The assay failed in three patients, two with subtype A and one with subtype D. This study found a significant inverse correlation between CD4 count and p24 from the first to the second sample. The initial change in p24 also correlated significantly and inversely with the CD4 change between samples one and three. Thus the initial p24 change predicted the overall CD4 change from the pretreatment baseline to sample three (the second treatment sample).

Other studies of the assay showed variable antigen detection rates in people infected with non-B virus. In a Swiss study the heat-denatured p24 assay detected 100% of subtypes G and O, but 70% of recombinant viruses, 60% of subtype A viruses, 38% of subtype C viruses, 30% of subtype E viruses, and no subtype D or F viruses. Researchers from the Institute of Tropical Diseases in Antwerp also initially found the assay weak in recognizing subtypes A, C, and D. And workers at the University of Witwatersrand in South Africa, where subtypes A and C



prevail, found appropriately high antigen levels in only three of eight samples.

Dr. Fiscus noted that skill in using the assay increases with time and results improve accordingly. For example, the Antwerp investigators repeated their tests of subtypes A, C, and D samples after receiving additional instructions and detected antigen more accurately. Technicians in her own lab also needed some time to get consistently good results with the assay.

Dr. Fiscus reached the following conclusion concerning the sensitivity of the heat-denatured p24 assay in detecting non-B viruses:

The HIV-1 Core Profile ELISA from PerkinElmer is an excellent test for detection and quantification of HIV-1 subtype B infections. However, the test, as it stands now, may have weaknesses regarding detection of certain subtypes prevalent in some African countries. This must be addressed carefully, and weaknesses should be eliminated before the test is transferred to these areas.

Quality Control, Quality Assurance, and Training

The PerkinElmer kit includes a negative control and a positive control from which the standard curve is prepared. A control with a known dissociation value will be included in the next version of the kit. Dr. Fiscus suggested that internal controls should be added, such as a high positive and a low positive that can be used to plot Levy-Jennings curves.

Jörg Schüpbach (University of Zurich) has suggested using three negative controls when someone is known to be infected and the assay is being used for patient monitoring. When serostatus is unknown and the assay is being used for diagnosis, he uses eight negative controls. Others have suggested that a different cutoff be calculated from a minimum number of negative controls. Dr. Fiscus noted that use of more controls increases the cost of each assay. The minimum required according to the package insert is two negative controls and one positive control.

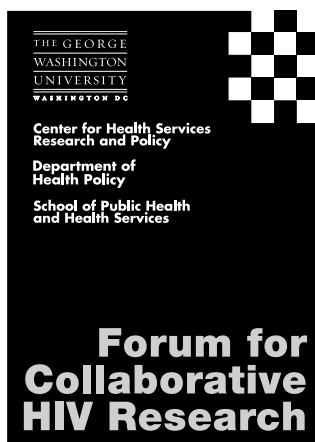
PerkinElmer has offered to handle logistics for training in use of the assay and has volunteered to help with a training and orientation program.

Viral Load Working Group

Susan Fiscus will chair a working group for further study and development of the heat-denatured p24 assay. Several meeting attendees volunteered for the working group, and others not present were nominated:

Candidate members:

Gunnel Biberfeld, Swedish Institute for Infectious Disease Control
 Bernard Branson, Centers for Disease Control and Prevention
 Isabel Cabruja, PerkinElmer Life Sciences
 Sharon Cassol, Africa Centre
 Suzanne Crowe, MacFarlane Burnett Center for Medical Research
 Robert Downing, Centers for Disease Control and Prevention
 Mark Rayfield, Centers for Disease Control and Prevention
 Richard Respass, Centers for Disease Control and Prevention
 Tobias Rinke de Wit, PharmAccess International



Christine Rouzioux, Hôpital Necker (ANRS representative)
 Jörg Schüpbach, University of Zurich
 Wendy Stevens, University of Witwatersrand
 Oliviero Varnier, University of Genoa

Dr. Fiscus proposed the following action checklist for the working group:

- Identify labs to be involved and specimens to be studied.
- Transfer technology to new labs.
- Develop a technology transfer assessment proficiency testing program.
- Train trainers.

She suggested that the following groups of specimens will be needed:

- HIV-1 subtype B specimens from patients with clinical endpoints so that data can be included in treatment guidelines
- Non-subtype B specimens from infants to test the assay in diagnosing infection
- Non-subtype B specimens to test the assay in clinical monitoring
- Non-subtype B specimens to test the assay for disease progression

Dr. Fiscus felt confident that cohorts of infants and people beginning antiretroviral therapy could be identified. Ideally, she said, the working group should try to identify a MACS-type cohort to study the assay in monitoring progression.

PerkinElmer will provide some funding for the working group, but Dr. Fiscus noted that other sources of financing will be needed. She added that advocacy for the assay will be needed because some workers have not had success with it. This problem, she argued, can be overcome with proper training and more experience using the assay.

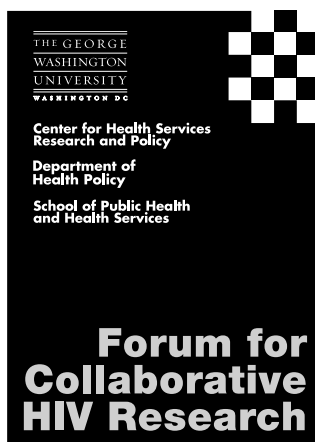
Appendix D includes all references for Dr. Fiscus's presentation.

Discussion of Heat-Denatured p24 Assay

Several issues emerged during the open roundtable following the presentation by Dr. Fiscus:

Assay accuracy

- A PerkinElmer representative agreed that the assay has not met expectations. She blamed inadequate technical support from PerkinElmer for a large proportion of assay failures, and she pledged that the company will improve its support.
- PerkinElmer will have several technical support teams in different geographic areas. If a problem cannot be resolved, PerkinElmer will replace faulty kits.
- Attendees stressed that technical support in developing countries will be critical in maintaining a high level of performance with such an assay.
- Attendees echoed the concern of Dr. Fiscus about still-scant data on non-subtype B virus.



Assay characteristics and technical issues

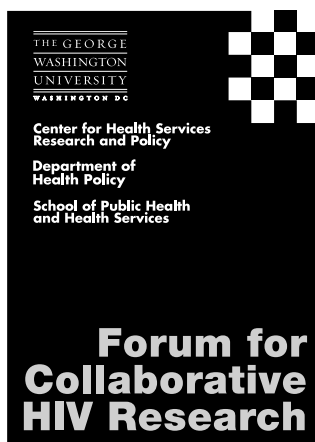
- The sample required for the kit is 50 μ L.
- The heat-denatured assay works well on samples that have been frozen for up to 10 years.
- PerkinElmer is studying assay performance on samples stored at different room temperatures to determine if results vary by storage temperature.
- The assay uses denatured epitopes. Different denatured proteins can have the same denatured epitopes, so care must be taken to avoid cross-reactivity.
- Kits currently have a 5-month expiration date. That seemed short to attendees worried about customs delays in many countries. PerkinElmer plans ahead to minimize customs delays, and a researcher who has used the kit in Africa confirms fast delivery.

Plans for future research

- One attendee stressed that all research from this point should use the same kit so that valid between-study comparisons can be made.
- Field testing in a nonresearch setting is essential.

Cost

- The current lab-in-a-box kit, including heat block, reader, and software, will cost US\$10 per test.



CD4 Assays: Cyto-Spheres and Dynabeads

Alan Landay (Rush Presbyterian-St. Luke's Medical Center, Chicago) summarized research on alternatives to flow cytometry for monitoring CD4 counts: Cyto-Spheres (Beckman Coulter) and Dynabeads (DynaL Biotech). He began the session by showing a table listing Cyto-Spheres and other alternative CD4-counting technologies reviewed at a World Health Organization meeting on April 16, 1992, almost exactly 10 years before the current meeting. Yet only now are interested parties making a concerted effort to transfer validated technologies to resource-limited countries, as attention on HIV in poorer nations has grown and antiretrovirals have become more affordable there.

Correlation With Flow Cytometry

Five of six studies reviewed by Dr. Landay showed excellent correlation between Cyto-Spheres and flow cytometry (Table 2). One study, however, showed a better correlation in Danish patients than in Ivory Coast residents (Gernow, 1995). And a study in Paris did not show a good correlation between the technologies (Didier, 2001). The authors of that study cite two characteristics of Cyto-spheres that may explain the poorer correlation in their study than in others: "the number of positive cells depended on how vigorously the samples were mixed during the experiment," and "the reading had to be carried out as soon as possible after labeling the cells" (Didier, 2001).

Table 2. Cyto-spheres correlation with flow cytometry in measuring CD4 count

Study	Site	Population	Correlation (r)
Landay, 1993	United States and Uganda	376 HIV-positive	0.912
Carella, 1995	United States	117 HIV-positive	0.93
Gernow, 1995	Denmark and Ivory Coast	123 HIV-positive 44 in Denmark 79 in Ivory Coast	0.93 0.74
Johnson, 1995	United States	46 HIV-positive	0.9
Zwerner, 1997	United States	140 HIV-positive	0.98
Didier, 2001	Paris	55 HIV-positive	0.453

Carella AV, Moss MW, Provost V, Quinn TC. A manual bead assay for the determination of absolute CD4+ and CD8+ lymphocyte counts in human immunodeficiency virus-infected individuals. *Clin Diag Lab Immunol* 1995;2:623-625.

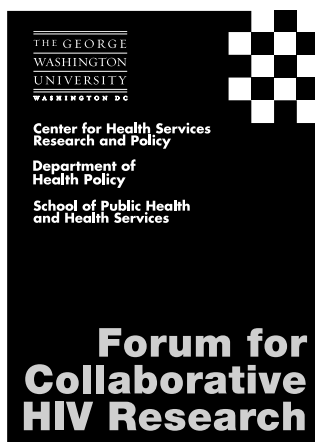
Didier JM, Kazatchkine MD, Demouchy C, et al. Comparative assessment of five alternative methods for CD4+ T-lymphocyte enumeration for implementation in developing countries. *JAIDS* 2001;26:193-195.

Gernow A, Lisse IM, Bottiger B, et al. Determination of CD4+ and CD8+ lymphocytes with the Cyto-Sphere assay: a comparative study with flow cytometry and the immunoalkaline phosphatase method. *Clin Immunol Immunopathol* 1995;76:135-141.

Johnson D, Hirschhorn D, Busch M, and the National Heart, Lung, and Blood Institute Retrovirus Epidemiology Donor Study. Evaluation of four alternative methodologies for determination of absolute CD4+ lymphocyte counts. *JAIDS* 1995;10:522-530.

Landay A, Ho HL, Hom D, et al. A rapid manual method for CD4+ T-cell quantitation for use in developing countries. *AIDS* 1993;7:1565-1568.

Zwerner RK, Hudson JC, Coulter WH, et al. A whole blood alternative to traditional methods for CD4+ T lymphocyte determination. *JAIDS* 1997;14:31-34.



In a study of samples from 83 people with CD4 counts at or below 200 cells/ μ L and 299 people with counts above 200 cells/ μ L, Cyto-Spheres had a sensitivity of 84% and a specificity of 98% (Landay, 1993). The assay's predictive value was 96% for CD4 counts above 200 cells/ μ L and 92% for counts at or below 200 cells/ μ L.

Three studies in Africa and Europe verified an excellent correlation between Dynabeads and flow cytometry (Table 3). A study of 98 people in Dar es Salaam showed that this correlation held true in different CD4 strata: 0 to 199 cells/ μ L, 200 to 499 cells/ μ L, and 500 or more cells/ μ L (Lyamuya, 1996).

Table 3. Dynabeads correlation with flow cytometry in measuring CD4 count

Study	Site	Population	Correlation (r)
Lyamuya, 1996	Dar es Salaam, Tanzania	91 HIV-negative	0.827
		98 HIV-positive	0.974
		0 to 199 cells/ μ L (n = 38)	0.898
		200 to 499 cells/ μ L (n = 55)	0.809
		>500 cells/ μ L (n = 25)	0.925
Didier, 2001	Paris	46 HIV-positive	0.913
Diagbouga, 2002	West Africa	301 HIV-positive	0.890

Diagbouga S, Chazallon C, Kazatchkine M, et al. Successful implementation in West African laboratories of an alternative method to accurately enumerate at lower cost circulating CD4+ T lymphocytes in HIV-infected patients. XIV International AIDS Conference. 2002, Barcelona. Accepted for oral presentation.

Didier JM, Kazatchkine MD, Demouchy C, et al. Comparative assessment of five alternative methods for CD4+ T-lymphocyte enumeration for implementation in developing countries. *J AIDS* 2001;26:193-195.

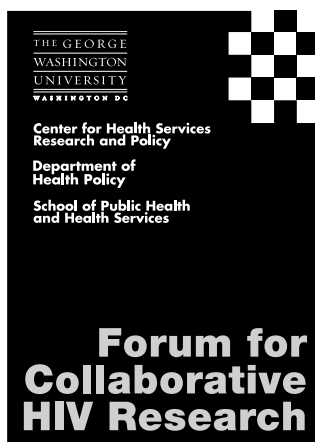
Lyamuya EF, Kagoma C, Mbena EC, et al. Evaluation of the FACScount, TRAx, CD4 and Dynabeads methods for CD4 lymphocyte determination. *J Immunol Methods* 1996;195:103-112.

The West African study by Serge Diagbouga (Centre Muraz, Bobo-Dioulasso) (Diagbouga, 2002) compared Dynabeads with flow cytometry in 657 pairs of values from 301 people with HIV infection. The study involved 45 technicians at six sites. Correlation between the two technologies was good ($r = 0.89$, $P < 10^{-4}$). The tests were 88.7% concordant when CD4 counts were under 200 cells/ μ L. Dynabeads and flow cytometry proved most concordant at lower CD4 counts:

Table 3. Median difference between Dynabeads and flow cytometry results

CD4 count (cells/ μ L)	Median difference between assays
<200	+7.5 cells
200 to 350	-23 cells
350 to 500	-43.5 cells

Source: Serge Diagbouga.



Laurence Weiss (Hôpital European Georges Pompidou, Paris) reported that the repeatability of the Dynabeads assay was also assessed in 130 blood samples at sites in West Africa. The coefficient of variation was 8.4%, ranging from 5.3% to 14.6% at different sites. In the same study the coefficient of variation for flow cytometry at the reference site was 8.3%.

CD4 count change over time was assessed in untreated HIV-infected individuals during five quality control checks at five West African sites. A consistent drop in CD4 cell count as measured by the Dynabead assay was reported between visits.

Cyto-Spheres and Dynabeads Performance Characteristics and Cost

The Cyto-spheres assay has a dynamic range of 30 to 2000 CD4 cells/ μ L. A pipetter, hemacytometer, light microscope, and lysing agent to inactivate HIV are needed to perform the assay. The cost for reagents is US\$10 per assay.

Thomas Denny (UMD-New Jersey Medical School) introduced Cyto-spheres technology in Elista, Russia several years ago. Training the technicians took time, he said, but they succeeded in getting reproducible results. Denny added that the FDA has approved Cyto-spheres for use in the United States. So for this assay “validation” means that its accuracy must be confirmed at resource-poor sites that may adopt it.

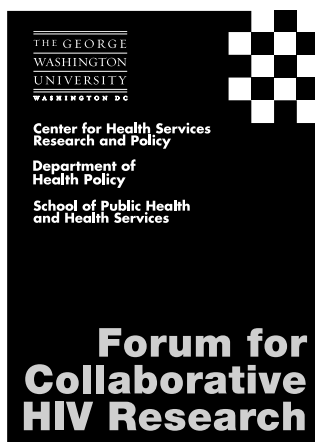
Dynabeads begin to underestimate CD4 counts at levels above 600 or 700 cells/ μ L. That would not present a clinical monitoring problem in poor or wealthy countries, except in children who tend to have high absolute CD4 counts. Even with Cyto-Spheres, Thomas Denny said, samples from children must be diluted or the count can be outside the test’s dynamic range.

Both assays are done at room temperature. Attendees noted, however, that “room temperature” means different things in northern Europe and sub-Saharan Africa. Laurence Weiss, who collaborated in studies in West Africa, said that samples can remain at room temperature there for 8 hours, even though the temperature is higher than the recommended 20 to 25° C.

With Dynabeads, the cost per CD4-cell assay averages US\$4.11, while the cost for a CD4- and CD8-cell assay comes to US\$5.86. One-time costs are US\$235 for a Dynal MPC-S magnet needed for the assay, US\$375 for a sample mixer, and the cost of a microscope. Dynal supports both fluorescent and light microscopy; a study with 60 samples showed good correlation between results with light and fluorescent microscopy.

Attendees suggested that per-assay costs for either test could be lowered by negotiating with Beckman Coulter and Dynal for batch purchase discounts. The cost of a microscope, on the other hand, could be a substantial hurdle in developing countries, especially tropical countries where rapid fungal growth can shorten microscopes’ life spans. Charles Gilks (World Health Organization) stressed that shortage of equipment considered standard in more affluent countries should not be underestimated in poorer countries. In central Malawi, he said, there is one working light microscope per 110,000 people.

See Appendix E for procedure principles from product literature on Cyto-Spheres and Dynabeads.



Quality Control

Laurence Weiss proposed that quality control of Dynabeads could be ensured by creating a network connecting Dynabeads sites to sites using flow cytometry. She is trying to set up such a network with African and Asian flow cytometry sites run by ANRS, the French national clinical trials group. But there is an obstacle to such a quality control network—lack of a blood stabilizer for samples assessed by Dynabeads. She called for tests of available blood stabilizers to determine which may work well with Dynabeads. Thomas Denny has identified blood stabilization reagents that work well with Cyto-Spheres, and he is now studying them with Dynabeads.

Moving Cyto-spheres and Dynabeads Testing Into the Field

Dr. Landay made the following suggestions for validation testing of Cyto-Spheres and Dynabeads in poor and developing countries:

- Flow cytometry should be the reference method.
- The tests should be validated in the context of an antiretroviral trial.
- In that context, the assays should ideally provide real-time results so that treatment decisions can be implemented without having to wait for the infected study participant to return.
- Quality assurance and quality control must be addressed in validation studies.
- Adequate training must precede the trials.

Beckman Coulter and Dynal agreed to supply free reagents for validation studies.

Already planned or ongoing trials may lend themselves to studies of Cyto-Spheres and Dynabeads. For example, Sharon Cassol (Africa Centre, Durban) mentioned two South African studies, one involving people coinfecting with HIV and Mycobacterium tuberculosis, the other involving people with HIV infection and Kaposi's sarcoma.

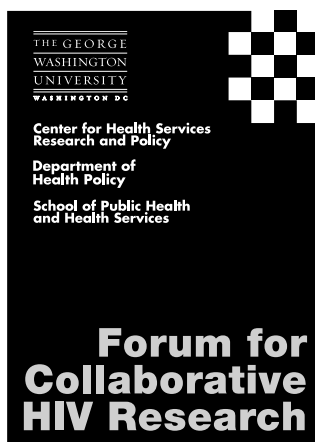
Attendees agreed on the need to identify cohorts in which HIV disease progression can be studied by tracking CD4 cells with Cyto-Spheres and Dynabeads.

CD4 Assay Working Group

Alan Landay will chair a working group for further study and development of alternative CD4 technologies. Several meeting attendees volunteered for the working group, and others not present were nominated:

Candidate members:

Gunnel Biberfeld, Swedish Institute for Infectious Disease Control
 Suzanne Crowe, MacFarlane Burnett Center for Medical Research
 Serge Diabougoua, Centre Muraz
 Berit Marie Eidal, Dynal Biotech
 Debbie Glencross, National Health Laboratory Service
 Daniella Livnat, National Institutes of Health
 Steve McDougal, Centers for Disease Control and Prevention



Pat Roth, Beckman Coulter, Inc
 Gaby Vercauteren, World Health Organization
 Kyle Webster, Becton Dickinson
 Laurence Weiss, Hôpital European Georges Pompidou (ANRS representative)

Discussion of Cyto-Spheres and Dynabeads

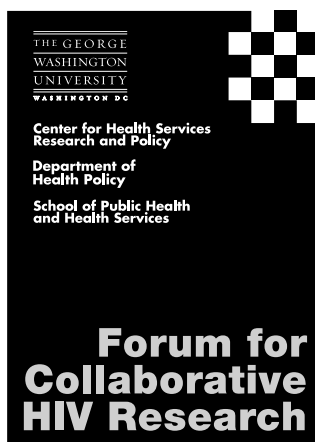
Several issues emerged during an open roundtable following Alan Landay's presentation on these CD4 assays.

Hidden costs, accuracy, and risks with an open counting system

- Participants were reminded that although nonautomated cell counting with technologies like Cyto-Spheres and Dynabeads are cheaper than automated flow cytometry, the cost of staff and training as well as the risk of error due to nonautomated counting needs to be considered.
- Others noted that the precision of flow cytometry is not essential for clinical management of people with HIV infection. Assays that can classify patients into ranges, such as less than 200 cells/ μ L and 200 to 350 cells/ μ L, would represent a huge advance in countries that now have no CD4 technology. But cutoffs using absolute CD4 counts, rather than CD4 percents, are not appropriate for pediatric management.
- A potential risk of an open cell-counting system is exposure of technicians to HIV. Inactivation of the virus must be ensured.

Implementation of alternative CD4 technologies

- An argument was made to focus first on essential questions in studies of CD4 technologies: (1) Is this person infected? (2) If yes, does this person need treatment? (3) If a person is being treated, is that person responding? (4) Is the treatment toxic?
- The World Health Organization is beginning to support HIV care and research in poor and developing countries. Initial efforts will focus on better equipped central hospitals. That raises the question whether programs should rely on a validated technology, like flow cytometry, or try less costly alternative technologies.
- The argument favoring less costly, though still unvalidated, CD4 assays is that district hospitals are not equipped to perform flow cytometry. Cyto-Spheres or Dynabeads using light microscopy could clearly have a role at the district level.
- Senegal has already begun using Dynabeads to monitor people taking antiretroviral therapy and is considering implementing Dynabeads monitoring across the country.
- Attendees suggested that hospitals already using flow cytometry could be used to validate the less costly assays, with the goal of moving the cheaper assays into less well equipped district centers.
- Attendees familiar with district-level labs warned that which assay to implement may be a moot question because the labs are so understaffed. Many have only one or two technicians already overburdened with assays for tuberculosis, malaria, and other endemic diseases. African technicians are becoming infected with HIV, so some labs have no regular technicians.



Meeting Overview: the Next Steps

Veronica Miller, director of the Forum for Collaborative HIV Research, assured attendees of the Forum's ongoing commitment to validation and transfer of technologies discussed at this meeting. She outlined three initiatives:

1. Writing a scholarly review of the topic for publication in a peer-reviewed journal. Such an article would raise awareness of the issues and could be cited in grant applications.
2. Organizing a satellite symposium on this topic at the XIV International AIDS Conference.
3. Facilitating communications among members of the two working groups established at this meeting.

The steering committee for the satellite symposium includes John Nkengasong (Centers for Disease Control and Prevention, Côte d'Ivoire), Gaby Vercauteren (World Health Organization), in addition to the Steering Committee for this project (listed on page 8)

Dr. Miller agreed that the Forum should consider workshops or round table discussions to consider three issues related to technology transfer:

1. Quality assurance and quality control of CD4 and p24 assays
2. Toxicity monitoring for trials in resource-limited settings

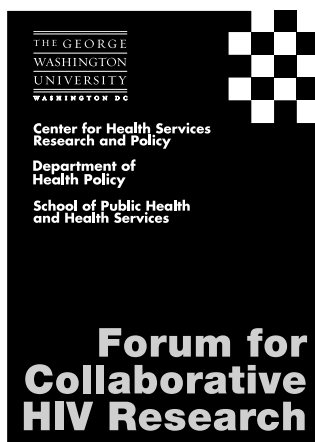
On the issue of quality assurance and control, Susan Fiscus (University of North Carolina) noted that some questions are specific to p24 assays and some to CD4 assays, but there are overarching questions that should be addressed. She proposed that a Forum meeting on this topic should be devoted mostly to these larger questions. Institutions like the CDC and the ANRS already have quality assurance and quality control programs. Dr. Fiscus recommended contacting those organizations to determine what programs are in place and which may be in development.

Attendees proposed inviting front-line clinicians from developing countries to these roundtables because they can bring a practical perspective that otherwise would be lacking.

Attendees discussed the need to address modified flow cytometry methods as alternatives to standard flow methods used in developed countries, and also in the role of reference and quality standards. Groups are already examining these issues, and the Forum will coordinate with them to determine whether additional input from the Forum would help in this worthwhile endeavor.

All agreed that the top priority is prompt action by the working groups with two primary goals in mind:

1. Identifying gaps in validating heat-denatured p24, Cyto-Spheres, and Dynabeads.
2. Determining what work that must be done to fill those gaps.



Susan Fiscus and Alan Landay, chairs of the two working groups, proposed the following first steps:

- Prioritize problems.
- Decide where testing of the assays and analysis of the results should be done.
- Establish programs for quality assurance and quality control.
- Work initially by e-mail and conference call, and convene working group meetings perhaps at the XIV International AIDS Conference in July.

Attendees made the following suggestions:

Where to start studies

Several resource-poor countries have a head-start in treating people with antiretrovirals. They would be the logical sites for studies of alternative technologies.

Toxicity monitoring

Toxicity monitoring should be considered in any validation study, but current toxicity monitoring systems at some potential sites cannot be relied on. In Malawi, for example, there are three tertiary care hospitals, and none do liver enzyme monitoring. Yet hepatotoxicity is common with a range of antiretrovirals.

A first step in toxicity monitoring is determining how much should be done. For hepatotoxic drugs, for example, is a full liver enzyme study needed, or just an alanine aminotransferase (ALT) and aspartate aminotransferase (AST)?

Toxicity monitoring at the level practiced in the most affluent countries may not be appropriate in the developing world. There may be a reasonable middle ground combining some lab tests and clinical evaluation. Physicians prescribing antiretrovirals in poor countries should be consulted to determine the current standard of care in toxicity monitoring.

The World Health Organization has proposed preferred and alternative antiretroviral regimens for the developing world. If those standards are implemented, toxicology monitoring could be focused mainly on the most-used drugs.

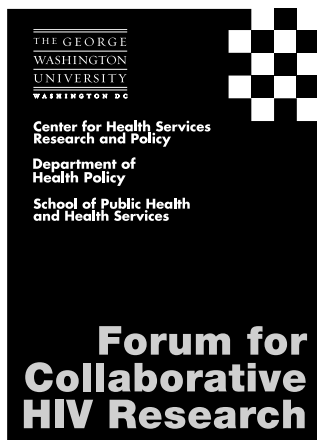
Whatever the approach, toxicity problems must be faced. In the developed world, toxicity is the most common cause of antiretroviral treatment failure. The same will likely prove true in the developing world.

Involving the private sector

Consideration should be given to the private sector in planning technology transfer. Can private labs use these technologies, and do they have to be validated there?

Private sector labs vary greatly in the quality of their work. They often fall outside the radar of government oversight. But if deficient private labs can be upgraded, a completely new testing system does not have to be built.

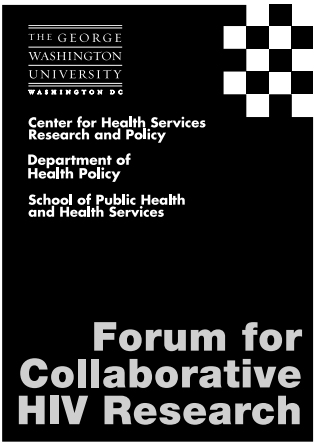
The private sector cannot be ignored in Africa, because most people getting antiretroviral treatment there now do so through the private sector.



CD4 assay guidelines

The National Committee of Clinical Laboratory Standards (NCCLS) developed guidelines for use of flow cytometry. The NCCLS would be the logical body to develop similar guidelines for other CD4 technologies.

The Forum for Collaborative HIV Research could support advocacy for guidelines, which take time to establish.



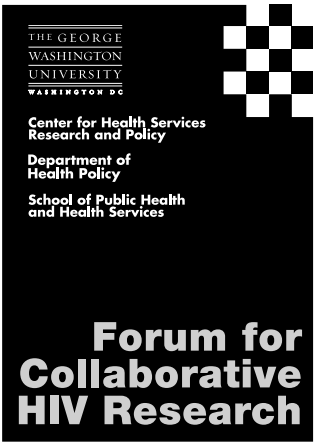
Appendix A

Appendix A

	Representative	Technology	Role in Transfer of Monitoring Technologies					Specific contribution	Specific Needs			
			Development	Validation	Quality Assurance/Quality Control	Training	General Process		from investigators	from industry/agency	other	at workshop
CDC	Branson	p24	implement pilot studies	coordination				site recruitment, support	continued collaboration			
CDC	Downing	p24, MFC*	development & adaptation	assay evaluation					participation in multicenter evaluations, serviceproviders participating in implementation of technology	cheaper, appropriate instrumentation		
USAID	Lans	Dynabeads, Cytospheres, MFC, p24					strategic planning, facilitating networking/linkages & collaborations between groups, leverage funding					
PerkinElmer	Cabruja	p24				yes	set up & logistics, technology support, communications	world-wide coverage for logistics, test availability, lab set up, other items related to assay performance	Organization & support		specific guidelines for test performance	
Dynal Biotech	Daly	Dynabeads					facilitator of contacts, communications, product needs	Background, product information, worldwide contact re: Dynabeads quant. T4-T8 protocol				
UMDNJ	Denny	Dybeads, Cytospheres, MFC		yes	establish Quality Assurance in Resource Poor Settings	train staff from Resource Poor Settings					support for training: educational material, support trainers and travel	
Centre Muraz	Diagbouga	Dynabeads			yes	yes		training & Quality Assurance, recruitment of pts, promotion of alternative tech use in central, regional and district level	participation in international networks		funding for material & reagents	exchange & collaboration
PharmAccess	Rinke de Wit	mod. Dynabeads & Set B					liaison person between PharmAccess and international partners using HAART	contact with HAART access projects, experience in building laboratories, immunological & virological research			Quality Control, scientific support for operational research on HAART access	non-competition; willing to collaborate for comparative studies
Harvard	Rodriguez	Set B tech						sense of what lies on the horizon for low-cost diagnostic, experience as physician	collaborations with clinical centers in developing countries for field testing	funding for research	foundations/organizations: prevent patent issues from delaying development	critical assessment of technology
Academic Alliance	Ronald	non specific						access to African patients and samples, population based studies			protocols, reagents, technology	
Univeristy of Zurich	Schupbach	p24				yes	technical advisor, testingservices under qual. Optimal conditions	testing of samples shipped to his lab: p24, HIV-1 RNA, subtype, training of technicians, ref. Lab	expert statistical evaluation		funding for shipment, p24 and RNA tests	continue existing collaborations
Johns Hopkins	Sterling	p24 & others?						data analysis, comparison of tests with standards	samples & populations from developing countries			continue collaborations with Tom Quinn & Jog Schupbach, willing to collaborate with others
Johannesburg National Health Service Unit	Stevens	p24 & MFC			national external Quality Control which could be expanded to include the assays under considerations and expand into other Resource Poor Settings	significant experience in training technologists & scientists, could provide training in Africa	access to clinics managing HIV	collective experience in diagnostics and cost-effective algorithms			additional funding,	access to international collaborations, exploring impact of subtype C
FHI	van Praag	non specific			Quality Assurance testing via collaborations with Royal Tropical Institute (Antwerp) & UNC		advising, guiding and assisting local government and NGOs	development of tools/guidelines		price reduction for Resource Poor Settings	regular technical validity updates, developing country adaptations of sophisticated Tools, waiver mechanism for USAID supported work,	
University of Genoa	Varnier	non specific	R&D, kit development, lab & field evaluations, techtransfer, pilot scale manufacturing, business development,			organize training course (already did in October 01)	will accept key role in a project aimed to transfer this technology to Resource Poor Settings	long time experience with p24 and dried blood filter paper, developed QuantiKin software for p24 assay, specific and extensive experience with US p24		funding for projects	guidelines for use of p24 (general acceptance)	collaborations with others wanting to use p24 assay
PATH	Tam	virologic assays		Evaluation and implementation through NIAID clinical trials network	evaluation of assay with Virology Quality Assurance contractor		patent/intellectual process issues, collaborations with industry in developed and developing countries	technological or programmatic resource in developing, identifying, evaluating, or adapting appropriate tests, plus any other in house expertise		grants or contracts for projects		
NIAID	Ussery	Cytospheres Dynabeads MFC	Industry/manufacturing representative		yes			funding of Small Business Innovation Research	coordinated planning of evaluations			
Becton Dickinson	Webster	Cytospheres Dynabeads						Scientist with 20+ years experience in the developing world; experience with most applications				

Appendix A, continued

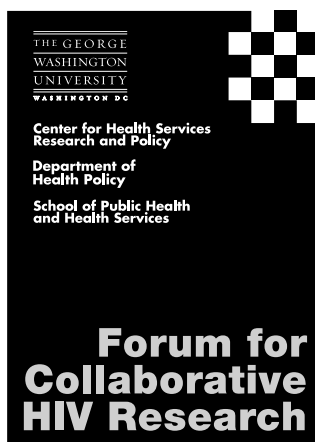
	Representative	Technology	Role in Transfer of Monitoring Technologies					Specific contribution	Specific Needs			
			Development	Validation	Quality Assurance/Quality Control	Training	General Process		from investigators	from industry/agency	other	at workshop
FDA	Dhawan	Dynabeads MFC p24						Efforts to develop sensitive diagnostic assays	Clinical specimens	Reagents		
Swedish Institute of Infectious Disease Control	Biberfeld	Cytospheres Dynabeads		Evaluation of modified p24 of Tanzanian women with subtypes A, C or D with known plasma RNA concentrations as measured by Amplicor 1.5				Evaluation of modified p24 for early diagnosis of Tanzanian infants with subtypes A, C or D		Manufacturer needs to standardize modified p24 assay. Should make test available on the market as a kit with all necessary reagents		
Agence Nationale de Recherches sur le SIDA	Weiss	Dynabeads		yes	Setting up users network connected to ANRS sites in Africa and Asia equipped with flow							
MacFarlane Burnett Center for Medical Research	Crowe	Cytospheres Dynabeads		Assays are performed in comparison to flow				Establish laboratory monitoring infrastructure in the Asia Pacific Region; Can contribute time, expertise and laboratory staff		Funding for studies		



Appendix B

Appendix B-1

Organization	Investigator	Completed Studies	Patient source & Sample size	Clinical Data	Funding & Sponsors	Collaborators	Planned Studies	Patient Source & Sample size	Access to stored samples with clinical data	Funding & Sponsors	Collaborators
CDC	Branson/Respress	correlation with quantitative PCR subtype B and a few non-B correlation with quant PCR from children born to HIV infected mothers		viral load & infection status	CDC limited funding	Fiscus	additional non B specimens from Thailand, Ivory Coast, South Africa. Cote D'Ivoire, Uganda, feasibility & reliability studies. Quality Control process	500 specimens		CDC	
CDC	Downing					Fiscus			aware of clinical data sets with stored clinical samples		
Perkin Elmer	Cabruja	Comparative with standard	500 world wide	no	Perkin Elmer			Europe, n TBD	yes	PerkinElmer	
UMDNJ	Denny								Serum samples		
University of Zurich	Schupbach	Published studies	x1000, multiple per patient, Swiss, Asian, US, African, Brazil	access to Swiss Cohort Study data	Swiss federal, Swiss HIV Cohort Study		Staccato: Thailand, pediatric HIV infection: Tanzania, Cambodia, NIH	x100 each	Swiss Cohort plasma samples (every 6 months) with quantitative RNA & complete clinical data available	Various	Thomas Quinn, Bernard Hirschel, Beat Richner, Inst. Of Tropical Medicine, Basel
Johns Hopkins	Sterling	comparative of p24 with bDNA	500 North American (ALIVE COHORT)	access to Swiss Cohort Study data	NIDA, Swiss federal off. Public Health	Schupbach	p24 on African samples	500 African	ALIVE Cohort samples	Swiss	Tom Quinn, Jorg Schupbach
Johannesburg, NHS	Stevens	40 samples from 8 pts on treatment, correlation with RNA	40 samples from 8 patients	access to	South African NHS				large number of samples		
University of Genoa	Varnier	Comparative to standard	>100, European & West Africa,x1000 samples	access to			Genova Study	100 patients			Varnier is coordinator
NIAID	Ussery	in process: laboratory and clinical studies		access to	NIAID		p24 and Cavid validation assays	Africa and US, size depending on results	ACTG, PACTG, CPCRA	NIAID	Virology Quality Assurance labs, NIAID funded clinical trials network, Jim Bremer
Swedish Institute of Infectious Disease Control	Biberfeld	early diagnosis of infants				Tanzania					



Appendix C

Specimen preparation and ELISA for the heat-denatured p24 assay, and principles of the procedure

Specimen preparation

Dilute 100 μ L of plasma with 500 μ L of 0.5% Triton X-100

Mix and incubate for 10 minutes at room temperature

Heat for 5 minutes at 100° C in dry heat block

Cool to room temperature

ELISA

OPD 30 minutes, RT

Streptavidin-HRP 15 minutes, RT

Biotinyl-tyramide 15 minutes, RT

Streptavidin-HRP 15 minutes, 37

Detector antibody-biotin 1 hour, 37

Antigen 2 hours with shaking RT

Capture antibody

Kinetic reading software (optional)

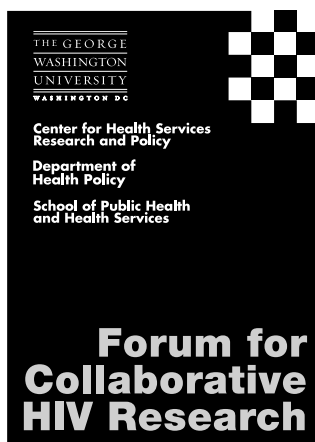
Kinetic reading increases dynamic range from about 1000 to 6,250,000 fg/mL

However, the endpoint assay appears to be more sensitive down to 200-500 fg/mL

Principles of the procedure (from PerkinElmer product literature)

The Ultrasensitive HIV-1 p24 Antigen Assay is accomplished using the PerkinElmer Life Science, Inc., Product Number: 302010 HIV-p24 Ultra Ag Assay, and Quanti-Kin Detection System software.

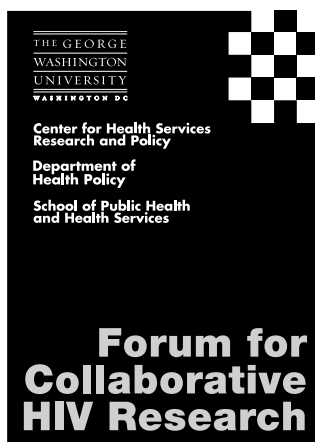
Immune complex disruption of plasma samples is done by using a 1:6 sample dilution in a dissociation buffer followed by boiling for 5 minutes at 100° C. Samples are cooled and transferred to microplate wells that are coated with a highly specific mouse monoclonal antibody to HIV-1 p24. The immobilized monoclonal antibody captures both free HIV-1 p24 and that which has been released upon disruption of immune complexes in the serum/plasma samples. The captured antigen is complexed with biotinylated polyclonal antibodies to HIV-1 p24, followed by a streptavidin-HRP (horseradish peroxidase) conjugate. Signal amplification is then done via incubation with the biotin tyramide solution, followed by streptavidin HRP (horseradish peroxidase) conjugate. Signal is detected by incubation with orthophenylenediamine-HCl (OPD) which produces a yellow color that is directly proportional to the amount of HIV-1 p24 core antigen captured. The absorbance of each well is determined using a microplate reader and calibrated against the absorbance of an HIV-1 p24 core antigen standard curve. Sample absorbance is measured in both kinetic and endpoint mode using the Quanti-Kin Detection System Software. The software allowed automatic computer-driven data collection from the plate reader and for quantitation of sample measurements.



Appendix D

References for the presentation of Susan Fiscus

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Appendix E

Technical notes on Cyto-Spheres and Dynabeads from product literature

Cyto-Spheres (Beckman Coulter)

The CD4 Cyto-Spheres Reagent is inert latex spheres coated with murine monoclonal antibody specific for the CD4 cell surface antigen. This assay depends on the ability of monoclonal antibody-coated latex spheres to bind to the surface of cells expressing discrete antigen determinants. When the CD4 coated latex spheres come in contact with a cell that has the CD4 cell surface antigen, the two bind forming a cell-latex spheres rosette that is readily recognized by light microscopy. The size of the CD4 coated latex spheres is from 1.8 to 2.4 microns in diameter which represents 0.9 to 2.4% of the depth of most hemacytometers. CD4 coated latex spheres also react with monocytes. In this procedure, the Coulter MY4 Cyto-Spheres Monocyte Blocking Reagent minimizes the reactivity of monocytes to the CD4 coated latex spheres. This reagent is a suspension of inert latex spheres coated with murine monoclonal antibody specific for the CD14 cell surface antigen MY4. After reaction with a monocyte, it has the effect of blocking the attachment of CD4 monoclonal antibody (the large latex spheres). The size of the MY4 coated latex spheres is from 0.55 to 0.65 microns in diameter. MY4 coated latex spheres are too small to be optically resolved in the hemacytometer chamber but, when attached to monocytes, given a golden-brown coloration to the cytoplasmic membrane. Cells having this appearance are not to be counted in the assay. The absolute CD4+ lymphocyte counts are performed after combining an aliquot of whole blood with the MY4 Cyto-Spheres Monocyte Blocking Reagent, adding the CD4 Cyto-Spheres Reagent, and mixing. An aliquot of this mixture is added to a Lysing Reagent to lyse erythrocytes. Crystal violet, a component of the Lysing Reagent, is used to stain the nuclear material of the leukocytes to facilitate identification in a hemacytometer. The combined diameter of a lymphocyte coated with CD4 coated latex spheres represents less than 30% of the depth of most hemacytometers.

Dynabeads (DynaL Biotech)

The Dynabeads used in this Dynabeads T4-T8 Quantification Protocol are monosized, superparamagnetic polymer beads containing a sealed iron oxide core. They are coated with monoclonal antibodies to provide a highly specific solid phase to isolate CD4+ and CD8+ lymphocytes directly from whole blood. Some monocytes express the CD4 antigen so highly efficient predepletion of monocytes with Dynabeads CD14 ensures accurate T4 counts even to the lowest range. The cell isolation procedure takes just 30 minutes and is performed at room temperature.

A simple, small magnet, the Dynal MPC-S, ensures safe and easy sample handling. The protocol is recommended to be used with fluorescence microscopy for cell counting and protocols for using this method are included.

