THE GEORGE WASHINGTON UNIVERSITY MEDICAL CENTER
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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

Review of CD4 Technologies

Suzanne Crowe

On behalf of

Members of the CD4 Working Group, Forum for Collaborative HIV Research

Ways of monitoring HIV infection in resource-poor countries

- **Viral load testing… currently rarely used**
- **CD4 testing… where resources are available**
- **Total lymphocyte counts… more easily obtained**
- **Other surrogate markers … not so useful**
- **Clinical assessment… the bottom line**

Reviewed in Crowe et al CID 2003 37(suppl 1) S25

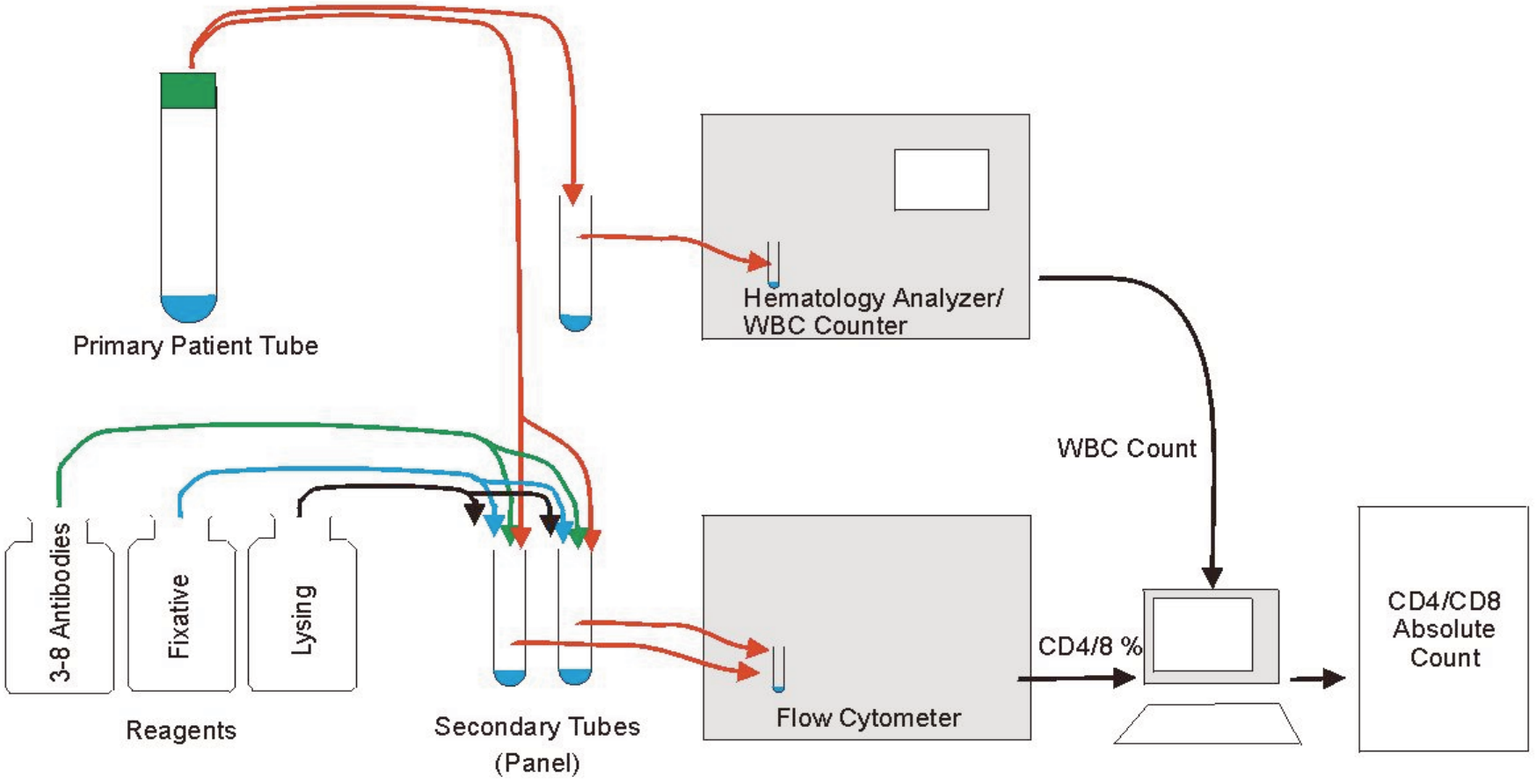
Available methods for CD4 testing

- **Flow cytometry (DP or SP) is the gold standard**
  - **easier/cheaper machines/platforms now available**
    - **Guava EasyCD4 System** ***(Abs cost $1, QC beads $3)***
    - **Partec CyFlow** ***(less than 2E,)***
    - **Pan*leuko*gating technology** ***(less than $6/test)***
    - ***PointCARE*** ***(less than $10/test)***
    - **FACSCount** ***(approx US$20-25/test)***

- **Non-cytofluorimetric methods**
  - **antibody-labelled beads** ***(approx $4-7 per test)***
    - **Dynabeads,**
    - **Coulter cytospheres**

Dual Platform Flow Cytometry

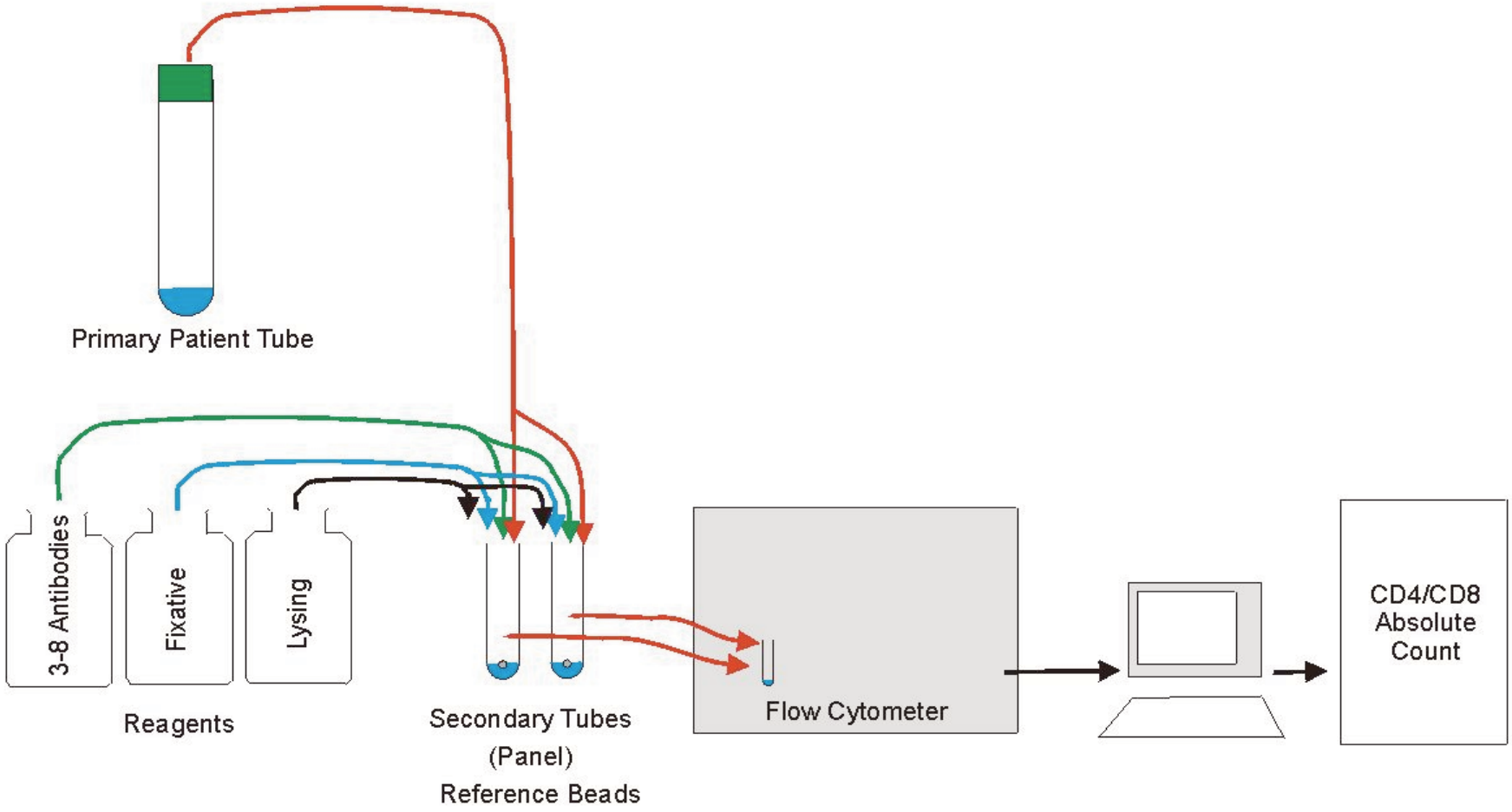
University of Münster



Slide courtesy of Roland Gohde

Single Platform Flow Cytometry

University of Münster



Slide courtesy of Roland Gohde

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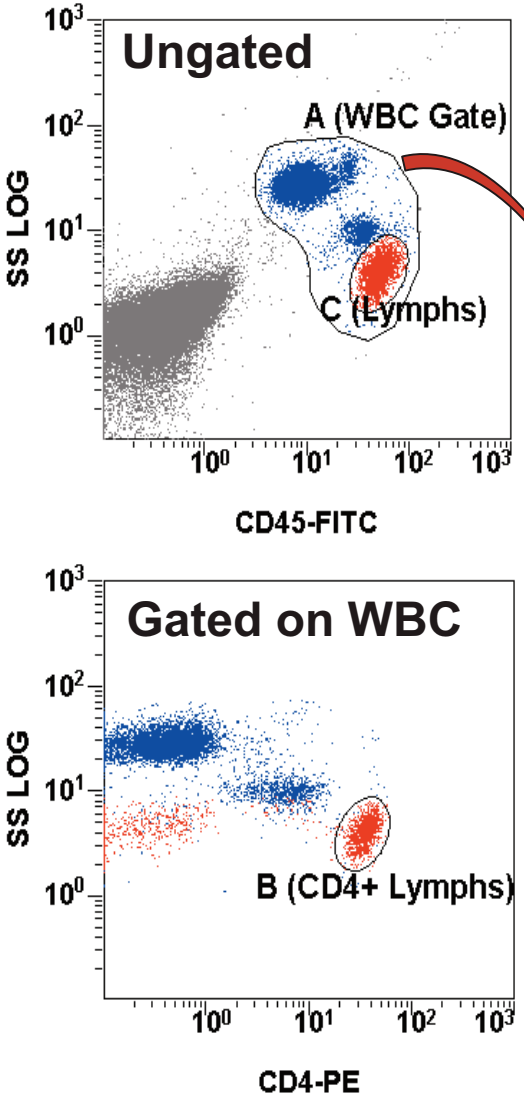
Pan-leukogate or PLG CD4 Methodology

Slides courtesy of Angela Vernon and Meryl Foreman, Beckman Coulter

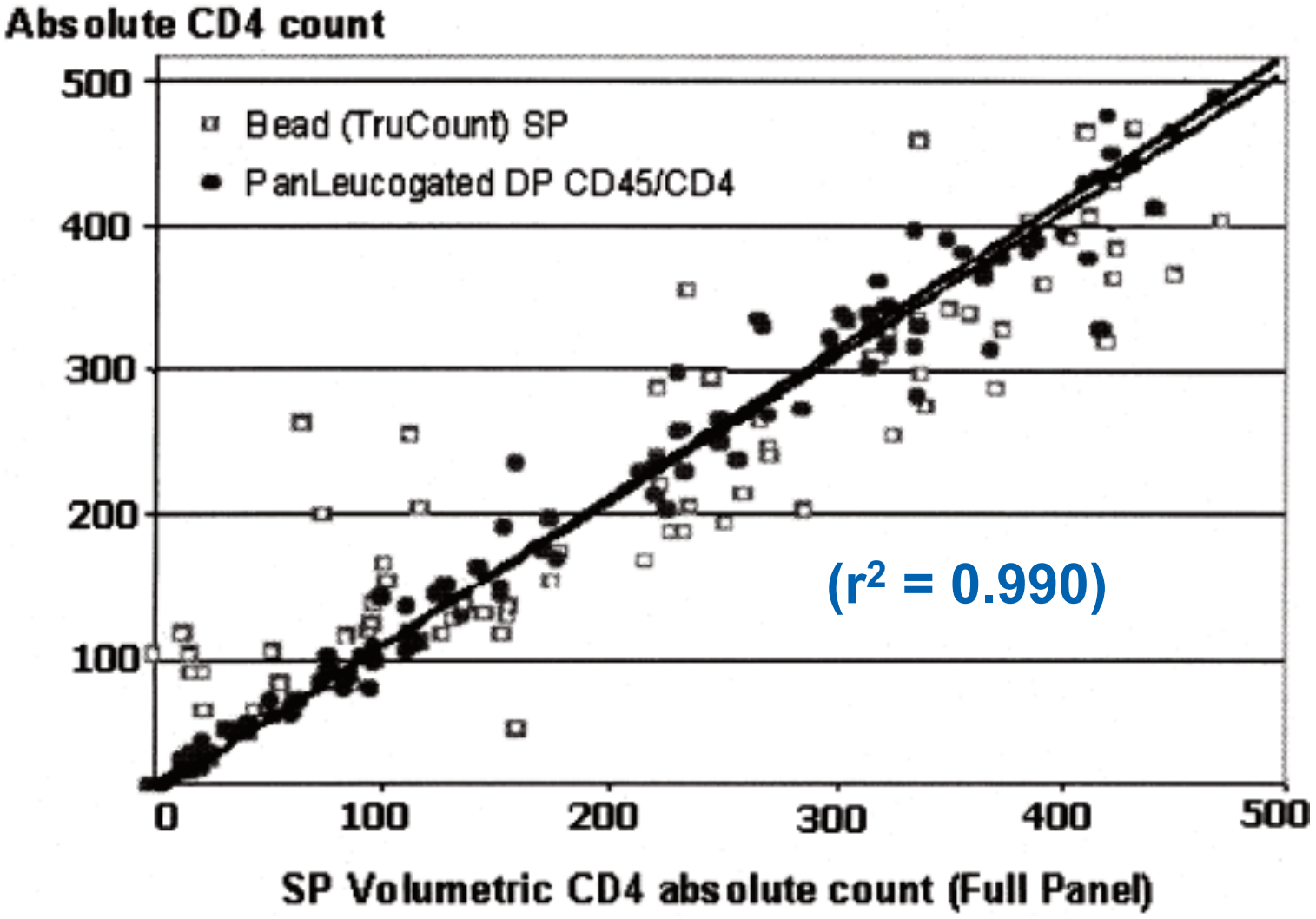
PLG CD4 Methodology



- Identifies CD4+ lymphocytes based on a pan-leucocyte count
- WBC count (cells/ul) X
  CD4 events from region B / CD45 events from region A
  = Absolute CD4
- The WBC gate is not affected by EDTA changes that occur with older specimens.
- Hematology lymph % is affected by EDTA, count not reliable beyond 24 hours.

Slide courtesy of Angela Vernon and Meryl Foreman, Beckman Coulter

PLG CD4 Count

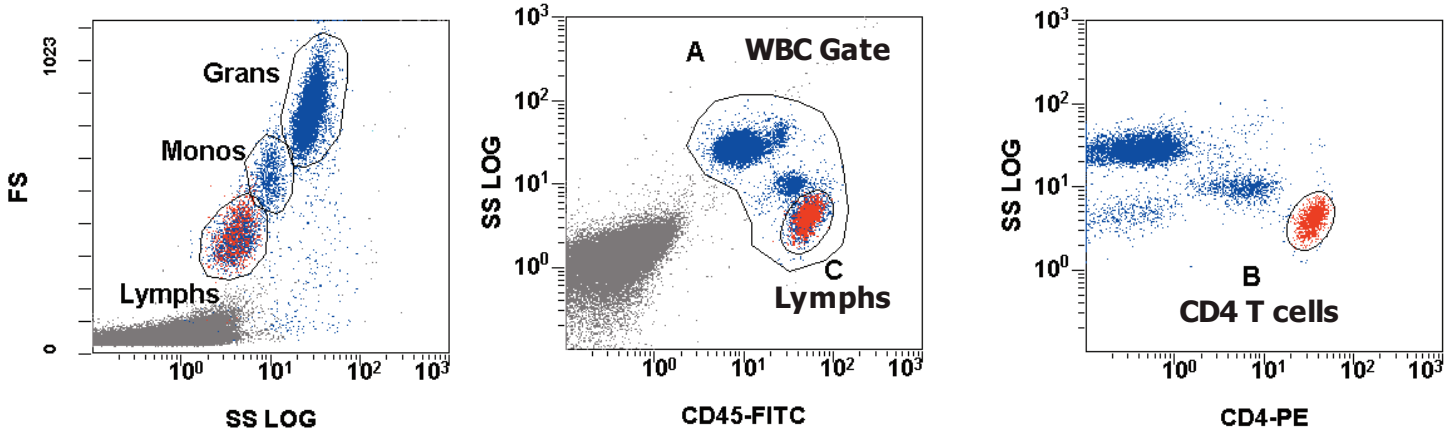


Glencross et al. Clinical Cytometry, 50:2, 2002

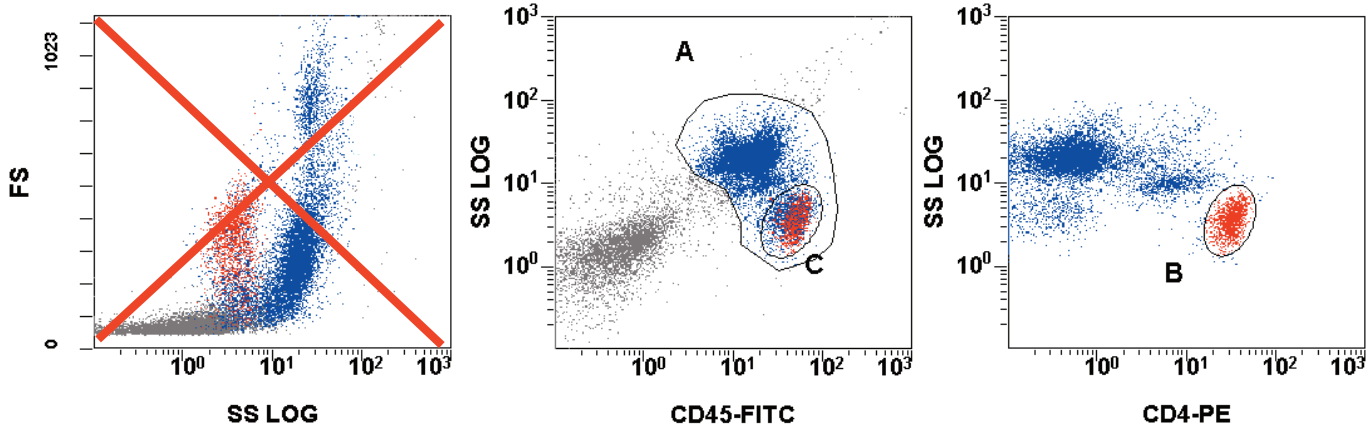
Aged Specimen Performance – Limitations of Scatter Gating

Forward Scatter cellular structure lost over time, results in inability to define appropriate gates using scatter alone

Day 1



Day 5

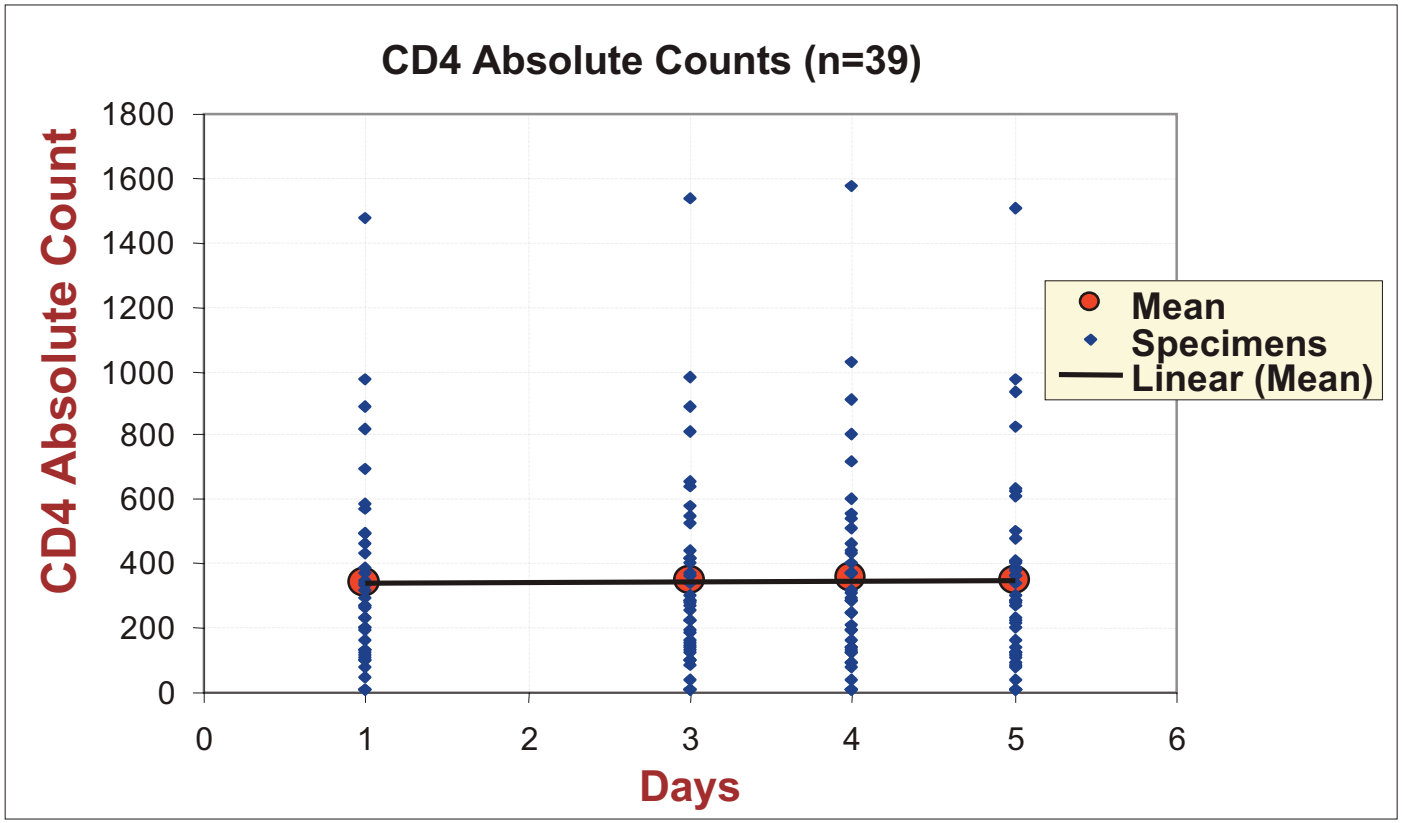


Slides courtesy of Angela Vernon and Meryl Foreman, Beckman Coulter

PLG: Aged Specimen Performance

Beijing, China

- **39 HIV+ donors, PLG CD4**



- **Mixed Model ANOVA for trend over time; p=0.8919**
- **CD4 Count Range: 7 – 1579 cells/Ml; Median CD4 count = 271 cells/µL**

Slide courtesy of Ank Gowans, Beckman Coulter and CDC Beijing

Summary: PLG CD4

- **New flow cytometry-based method**
  - **Based on a pan-leukocyte marker**
  - **uses a 2-color pre-optimized reagent**
  - **provides both CD4% and absolute counts**
  - **extends sample age beyond 24 hrs to up to 5 days**
  - **good correlation to 3 & 4 color "gold standard" flow**
  - **compatible with most flow cytometers**
    - ***with 2 color capability & 488 nm laser line***
  - **<$6 per test**

- **Licensed by Beckman Coulter from NHLS, South Africa**
- **High capacity: good for high volume centralized labs**

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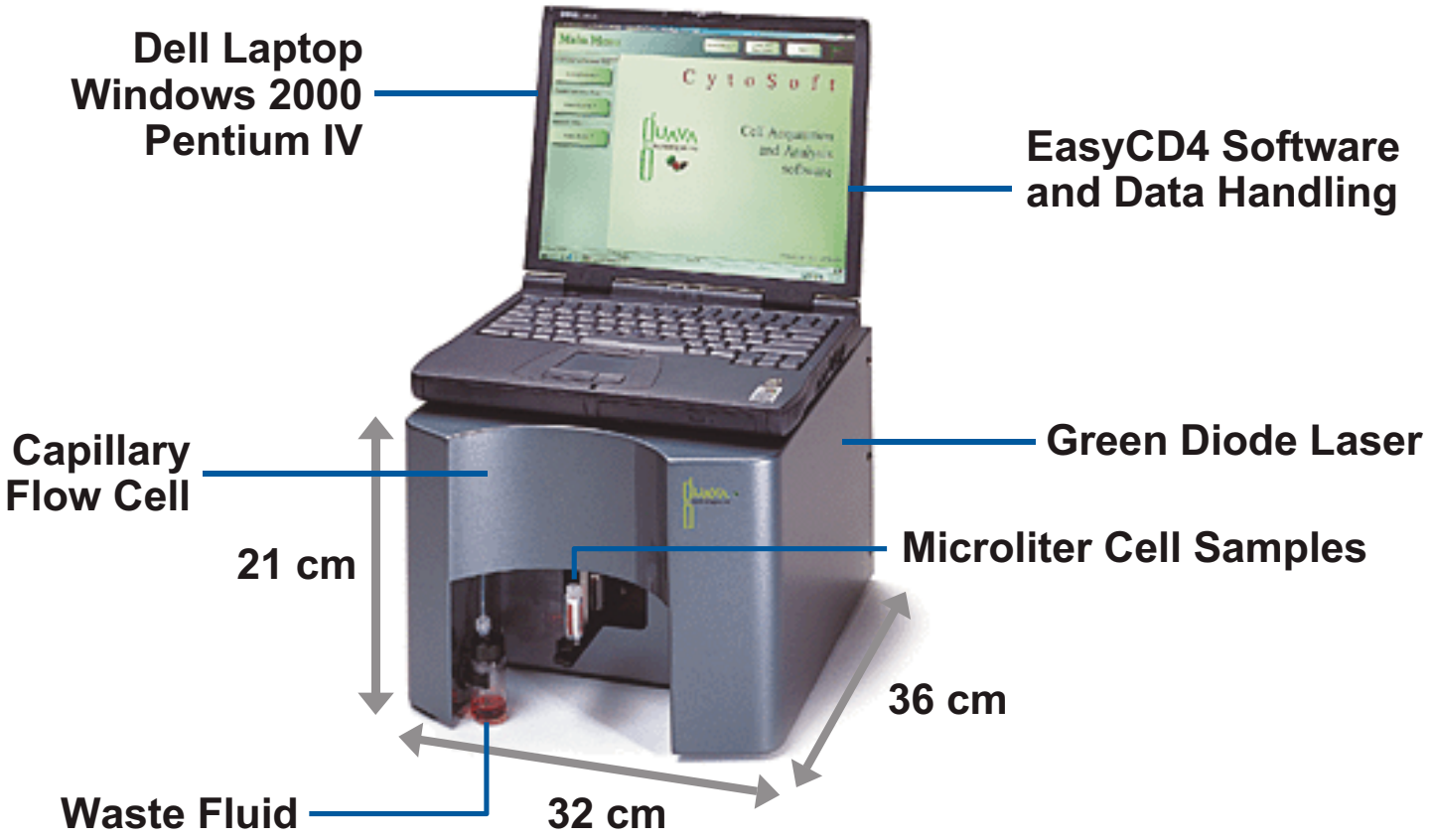
Guava EasyCD4

Slides courtesy of Jeff Harvey, Tina Baumgartner, Leonard Buchner

Guava EasyCD4

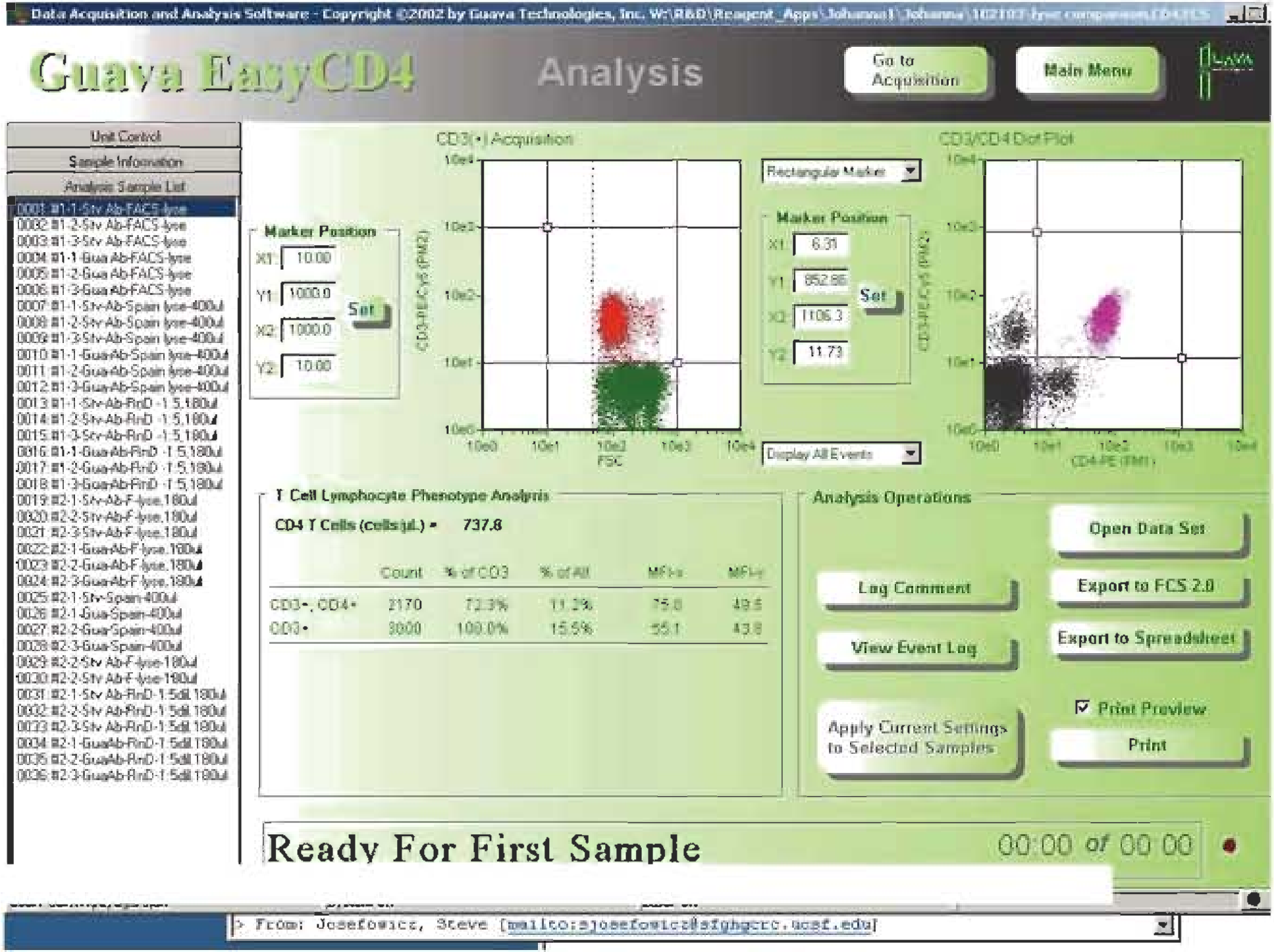
- Measures absolute CD4 (can measure CD8)
- Sample volume:
  - 10 μL of whole blood (EDTA)
- Reagents
  - 10 μL of antibody cocktail
    - Anti-CD3-PE-Cy5
    - Anti-CD4-PE
  - 180 μL of Lyse-Fix solution
- Components/Software
  - Dell LapTop computer included
  - Software includes instrument set-up, data acquisition and analysis

The Guava EasyCD4 System:



15.9 kilos with PC

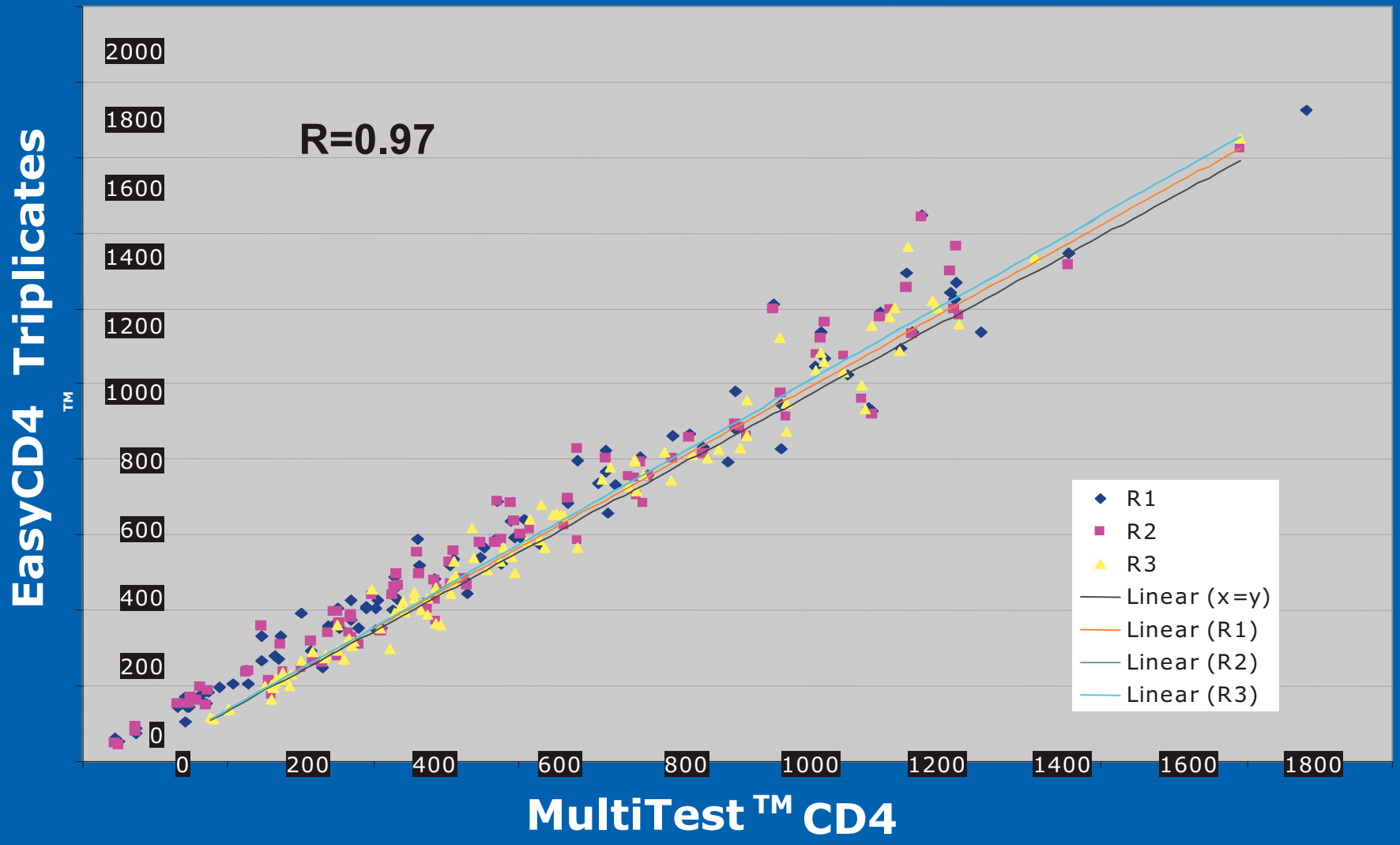
Slide courtesy of Jeff Harvey, Tina Baumgartner, Leonard Buchner


Data Acquisition and Analysis Software - Copyright ©2002 by Guava Technologies, Inc.
Guava EasyCD4
Analysis
Go to Acquisition
Main Menu
Unit Control
Sample Information
Analysis Sample List
0001 #1-1-Stv Ab-FACS-lyse
0002 #1-2-Stv Ab-FACS-lyse
0003 #1-3-Stv Ab-FACS-lyse
0004 #1-1-Gua Ab-FACS-lyse
0005 #1-2-Gua Ab-FACS-lyse
0006 #1-3-Gua Ab-FACS-lyse
0007 #1-1-Stv-Ab-Spain lyse-400ul
0008 #1-2-Stv-Ab-Spain lyse-400ul
0009 #1-3-Stv-Ab-Spain lyse-400ul
0010 #1-1-Gua-Ab-Spain lyse-400ul
0011 #1-2-Gua-Ab-Spain lyse-400ul
0012 #1-3-Gua-Ab-Spain lyse-400ul
0013 #1-1-Stv-Ab-RnD -1:5,180ul
0014 #1-2-Stv-Ab-RnD -1:5,180ul
0015 #1-3-Stv-Ab-RnD -1:5,180ul
0016 #1-1-Gua-Ab-RnD -1:5,180ul
0017 #1-2-Gua-Ab-RnD -1:5,180ul
0018 #1-3-Gua-Ab-RnD -1:5,180ul
0019 #2-1-Stv-Ab-F-lyse,180ul
0020 #2-2-Stv-Ab-F-lyse,180ul
0021 #2-3-Stv-Ab-F-lyse,180ul
0022 #2-1-Gua-Ab-F-lyse,180ul
0023 #2-2-Gua-Ab-F-lyse,180ul
0024 #2-3-Gua-Ab-F-lyse,180ul
0025 #2-1-Stv-Spain-400ul
0026 #2-1-Gua-Spain-400ul
0027 #2-2-Gua-Spain-400ul
0028 #2-3-Gua-Spain-400ul
0029 #2-2-Stv Ab-F-lyse-180ul
0030 #2-2-Stv Ab-F-lyse-180ul
0031 #2-1-Stv Ab-RnD-1:5dil 180ul
0032 #2-2-Stv Ab-RnD-1:5dil 180ul
0033 #2-3-Stv Ab-RnD-1:5dil 180ul
0034 #2-1-GuaAb-RnD-1:5dil 180ul
0035 #2-2-GuaAb-RnD-1:5dil 180ul
0036 #2-3-GuaAb-RnD-1:5dil 180ul
Marker Position
X1: 10.00
Y1: 1000.0
X2: 1000.0
Y2: 10.00
Set
CD3(+) Acquisition
CD3-PECy5 (FM2)
FSC
Rectangular Marker
Marker Position
X1: 6.31
Y1: 852.86
X2: 1106.3
Y2: 11.73
Set
Display All Events
CD3/CD4 Dot Plot
CD4-PE (FM1)
T Cell Lymphocyte Phenotype Analysis
CD4 T Cells (cells/µL) = 737.8
Count
% of CD3
% of All
MFI-1
MFI-2
CD3+, CD4+ 2170 72.3% 11.2% 75.0 49.5
CD3+ 3000 100.0% 15.5% 55.1 43.0
Analysis Operations
Open Data Set
Log Comment
Export to FCS 2.0
View Event Log
Export to Spreadsheet
Print Preview
Apply Current Settings to Selected Samples
Print
Ready For First Sample
00:00 of 00:00
From: Josefowicz, Steve [mailto:sjosefowicz@ghpccc.ucsf.edu]

Guava EasyCD4 Protocol

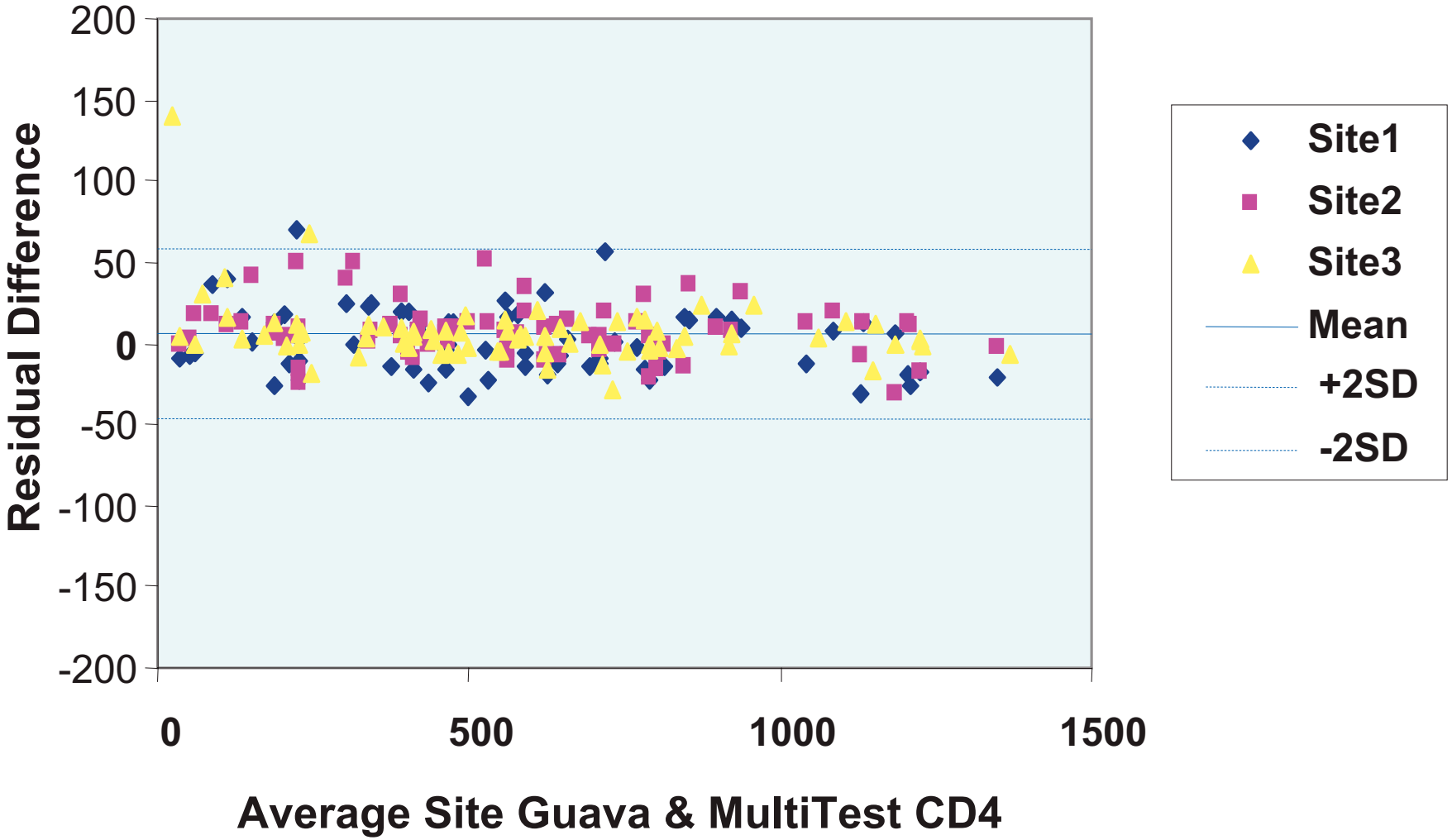
- **Add 10uL of antibody cocktail to each tube**
- **Add 10uL EDTA whole blood to each tube, vortex, incubate 15min**
- **Add 180uL of Lyse/Fix solution, incubate 15min**
- **During sample incubation, turn on power and allow 10 minute warm-up**
- **Run Guava Check QC procedure (5 min)**
- **Adjust (or recall) instrument settings**
- **Acquire samples; Analyze results**

EasyCD4 vs MultitestCD4



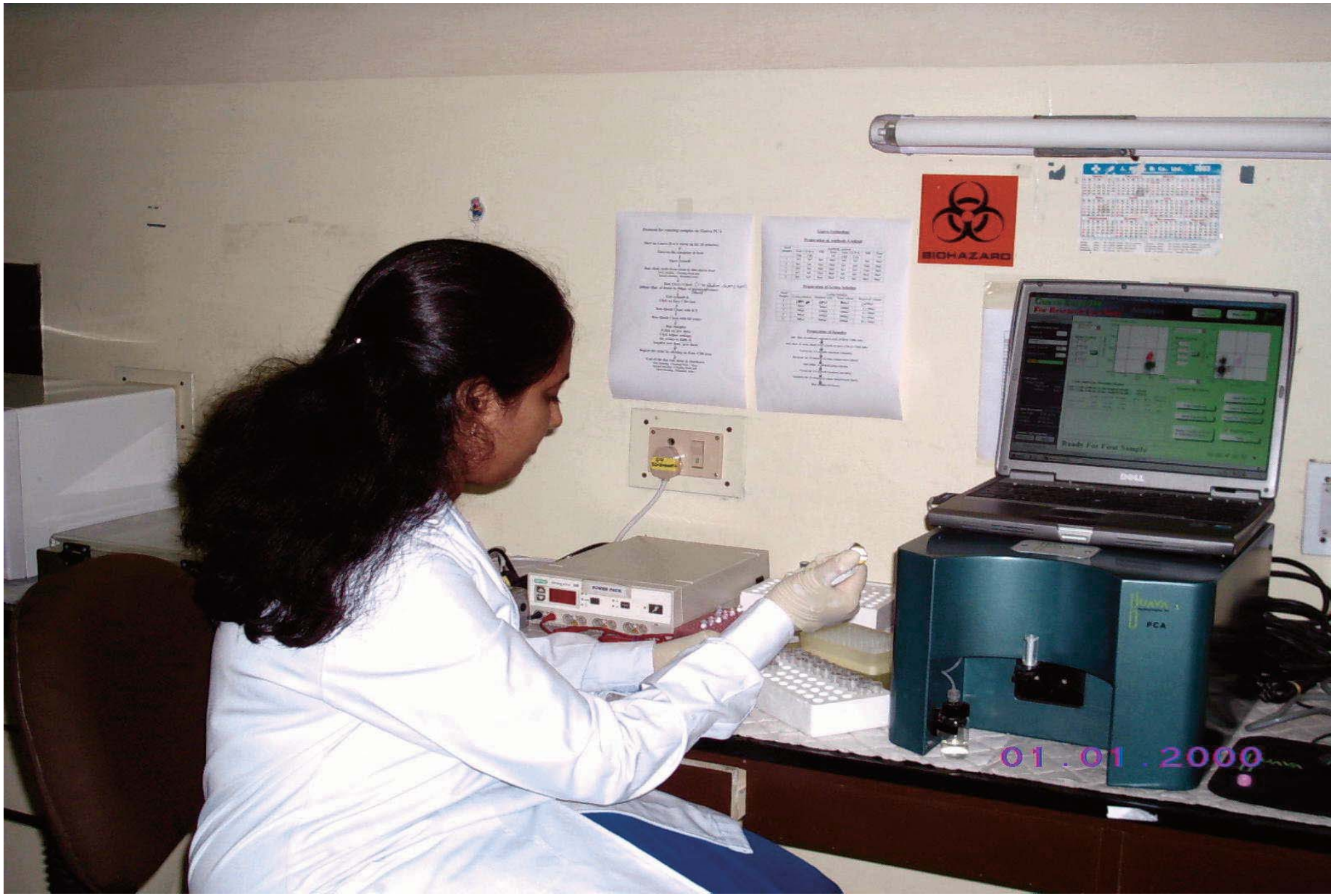
UCSF-GCRC/GIVI-CFAR Core Immunology Laboratory

North America – California 3 Site Trial



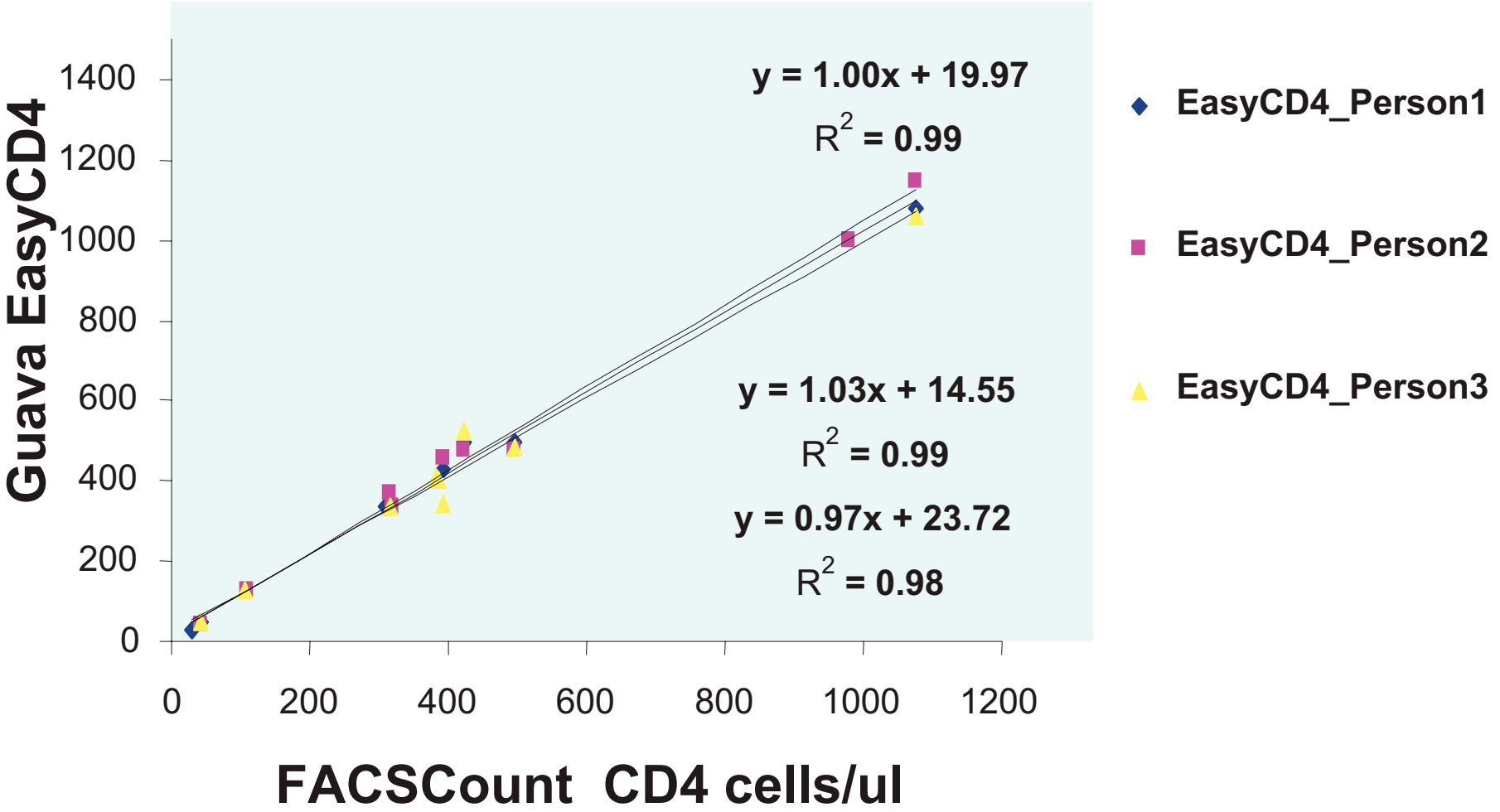
Slides courtesy of Jeff Harvey, Tina Baumgartner, Leonard Buchner

Guava EasyCD4 at YRG CARE



YRGCare (Chennai) Study

FACSCount vs Guava (Operator-to-operator variability)



Slide courtesy of Dr Balakrishnan, YRGCare, Chennai

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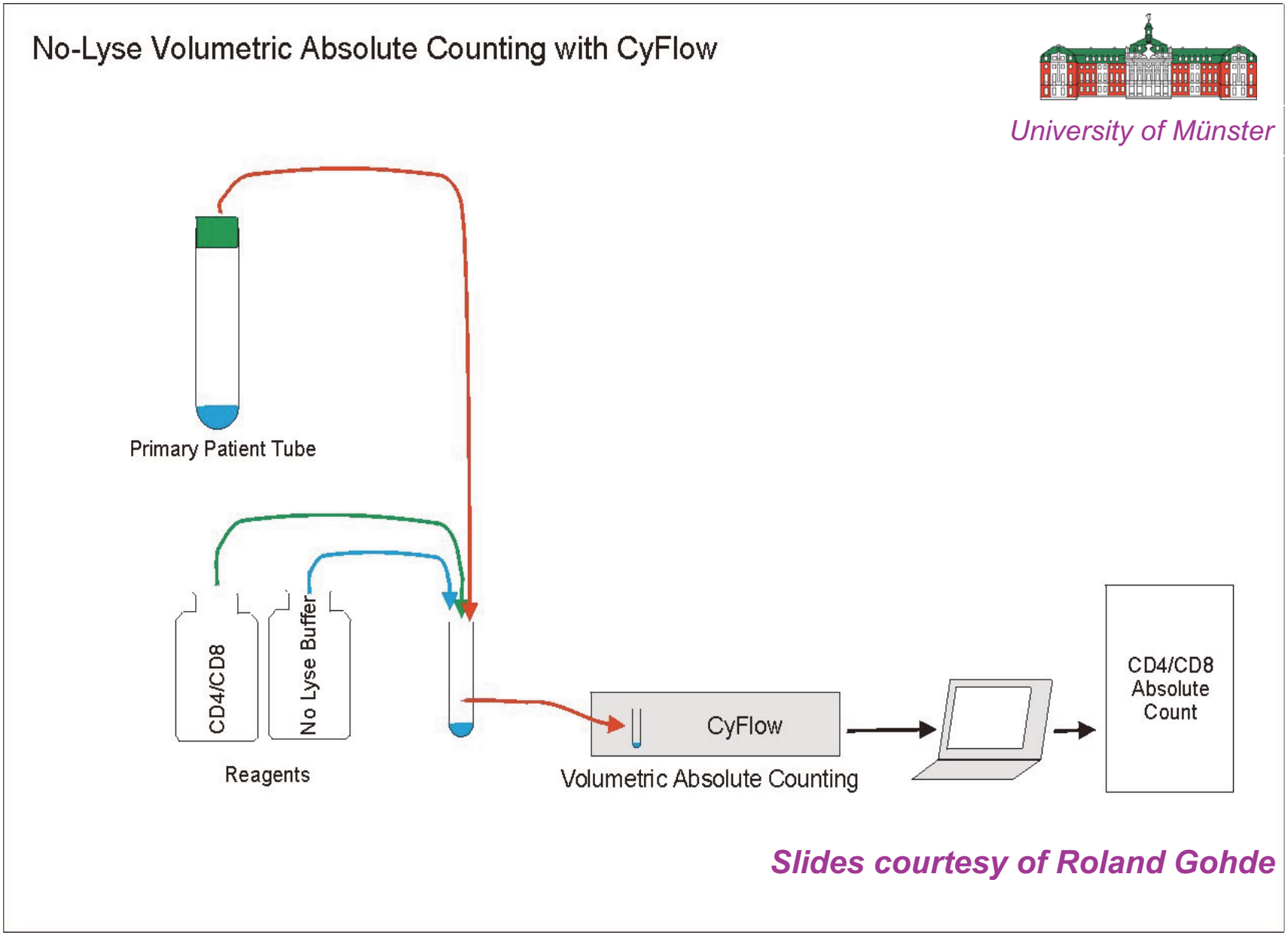
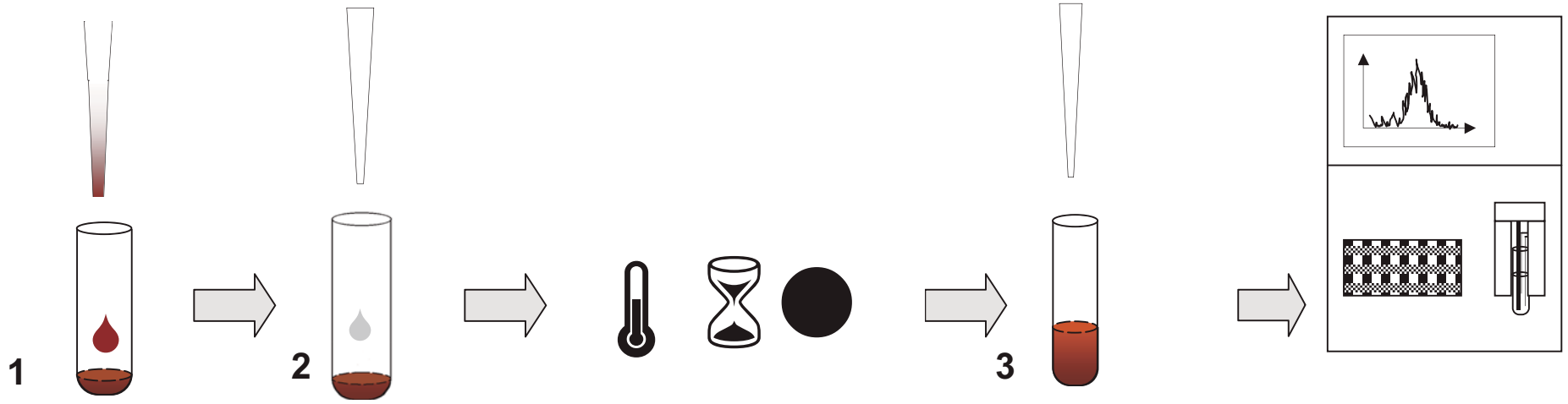
Partec Cy-Flow

Slides courtesy of Roland Gohde

Volumetric Single Platform Flow Cytometry



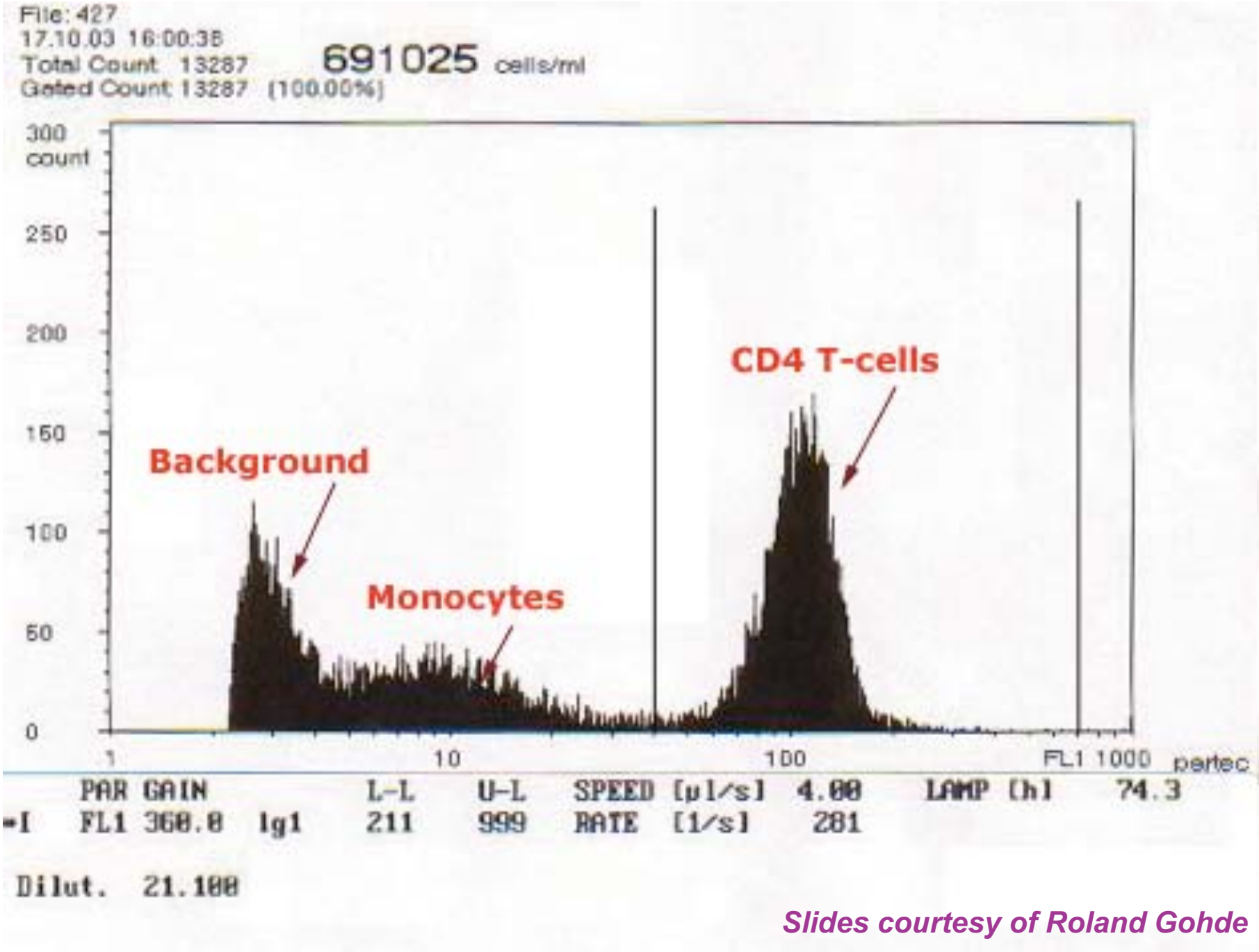
Cy-Flow
no lyse - no wash CD4 protocol



3-Step Protocol

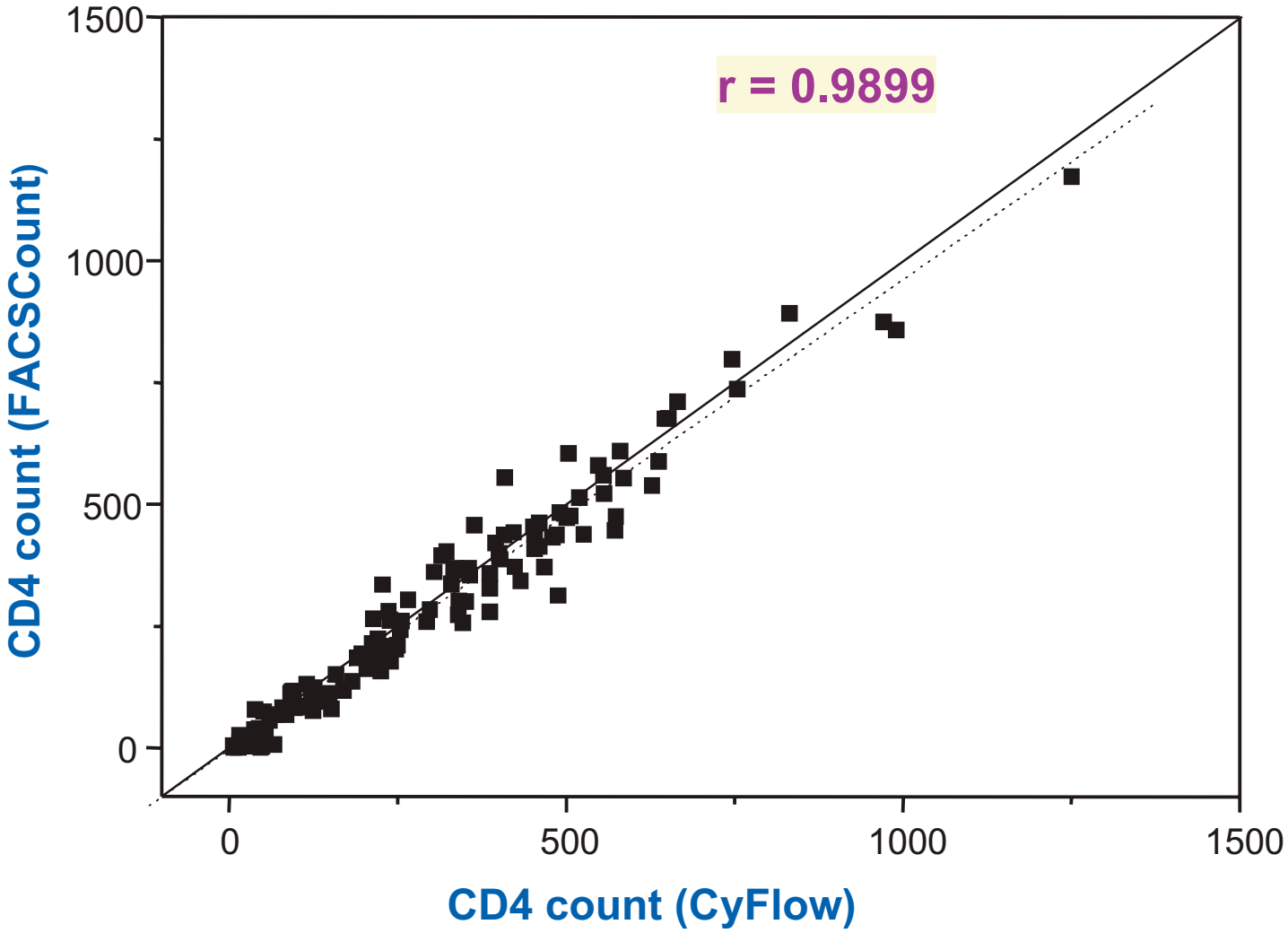
- 50µl blood from the patient into a sample tube
- add 10µl of CD4-PE and incubate for 10 minutes at RT in the dark
- add 850µl of the no lyse dilution buffer

University of Münster



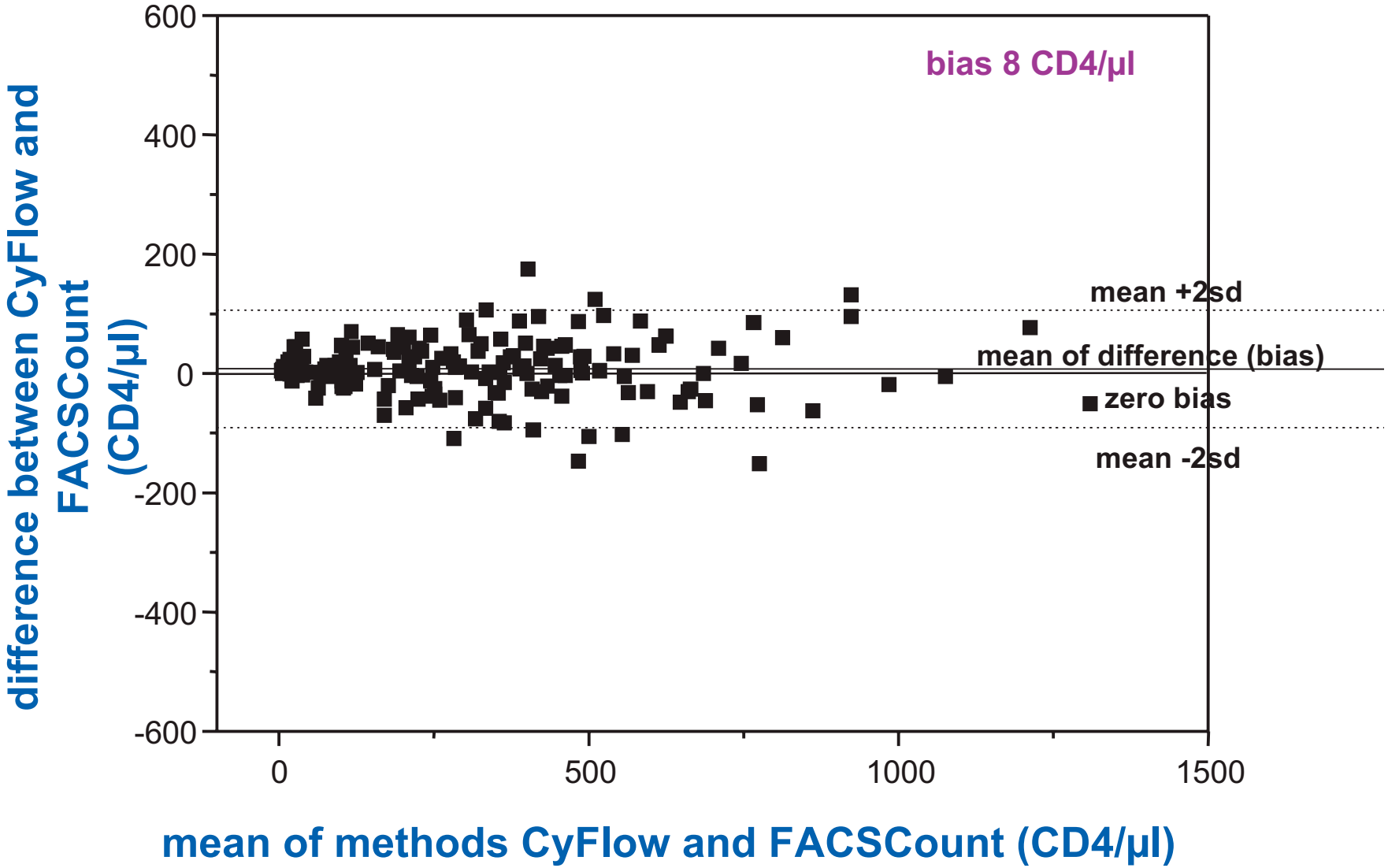
Slides courtesy of Roland Gohde

Cameroon: CD4 Counting - CyFlow vs. FACSCount



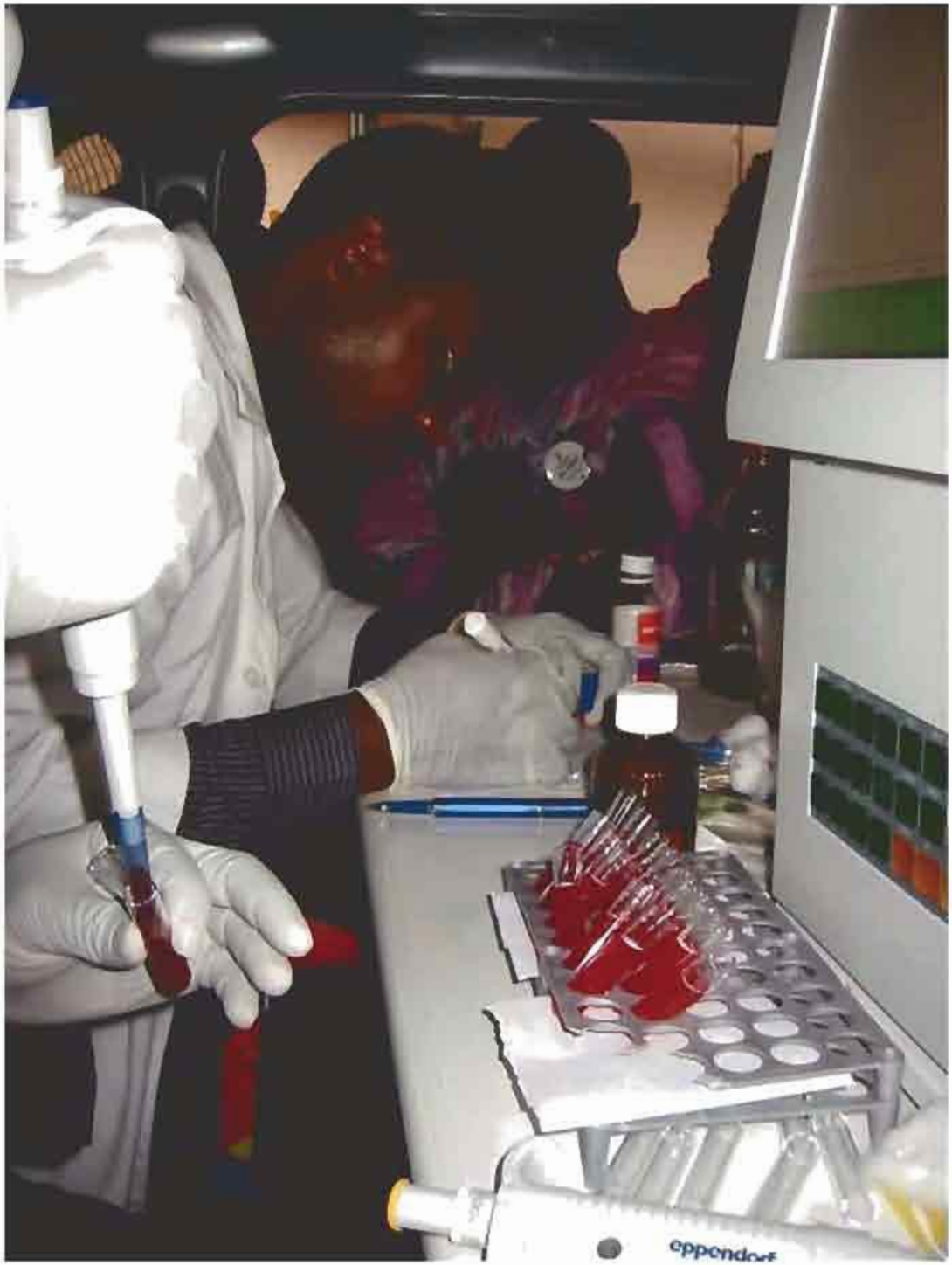
Douala and Marua, Cameroon

Cameroon: CD4 - CyFlow vs. FACSCount
Bland-Altman Plot



Douala and Marua, Cameroon

Partec – Mobile CyFlow® Immunology Lab


eppendorf

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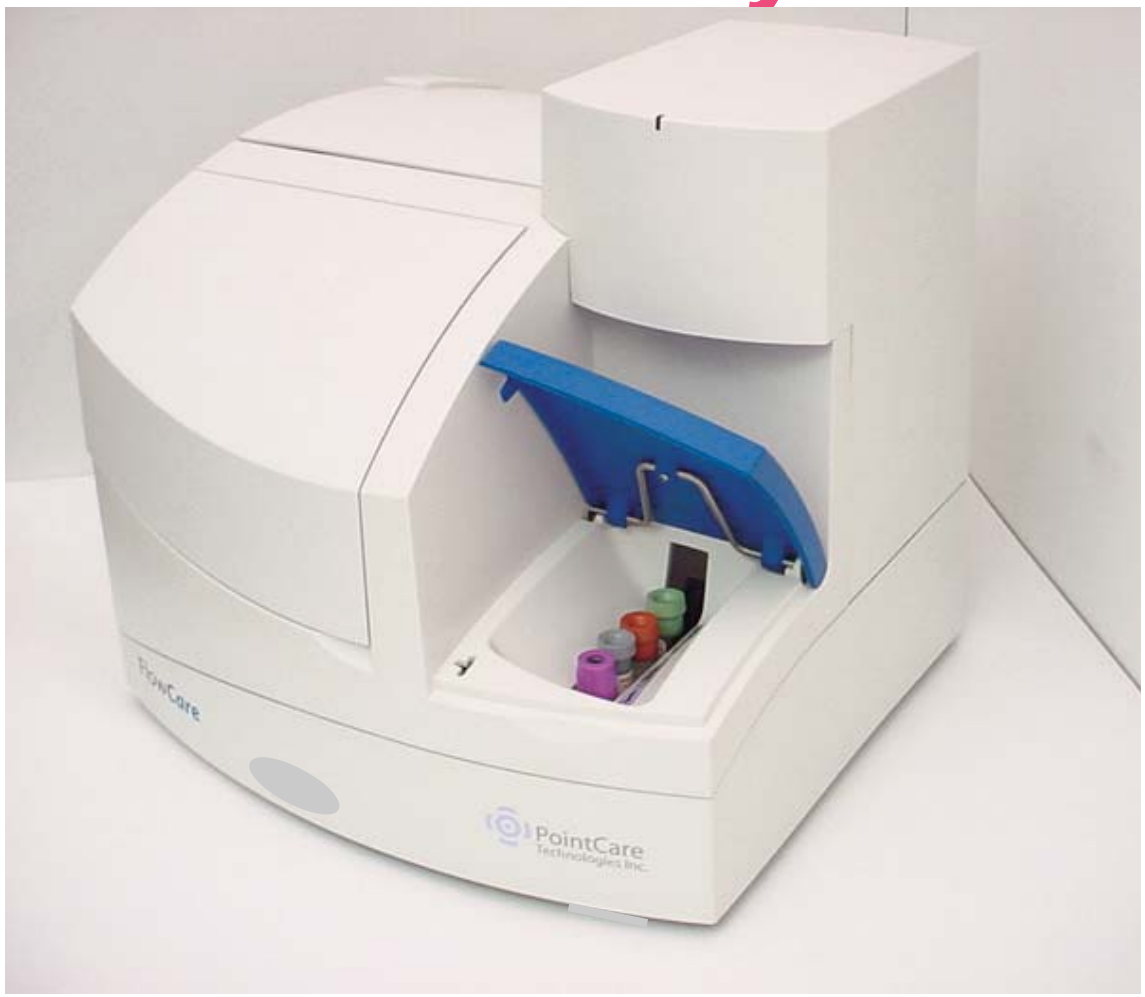
PointCARE

Slides courtesy of Cecil Sherrer

PointCARE System



%CD4 and Absolute CD4
WBC, LY% and count
Mobile; battery backup
Room temperature reagent storage and operation

Closed- tube operation – biohazard containment via cap piercing

4. Lysing Reagent Tube or Cleaning Solution Tube

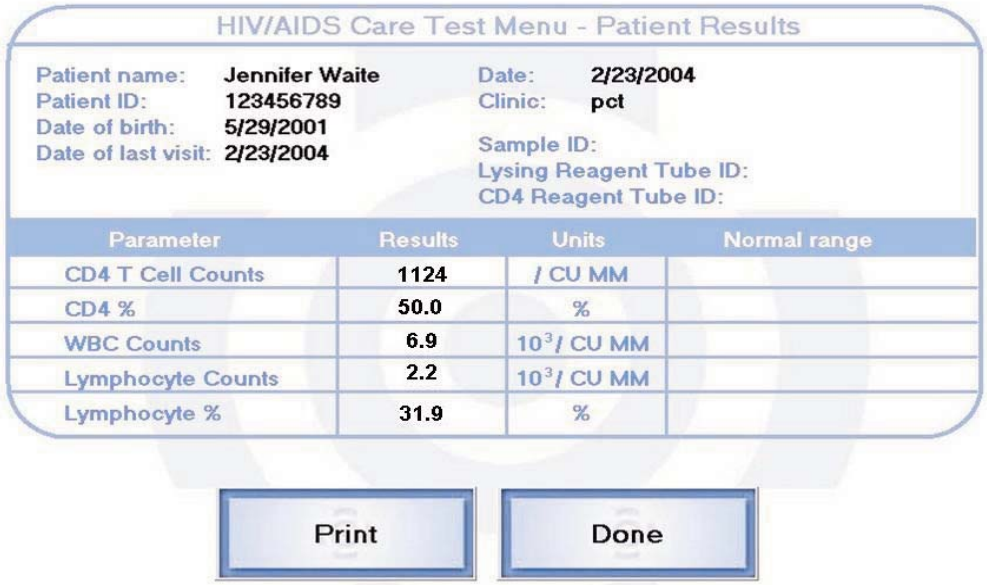
3. Rinse Tube

2. CD4 Reagent Tube

1. Patient Whole Blood Sample Tube

- Patient sample and reagents bar-code are tracked in the instrument.
- Ideal for low-volume, decentralized labs

Automated Patient Results

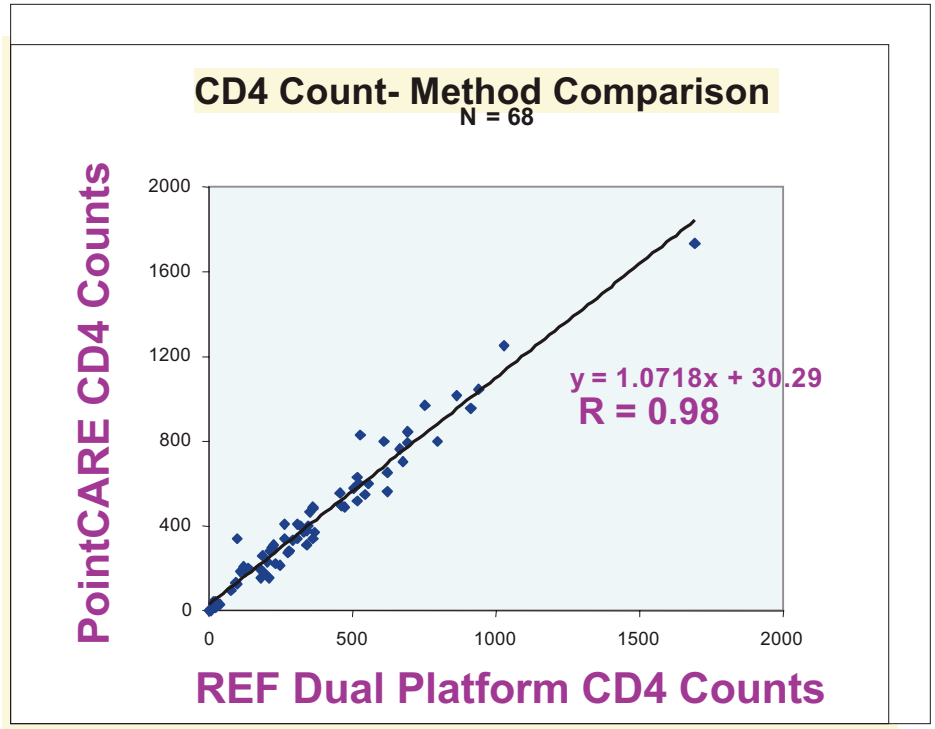


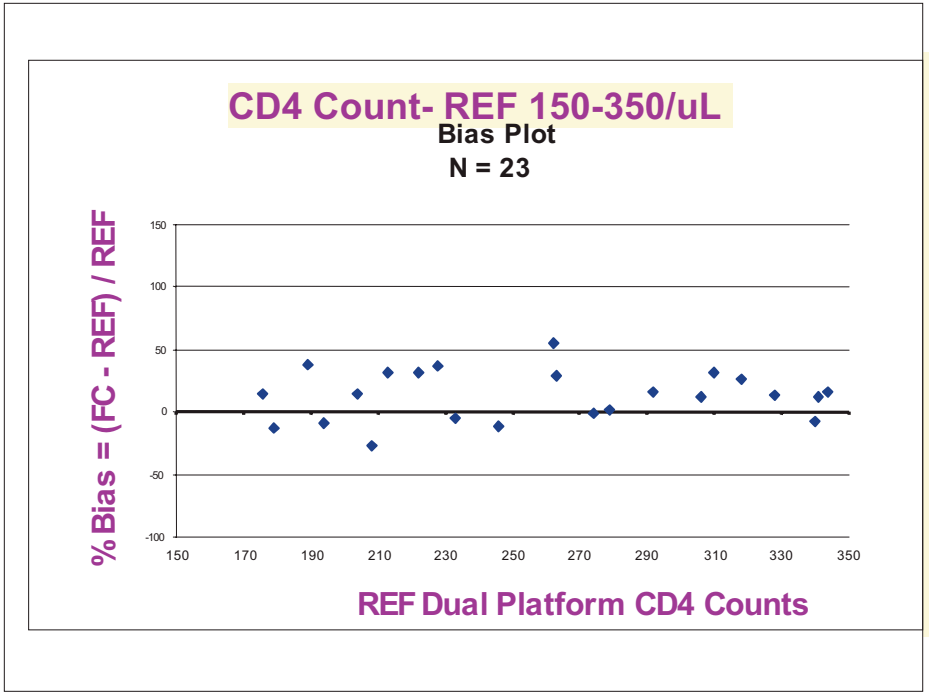
- **Both CD4% & Absolute CD4**
  - **without beads**
  - **critical for pediatrics**

- **Depending on test volume, cost of patient result is under US$10**
- **Cost of patient result includes:**
  - **All reagents and disposables**
  - **Operator time**
  - **CD4, CD4%, WBC, LY, LY%**
  - **Service**

Slides courtesy of Cecil Sherrer

PointCARE comparison with DP Flow





Slides courtesy of Cecil Sherrer

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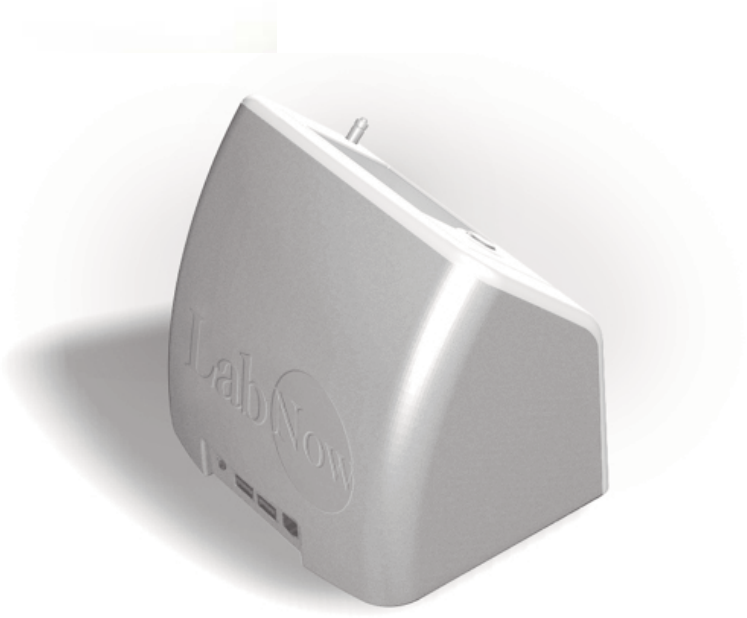
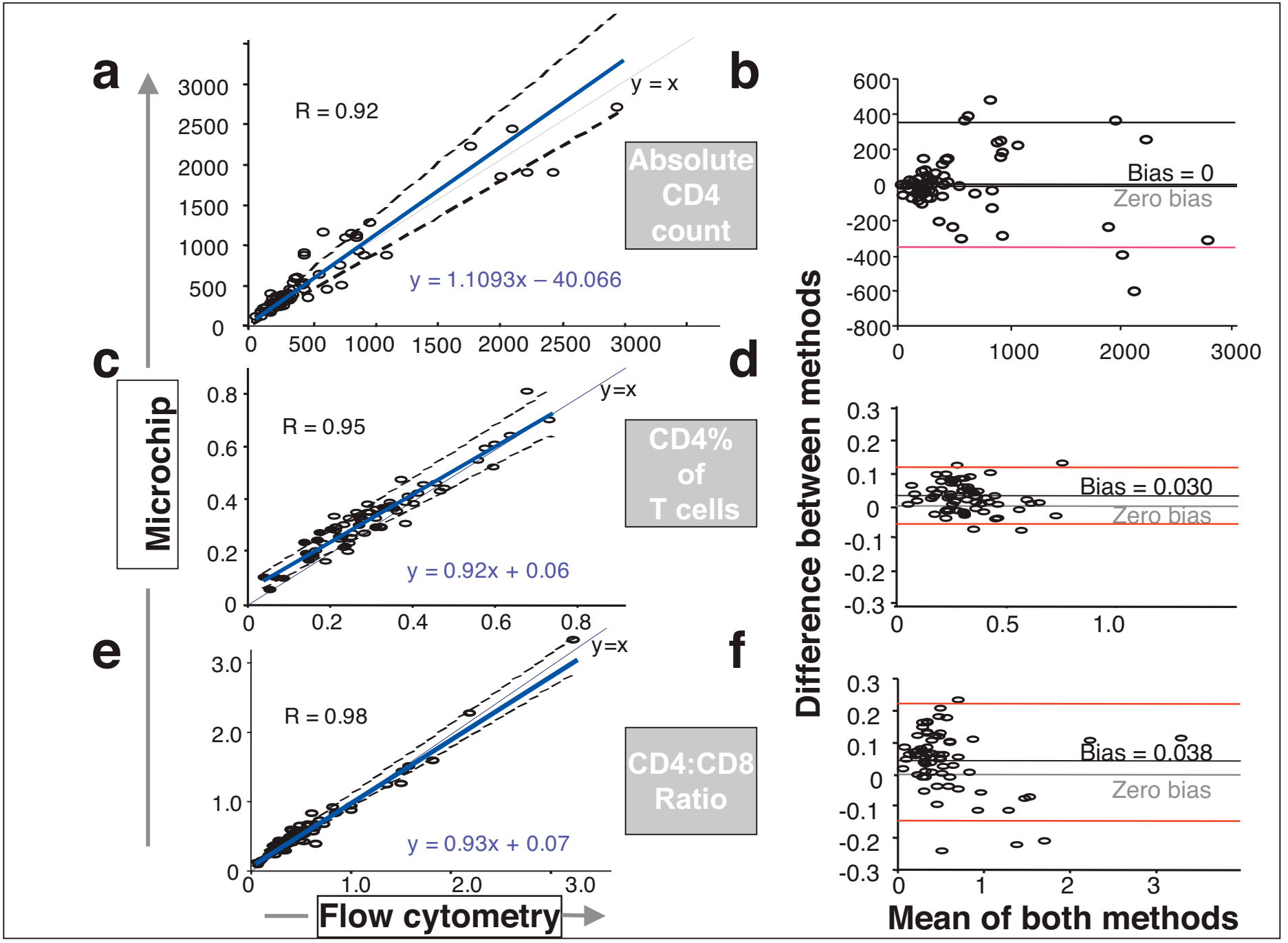
Microchip Technologies for CD4 Counts and HIV Diagnostics

LabNow

Data from Bill Rodriguez

Commercialization – LabNow Corp (Austin, Teas)




a
b
c
d
e
f
Microchip
Flow cytometry
R = 0.92
y = x
y = 1.1093x – 40.066
Absolute CD4 count
R = 0.95
y=x
y = 0.92x + 0.06
CD4% of T cells
R = 0.98
y=x
y = 0.93x + 0.07
CD4:CD8 Ratio
Bias = 0
Zero bias
Bias = 0.030
Zero bias
Bias = 0.038
Zero bias
Difference between methods
Mean of both methods

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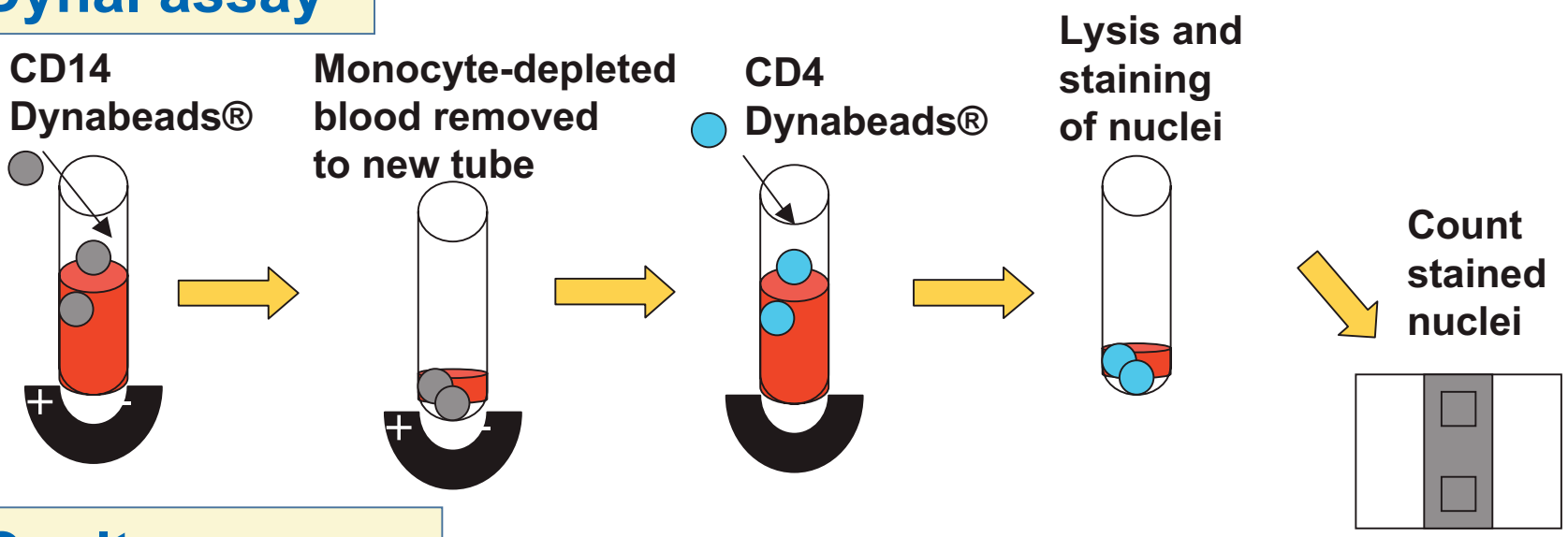
Manual low cost assays for monitoring CD4

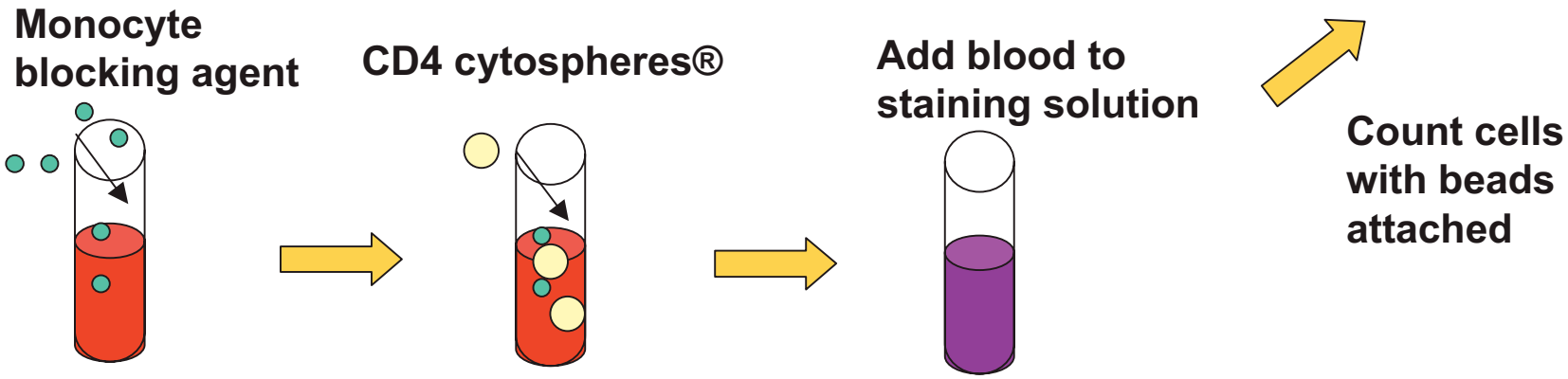
Data from Crowe lab, Burnet Institute Melb and
Dr Bala's lab, YRG Care Chennai
Arlene Darmanie, Cecile Goddard Vidal, Omah Mooleedhar, Shahir Ali, CAREC

CD4 manual methods

Dynal assay



Coulter assay



Crowe, et al. CID 2003: 37 (suppl 1) S25-35

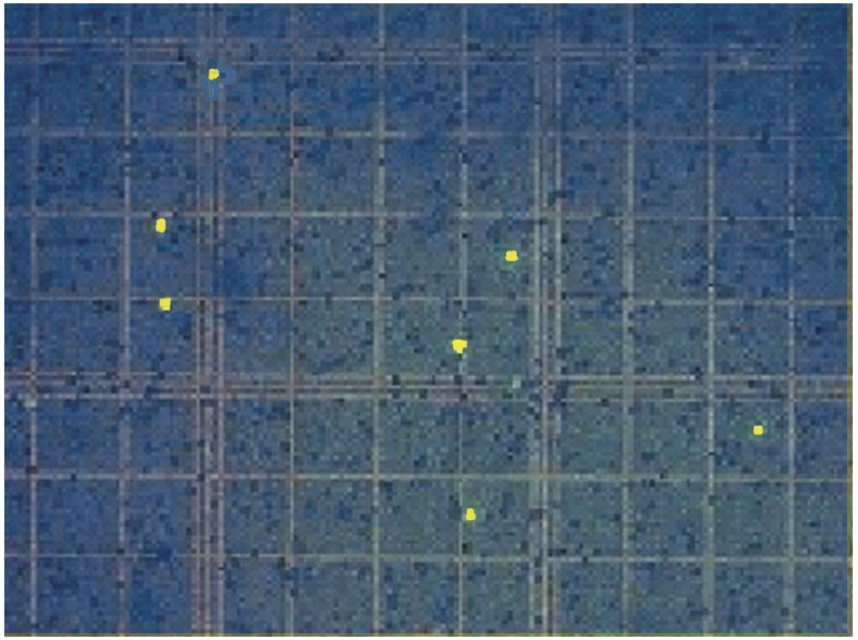
What equipment is needed for these manual CD4 assays?

- **Microscope with 40x objective**
- **Hemocytometer 0.1 mm deep**
- **Manual counter**
- **Tubes**
- **Pipettes**

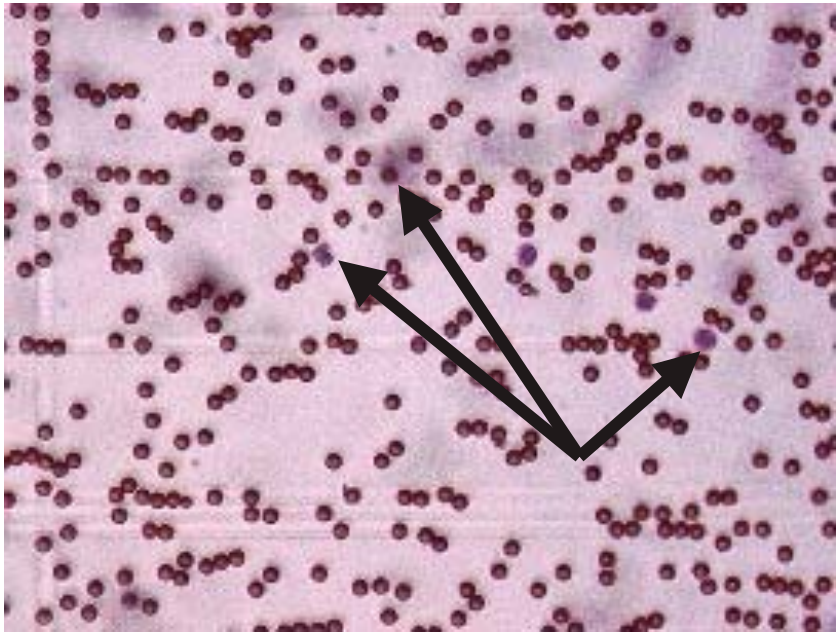
➢ **Plus rotating wheel and magnet for Dynal assay**

Dynal assay
Counting by fluorescence vs light microscopy

Fluorescence

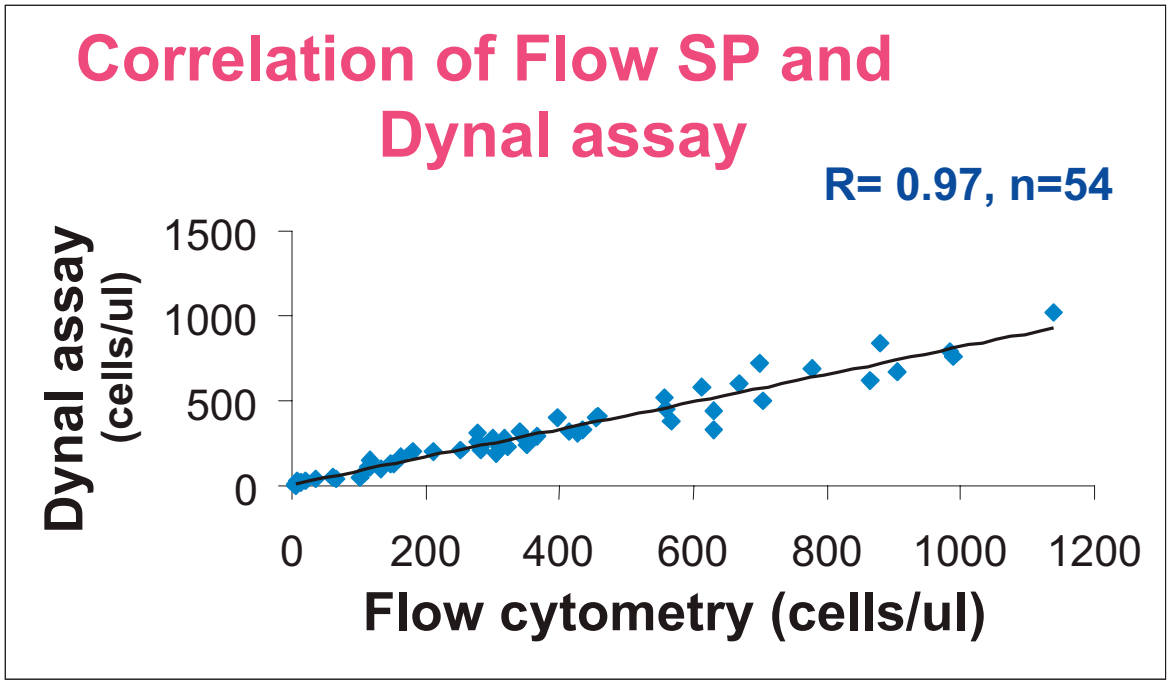


Light

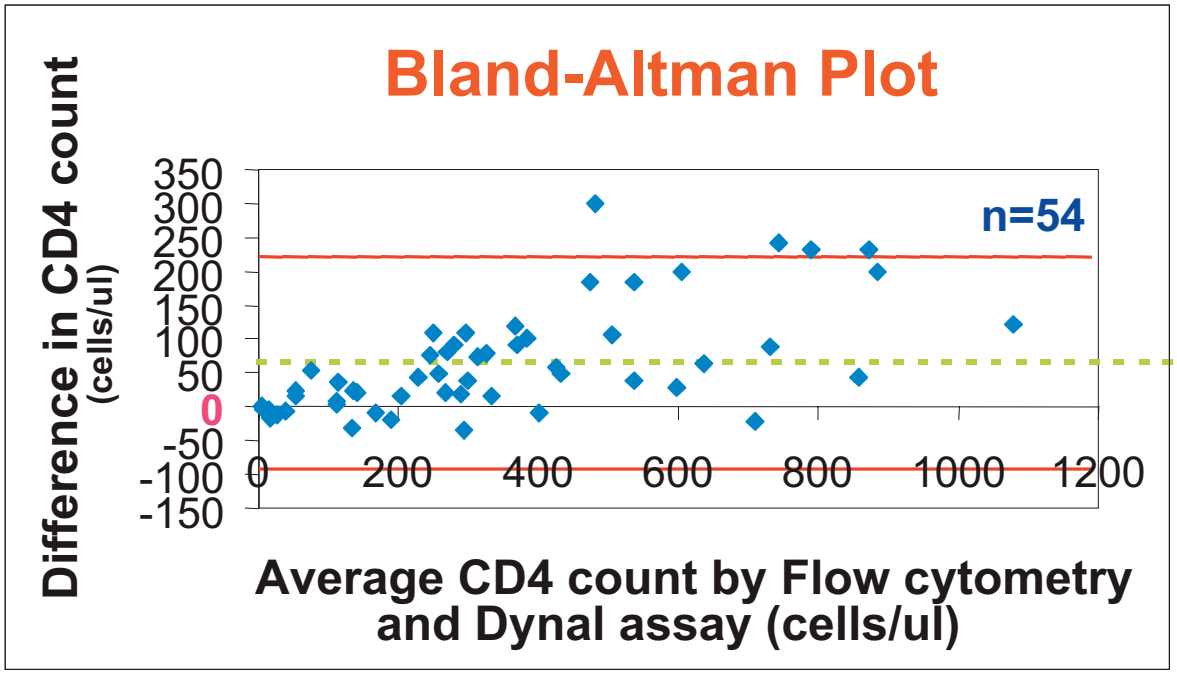


No significant difference

Crowe lab 2003



Dynal assay shows excellent association with flow cytometry

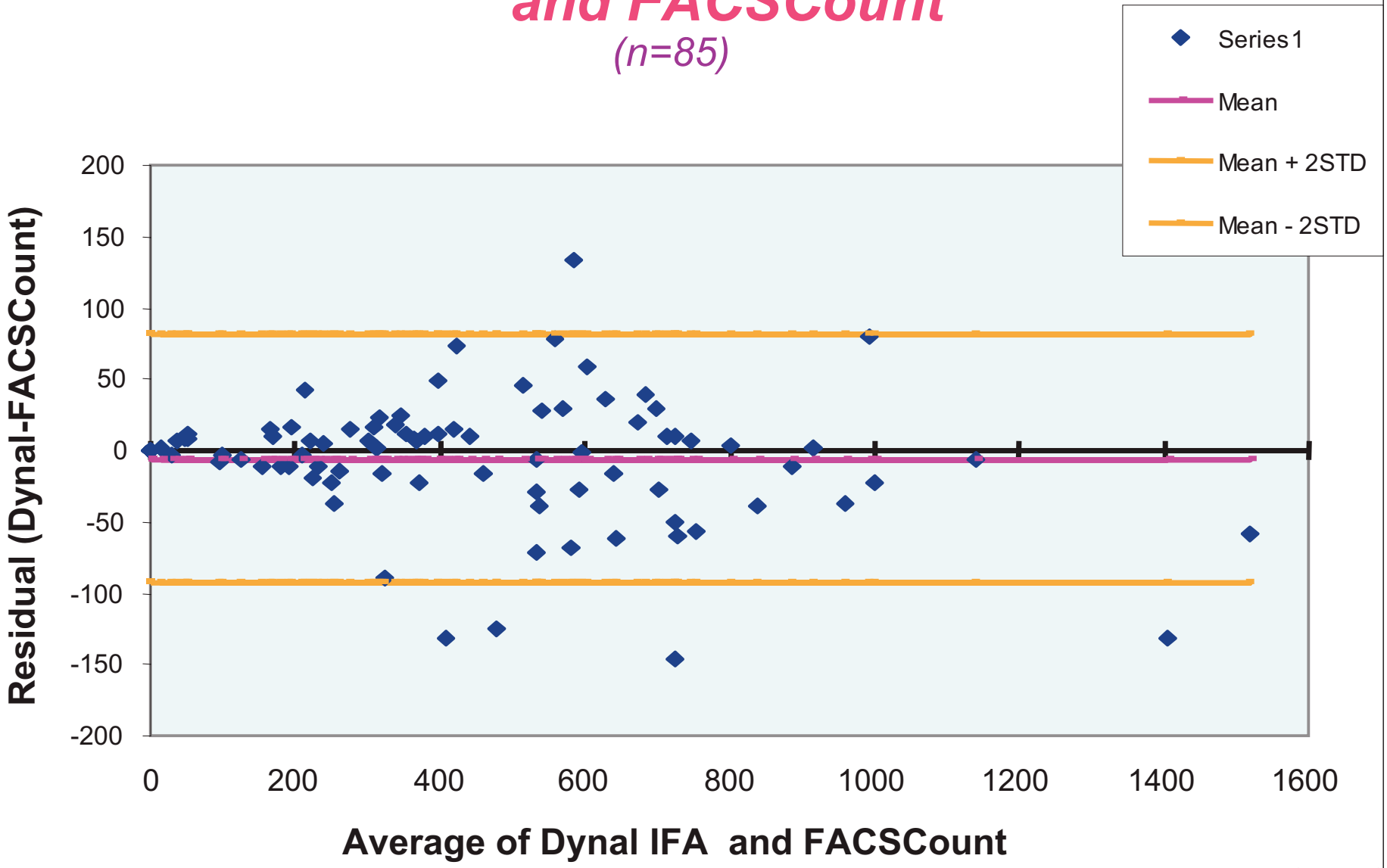


Average Flow cytometry result is 65 cells/µl higher than Dynal assay

Crowe lab 2003

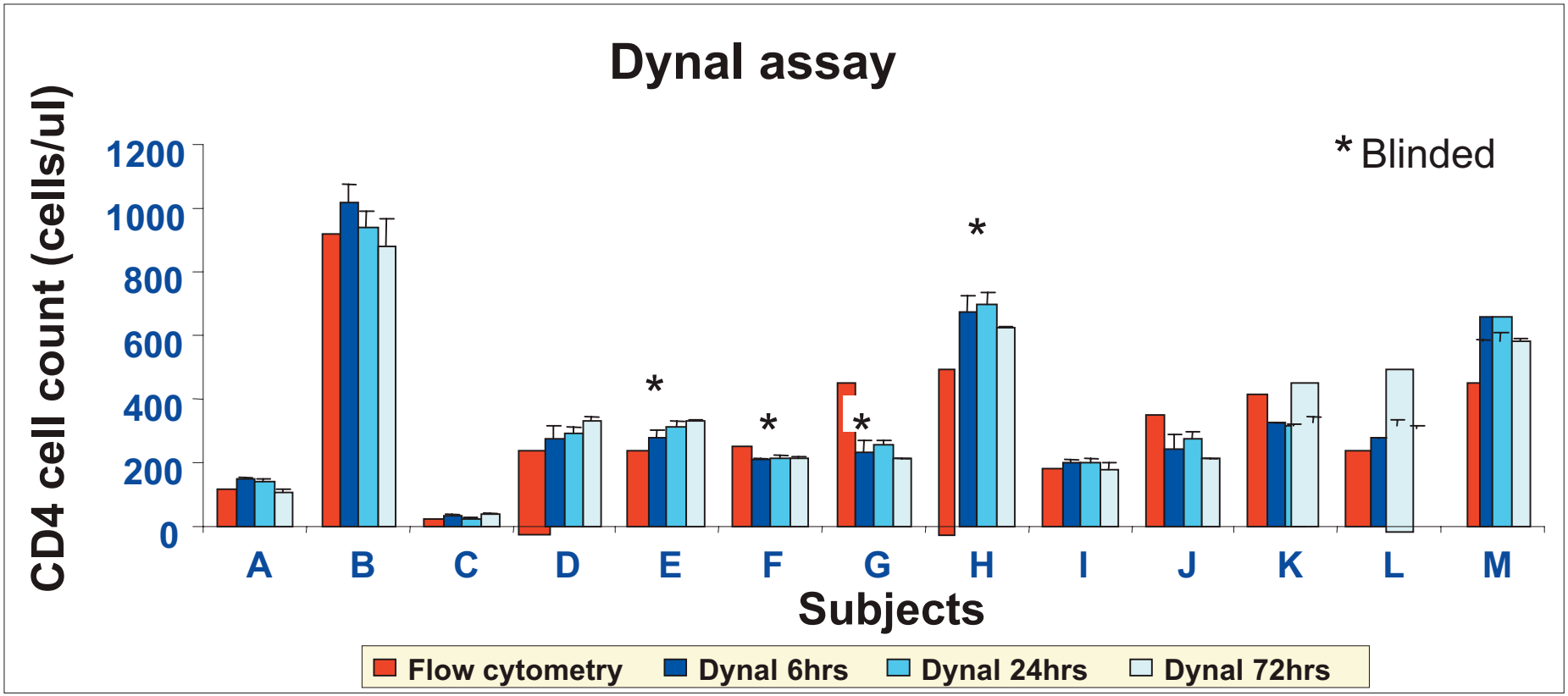
Comparison of CD4 Count between DYNAL and FACSCount

(n=85)



Arlene Darmanie, et al CAREC

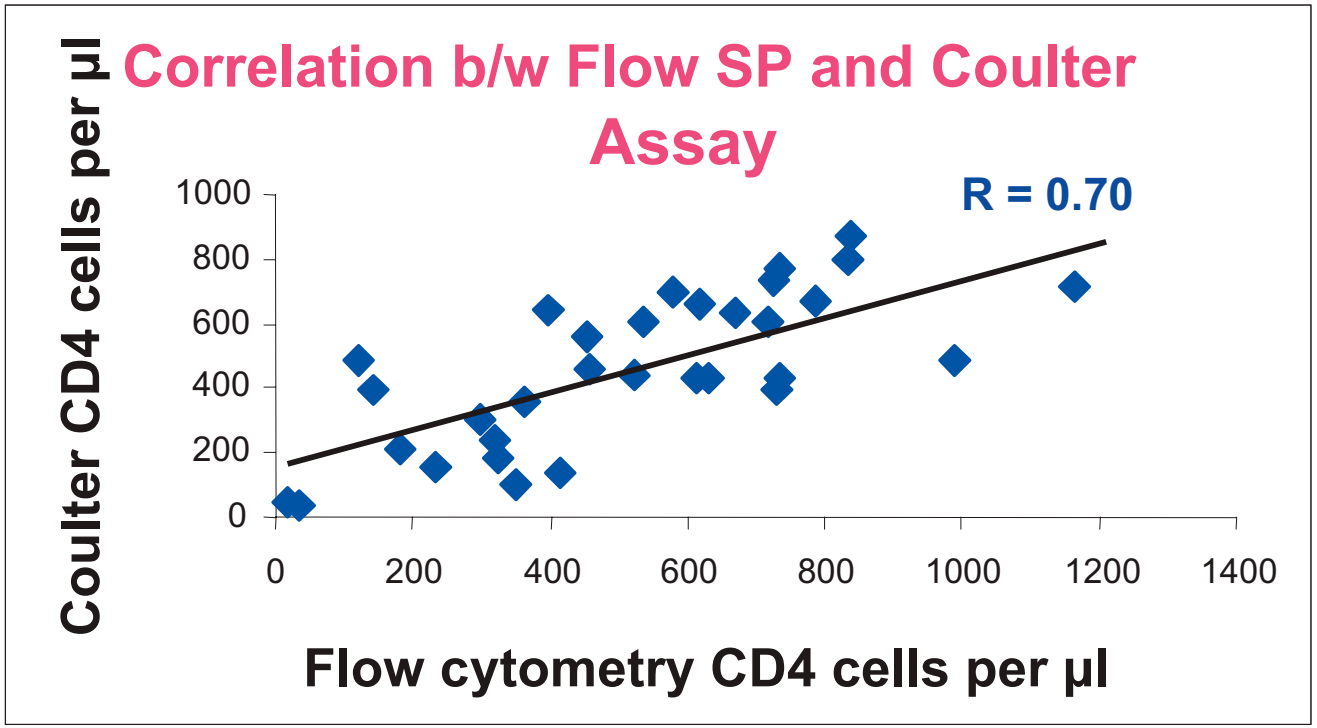
CD4+ T lymphocyte cell counts do not significantly differ over 72 hrs



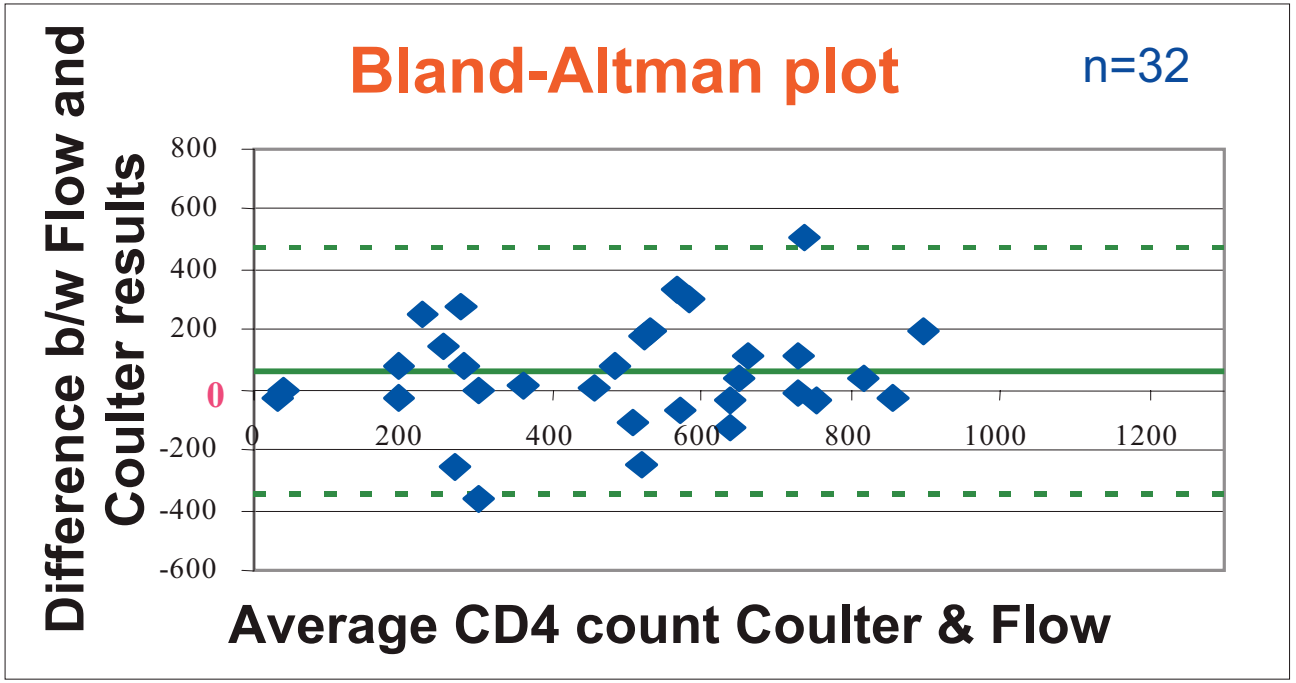
One way repeated measures ANOVA

- no significant difference between testing at different time-points (p=0.202)

Crowe lab 2003



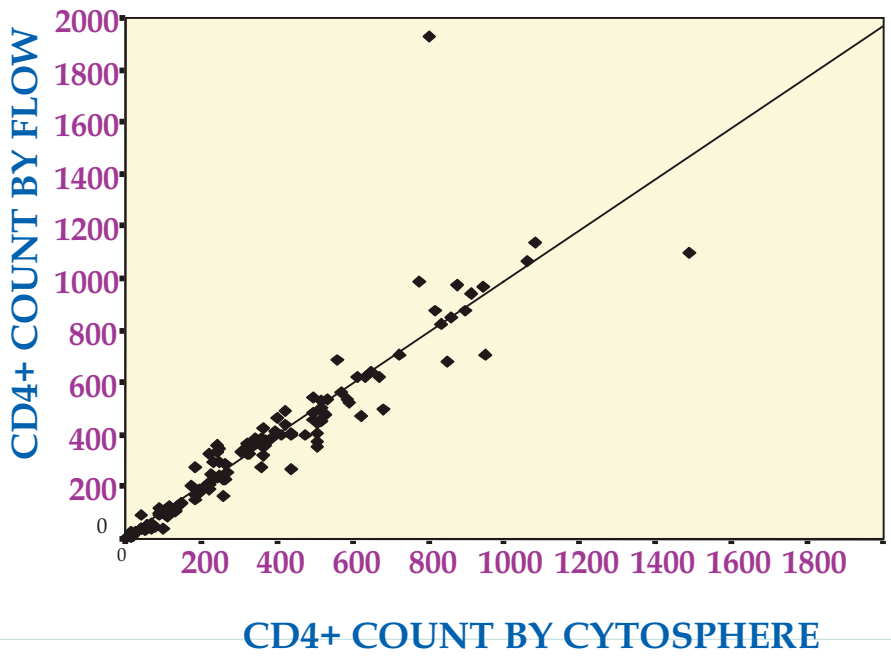
Coulter assay shows a high association with flow cytometry



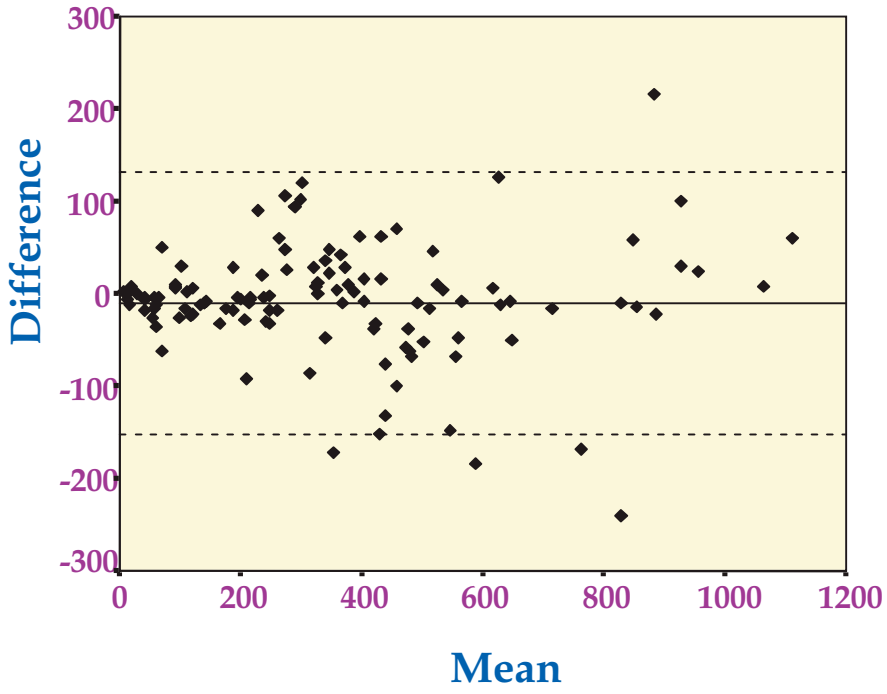
Average Flow cytometry result is 63 cells/µl higher than Coulter assay

Crowe lab 2003

CD4+ T-cell counts by flow cytometry and Coulter assay, YRGCare Chennai



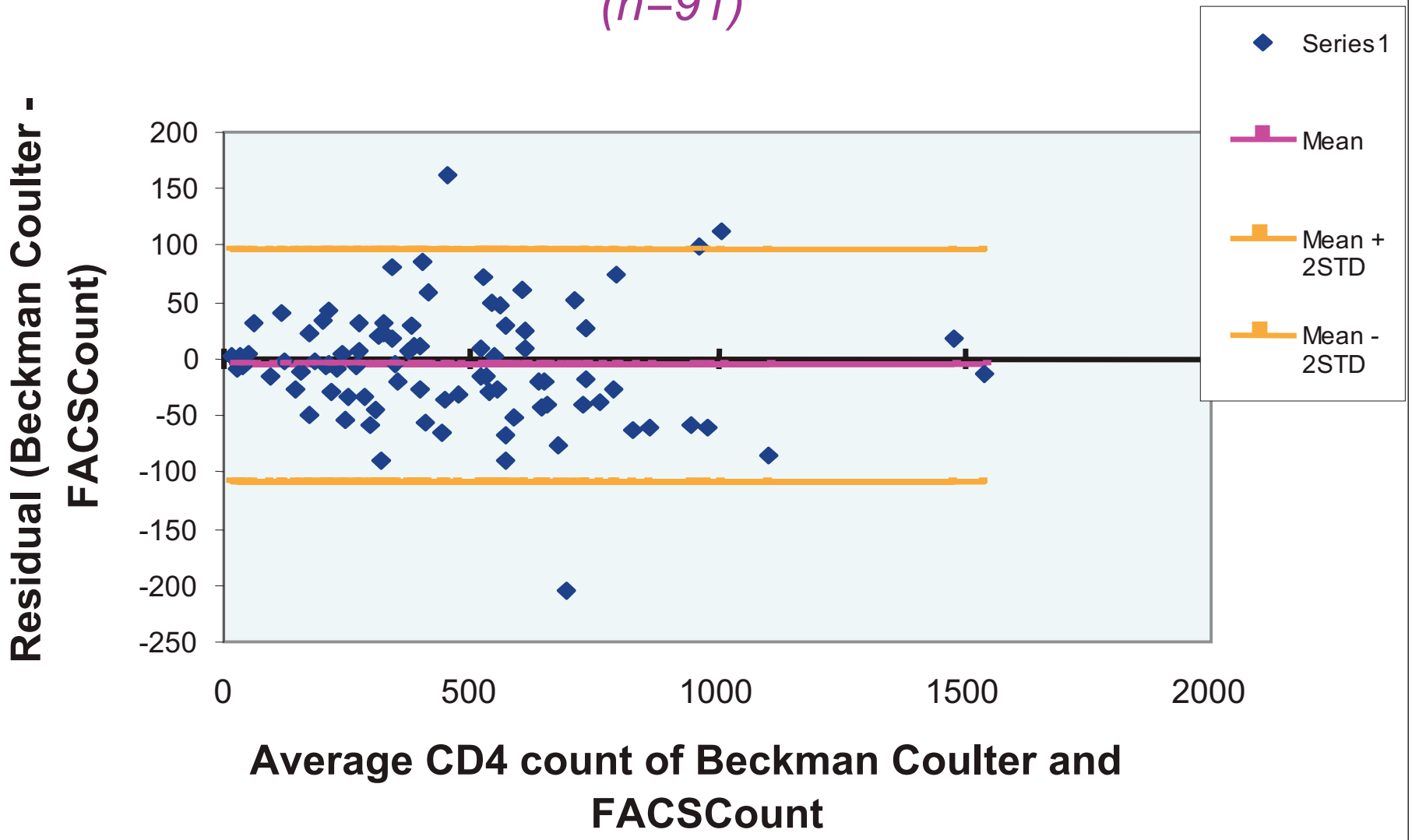
(n=123).



Slide courtesy of Dr Balakrishnan, YRGCare, Chennai

Comparison of CD4 Count between Beckman Coulter Manual and FACSCount

(n=91)



Arlene Darmanie, Cecile Goddard Vidal, Omah Mooleedhar, Shahir Ali, CAREC

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Blood stabilizers

Slides courtesy of Viv Granger and Dave Barnett, NEQAS UK

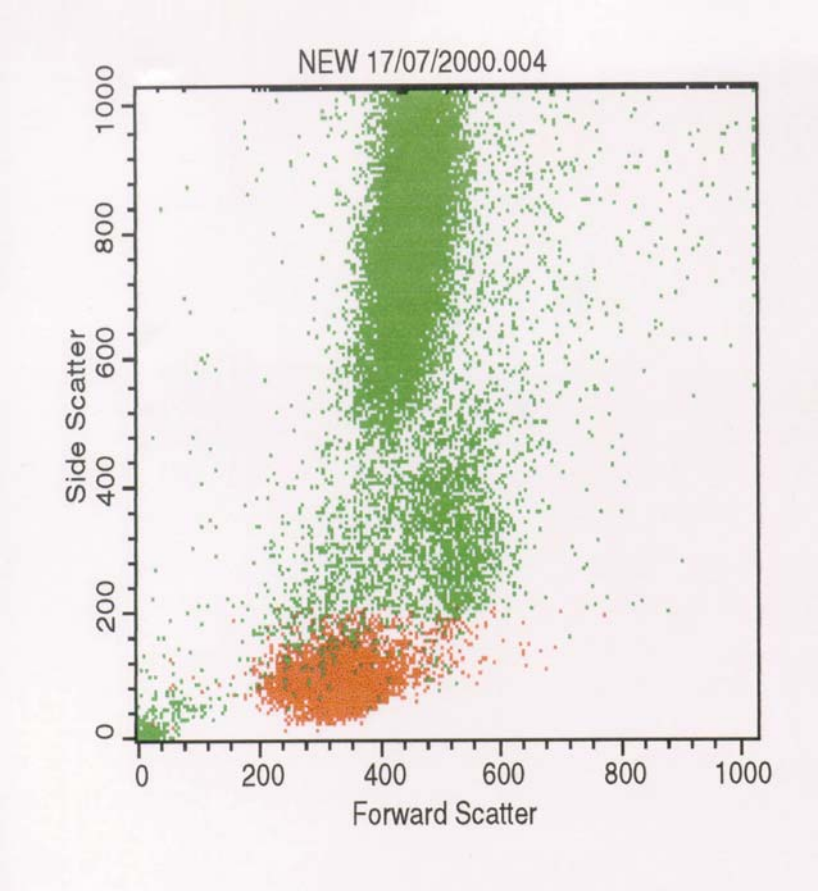
Reagents for stabilizing blood samples

- **Guidelines for $CD4^+$ T lymphocyte counting state that analysis must be complete within 18 hours**
  - Most haematology analysers will have difficulty producing a differential after 24 hours
- **CytoChex™ (Streck laboratories)**
  - **Member of family of non cross-linking fixatives**
  - **Designed to preserve WBCs in whole blood (1:1)**
  - **For up to 7 days at 4^0 C**
- **NEQAS (UK)**
  - **Stabiliser 1 that lasts up to 300 days: good for External QA**
  - **Stabilizer 2 (TransFix™) that lasts >10days, (1:10), <25^0 C**
  - **Termed Transfix because it allows transportation of fixed samples**
- **Both compatible with flow technology**
  - **No data on stabilized blood and manual CD4 counts**

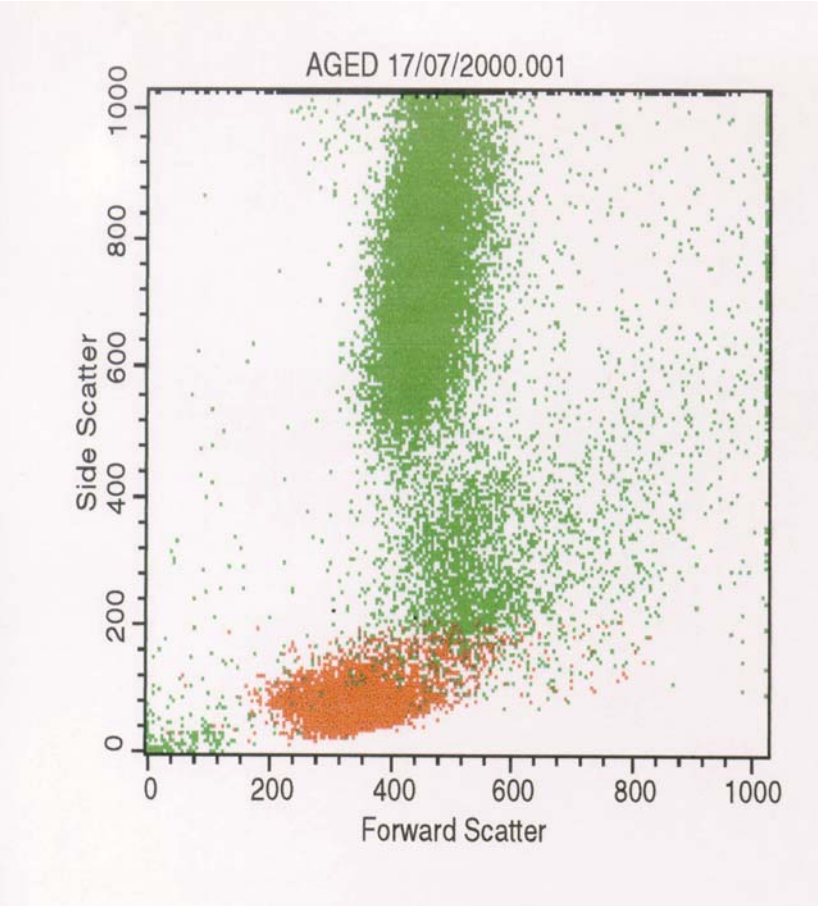
Turpen & Collins Amer Clin Lab 1996 15:30; Barnett et al Cytometry 1996 26:216
Jani et al J Imm Meth 2001 257:145

Flow Cytometric Analysis

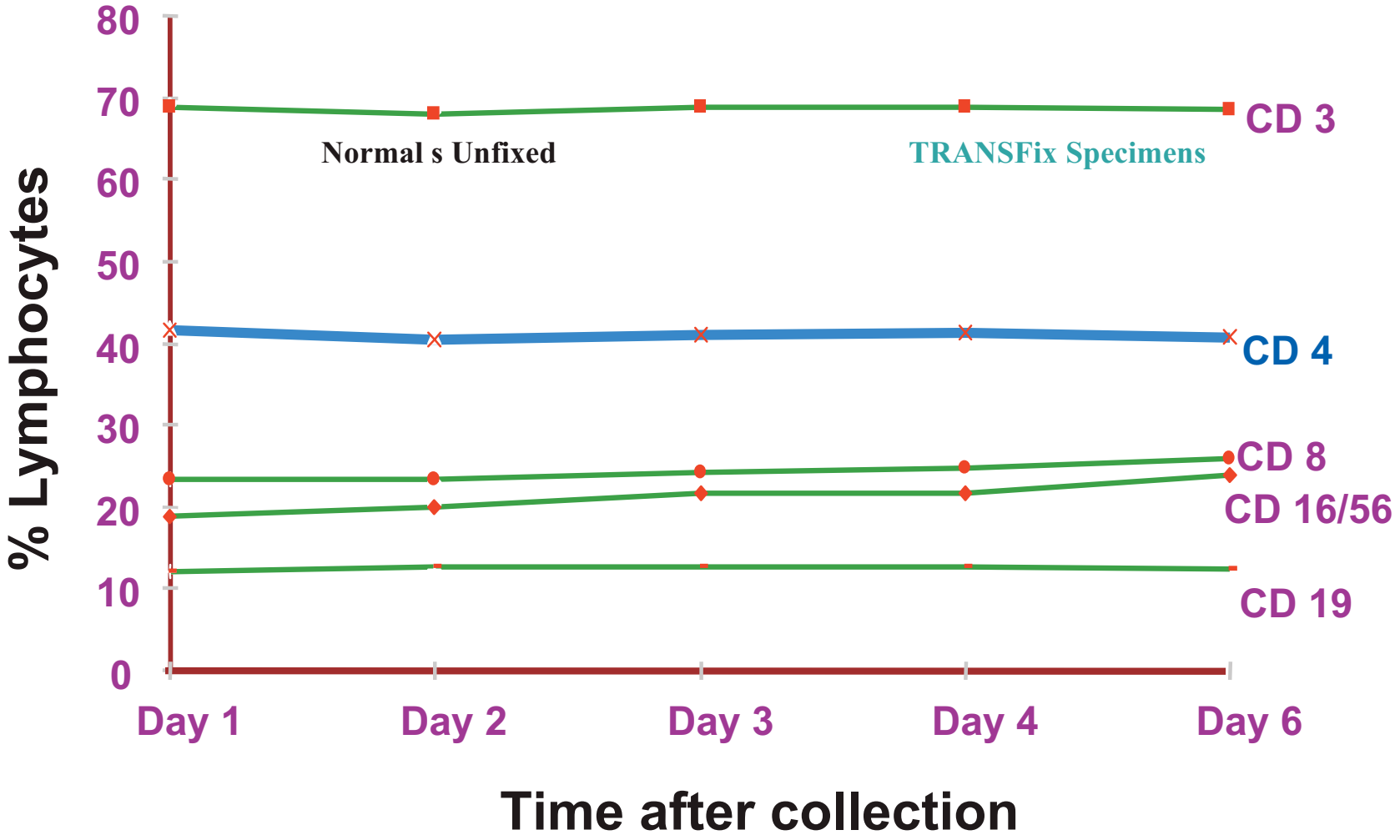
Fresh



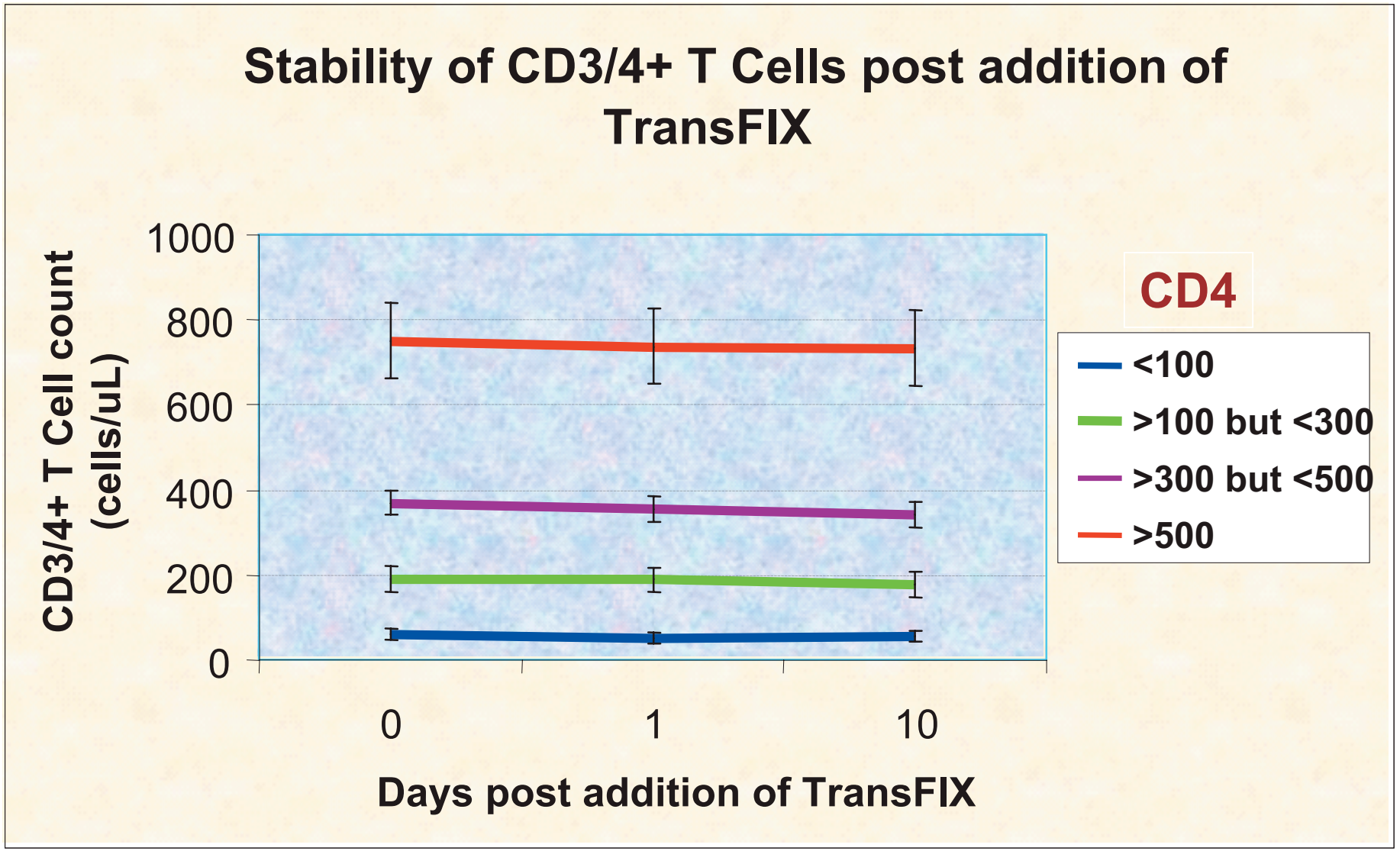
Day 7



Preservation of Lymphocyte Subsets with TransFIX



Stability of CD3/4+ TCells post addition of TransFIX



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Which low-cost CD4 assay to introduce?

Depends on

- Number of samples per day
  - Low throughput, manual may be most cost-effective
  - High throughput, flow method most cost effective (and definitely more practical)
- Sophistication of lab
  - Coulter and Dynal manual assays easy
  - Flow-based assays now relatively easy
- Availability of technical support
  - A key issue for flow methods…needs discussion+++
  - Remote area, opt for manual or ship samples
- Cost
  - More samples/day → lower cost for flow methods
  - Initial cost of flow equipment may be high
- Quality assurance and quality control critically important
- Chosen assay MUST have undergone rigorous comparative analyses in well designed independent studies

Where are we up to?

- All assays/methods are undergoing in-country analyses
- Rigorous independent evaluation required, including large clinical trial evaluation
- Some technologies recently licensed
- None have formal approval…. All are emerging technologies
- QA participation should be part of the deal

Final thanks to

- **Forum for Collaborative Research**
  - Ben Cheng
  - Houtan Movafagh
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