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Review of CD4 technologies

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On behalf of
Members of the HIV Monitoring Technologies Working Group

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Considerations when choosing CD4 technology



Operational characteristics

- Environmental requirements of the technology: instrumentation, power supply, refrigeration of reagents, robustness to heat and humidity
- Parameters measured
- Ease of use, training required, degree of automation
- Cost
- Throughput
- Compatibility with EQA programmes and availability of QC reagents
- Compatibility with stabilised specimens
- Optimum operational characteristics may be different for different levels of healthcare system

Performance

- Accuracy: how similar are the results obtained with the new technology to results of a reference technology? Includes bias and misclassification. May vary with magnitude of result, most important to know the accuracy over the clinically important range and around treatment cut-offs
- Precision: How close are the results of replicate specimens using the same new technology



Available CD4 technology

- Flow cytometry
 - Works on the principle of light scatter (due to different size or granularity of the cell) combined with fluorescence of cells after staining with monoclonal antibodies to cell surface markers tagged to fluorescent dyes
 - Population of interest can be identified and gated
 - Percentage of CD4 T cells can be calculated (% of lymphocytes, or % of leucocytes)
 - Absolute CD4 count can then be determined using either dual- or single-platform methodology



- Dual platform:
 - Uses a haematology analyser-generated wbc count and differential
 - Combining this with the CD4% gives absolute CD4 count
- Single platform:
 - Derive absolute counts directly from flow cytometer without need for haematology analyser
 - Either by counting CD4 T cells in a precisely determined blood volume or by using a known number of fluorescent beads mixed with a known volume of blood



- Single platform may be achieved by modified use of a 'traditional' flow cytometer, by the addition of beads (eg TruCount, FlowCount or Perfect Count beads)
- Alternatively, several dedicated single platform cytometers have been developed eg FACSCount (bead-based), Guava, CyFlow (both volumetric)



Manual methods

- Microscope based
 - Cytospheres: latex spheres coated with monoclonal antibody form rosettes on contact with CD4 cells which are visible by light microscopy
 - Dynabeads: magnetic particles coated with monoclonal antibody allow isolation of CD4 cells, which are lysed and stained nuclei counted using fluorescent microscope



Available CD4 technology

Technology for which independent, peer-reviewed performance evaluation data is available:

Flow cytometry

- Dual platform
- Single platform bead-based technology on standard flow cytometer:
 - TruCount beads
 - FlowCount beads
 - Perfect Count
- Single platform dedicated CD4 flow systems:
 - FACSCount
 - Guava Easy CD4
 - Partec CyFlow Counter, Partec CyFlow SL_3

Manual technologies

- Cytospheres
- Dynabeads

Technology in use but for which no peer-reviewed independent performance evaluation data is available:

- PointCare NOW
- Guava Auto CD4
- CD4 select
- Sysmex poch-100i



Operational characteristics of available CD4 technologies

FLOW CYTOMETRY				
	Dual platform	Bead-based single platform technology on conventional flow cytometer		
Assay name	Various	TruCount beads	FlowCount beads	Perfect Count
Manufacturer	Not applicable	Becton Dickinson	Beckman Coulter	Cytognos
		Compatible with various reagent systems and flow cytometers		
Instrumentation	Flow cytometer plus haematology analyser	Flow cytometer		
Assay principle	Absolute count calculated using results from flow cytometry together with the total wbc count or lymphocyte count from haematology analyser	Absolute CD4 counts determined using ratio of CD4 to a known quantity of fluorescent beads – no need for haematology analyser		
		TruCount tubes contain premeasured quantity of lyophilised beads	Liquid beads need accurate pipetting by operator	Two different bead populations allow detection of inadequate mixing
Parameters measured	Absolute CD4 and CD4% Others depend on reagent kits/methods	Absolute CD4 and CD4%		
Specimen	Whole blood in EDTA	Whole blood in EDTA		
Throughput	Up to 250/day	Up to 250/day		
Compatible with independent EQA/PT programmes?	Yes	Yes	Yes	Yes
Is there access to compatible QC reagent?	Yes	Yes	Yes	Yes
Maximum length of time from blood draw to testing	24 hours	5 days for PLG, otherwise 24 hours		
Does the test perform on fixed/ stabilised blood	Yes	Yes	Yes	Yes
Complexity/training required	Complex. Significant training required.	Complex. Significant training required		
Environmental/energy issues	Requires uninterrupted mains electricity	Requires uninterrupted mains electricity		
Robustness to heat/humidity	Climate control recommended	Climate control recommended		

DEDICATED SINGLE PLATFORM CD4 SYSTEMS

Assay name	FACSCount	Guava Easy CD4	Partec CyFlow Counter or CyFlow SL_3
Manufacturer	Becton Dickinson	Guava Technologies	Partec
Instrumentation	Flow cytometer	Flow cytometer	Flow cytometer
Assay principle	Dedicated single platform bead-based flow cytometer, two colour, no-lyse no-wash	Single platform, two colour, volumetric flow cytometer. Microcapillary flow cell.	Volumetric SP flow cytometer. Up to 3 parameters (2 colour plus SSC). Simplest is single parameter CD4 (CD4 easy count kit). For CD4%, 2 colour (CD45/4) + SSC.
Parameters measured	Absolute CD4, CD8 and CD3 counts, CD4/8 ratio Newer reagents give absolute CD4 count and CD4%	Absolute CD4, CD8,CD3 Easy CD4% assay measures CD4%	Absolute CD4, CD4%
Specimen	50µl whole blood in EDTA	10µl whole blood in EDTA	20µl whole blood in EDTA
Throughput	≥15 pairs per hour, at CD4 counts≥200	100-150 specimens per day	250 tests/day, 400/day with automat
Compatible with independent EQA/PT programmes?	Yes	Yes	Yes
Is there access to compatible QC reagent?	No manufacturer-produced full process control	No manufacturer-produced full process control	No manufacturer-produced full process control
Maximum length of time from blood draw to testing	Stain within 48 hours of draw (24 hours for CD4%) (store at 20-25°C), analyse within 48 hours of staining.	48 hours	48 hours
Does the test perform on fixed/stabilised blood	Yes	Yes	Yes
Complexity/training required	One day training	One day training	Complex, significant training
Environmental/energy issues	Requires uninterrupted mains electricity	Less biohazardous waste (reduced by 100 fold) Requires mains electricity	Mains electricity or car battery or solar panels
Robustness to heat/humidity	10-35°C, 5-95% non-condensing humidity. Reagents require refrigeration.	Instrument: up to 35C. 10-90% non-condensing relative humidity. Reagents need refrigeration.	Solid state laser more stable than water or air cooled gas lasers at high temperatures. Reagents need refrigeration, although dry reagent kits available which can be stored at up to 60°C in dark for up to 12 months

MANUAL METHODS

Assay name	Coulter Manual CD4 Count Kit (Cytospheres)	Dynal T4 Quant Kit (Dynabeads)
Manufacturer	Beckman Coulter	Invitrogen/Dynal biotech
Instrumentation	Light microscope, haemocytometer	Fluorescent or light microscope, haemocytometer, magnet
Assay principle	Inert latex spheres coated with monoclonal Ab form rosettes on contact with CD4 cells - readily visible by light microscopy	Magnetic polystyrene beads, coated with mouse monoclonal Ab, allow isolation of CD4+ T cells followed by counting using fluorescent (preferable) or light microscope
Parameters measured	Absolute CD4 count	Absolute CD4 count
Specimen	100µl whole blood in EDTA	125µl whole blood in EDTA or ACD
Throughput	10 per day. Operator fatigue limiting. Suggest stop counting at 500 cells. Batch size limited to 2 samples	10 per day. Operator fatigue limiting. Suggest stop counting at 500 cells. Batch size limited to 6 samples
Compatible with independent EQA/PT programmes?	No	No
Is there access to compatible QC reagent?	No	No
Maximum length of time from blood draw to testing	72 hours at 20°C	72 hours at 20°C
Does the test perform on fixed/stabilised blood	No	Compatible with Cyto-Chex stabilised refrigerated samples up to 9 days after collection. Not compatible with TransFix.
Complexity/training required	1-3 days training	1-3 days training
Environmental/energy issues	Power for microscope	Power for microscope
Robustness to heat/humidity	Reagents need refrigeration	Reagents need refrigeration

Technologies in use but for which no peer-reviewed independent evaluation data is available				
Assay name	PointCare NOW	Guava Auto CD4	POCH-100	CD4 Select
Manufacturer	PointCare technologies	Guava technologies	Symex	i+MED Laboratories Co. Ltd.
Instrumentation	POC instrument	Flow cytometer	Haematology analyser	Haematology analyser, magnet, rotator, computer
Assay principle	CD4 and haematology contained in one unit. Gold nanoparticles conjugated to CD4 antibodies (no fluorescence). LED light source.	Auto CD4 reagents replacing Easy CD4. Run on same cytometer (Guava PCA)	CD4 cell isolation using Dynabeads, counting using haematology analyser	Ferrous beads coated with MT4 mAb bind to CD4 T cells, which are then removed using magnet. CD4-deplete blood counted on haematology analyser. Software generates count and %
Parameters measured	Absolute CD4, CD4%, Hb, differential white cell count	Absolute CD4 count and CD4%	Absolute CD4 count, CD4%, CBC, 3 part WCC differential	Absolute CD4 and CD4%
Specimen	40µl whole blood in EDTA	10µl whole blood in EDTA	Whole blood in EDTA	400µl whole blood in EDTA
Throughput	50/day	100/day	12 per hour	?
Compatible with independent EQA/PT programmes?	No	Yes	No EQA evaluation data available	No EQA evaluation data available
Is there access to compatible QC reagent?	No data available	No data available	No data available	No data available
Maximum length of time from blood draw to testing	8 hours	48 hours	24 hours at 4 or 20°C	No data available
Does the test perform on fixed/stabilised blood	No data available	Manufacturer report reliable results with Transfix up to 11 days	No data available	No data available
Complexity/training required	Fully automated. Intended for POC use	Two days training	Moderate complexity	
Environmental/energy issues	Mains or battery power	Mains electricity required, UPS option	Power for haematology analyser	Power for haem analyser, rotator and computer
Robustness to heat/humidity	Reagents do not need refrigeration. Instrument: 18-34C, <80% relative humidity, non-condensing	Instrument: up to 35C. 10-90% non-condensing relative humidity. Reagents need refrigeration.	No data available	No data available



Performance

Accuracy

- No gold standard technology or internationally recognised reference preparation exists for CD4. This makes it hard to obtain the ‘truthful’ result against which to compare a new methodology
- Correlation alone is insufficient
- Bland-Altman plot alone (with or without “limits of agreement”) is insufficient
- Misclassification probabilities provide more clinically useful information about the test under evaluation
- Two types of misclassification can be defined — upward misclassification probability and downward misclassification probability.
- Upward misclassification around a treatment threshold may be most clinically important (leading to delay of start of ART or prophylactic treatment in some patients).
- Downward misclassification may result in the decision to treat large numbers of additional patients who have CD4 counts above the guideline threshold when using the reference test.



Performance cont.

Precision

- Reproducibility of the new test when repeated on the same specimen.
Includes within-run, between-run, between-operator, between-laboratory
- Usually measured as coefficient of variation (CV)
- Badly underestimated if based on too few replicates

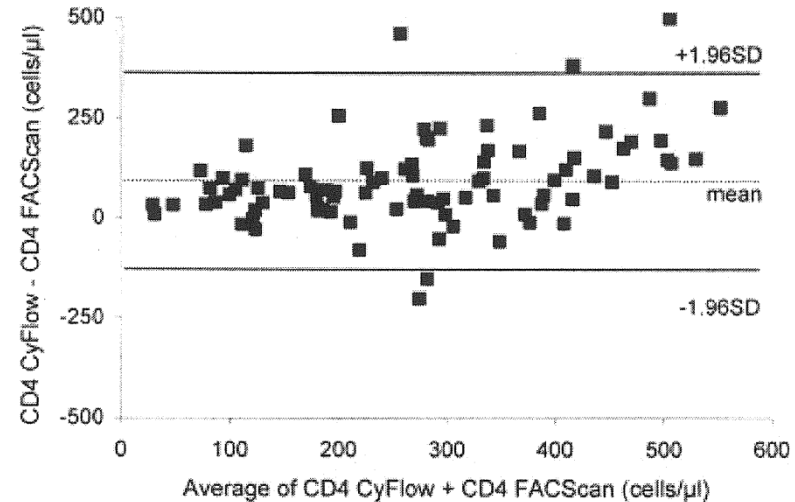
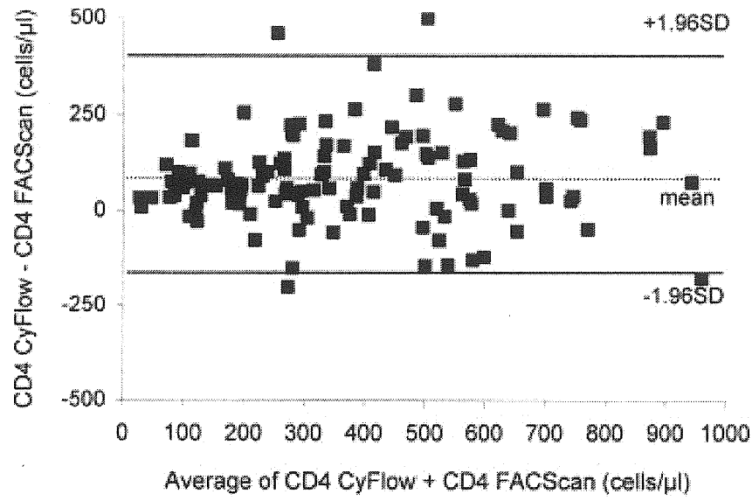
- In evaluating a particular technology, we need to know about both its accuracy and its precision



Literature review of performance of CD4 technologies

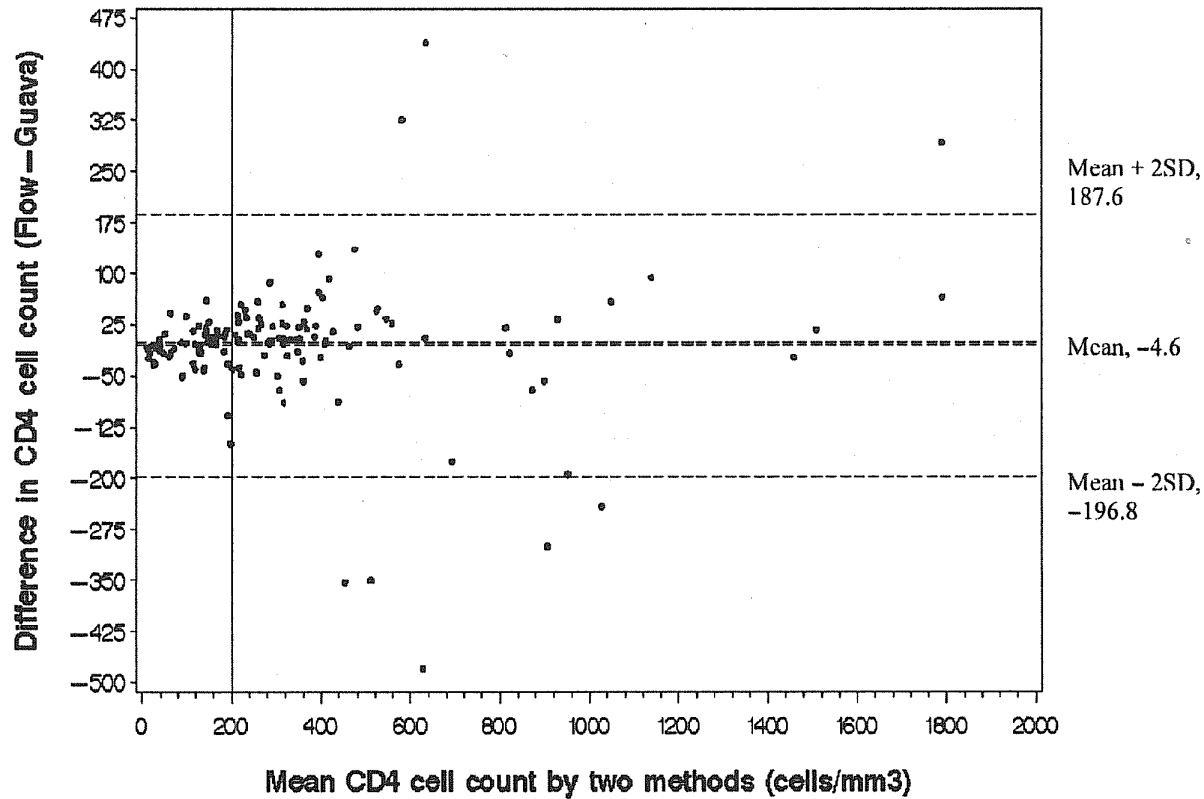


- Systematic review underway
- What is already clear is that clinically relevant questions are difficult to answer from literature
- Studies often conclude that a method is an acceptable alternative to a reference method based on correlation alone, or based on a 'mean difference' between the two, which gives no indication of maximum differences seen (which may be large, despite a small mean difference), and which is often different at different levels of CD4, even within the clinically important range



From Karcher et al 2006 Cytometry
Part B 70B:163-169

On correlation analysis, $r=0.929$
However, 29% of specimens with $CD4 < 350$ using FACScan
misclassified as > 350 when using CyFlow



Mean difference minimal (4 cells/ μ l).
But maximum differences large (-500 to + 400)

From Spacek et al. J Acquir
Immune Defic Syndr Vol
41, 5, 2006

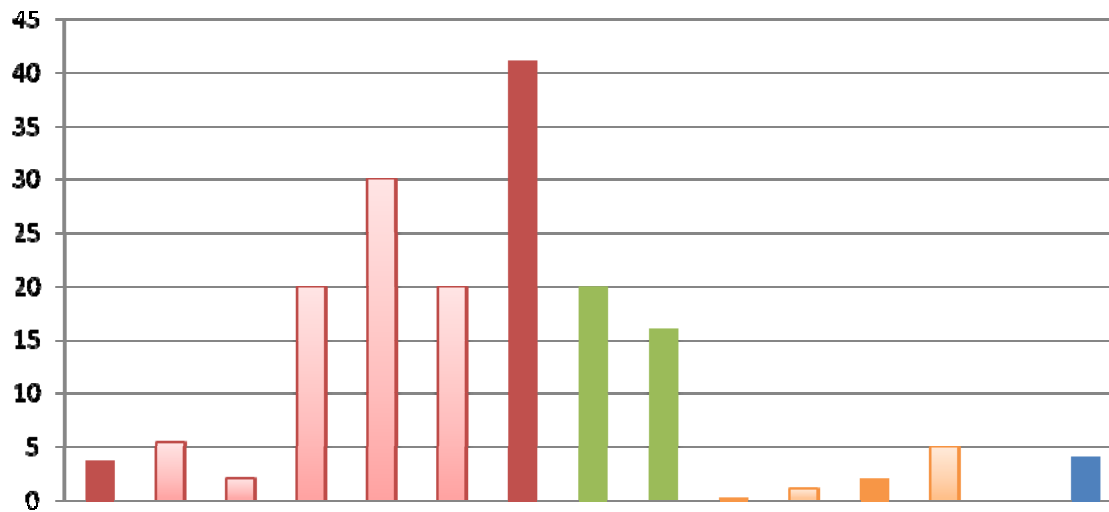
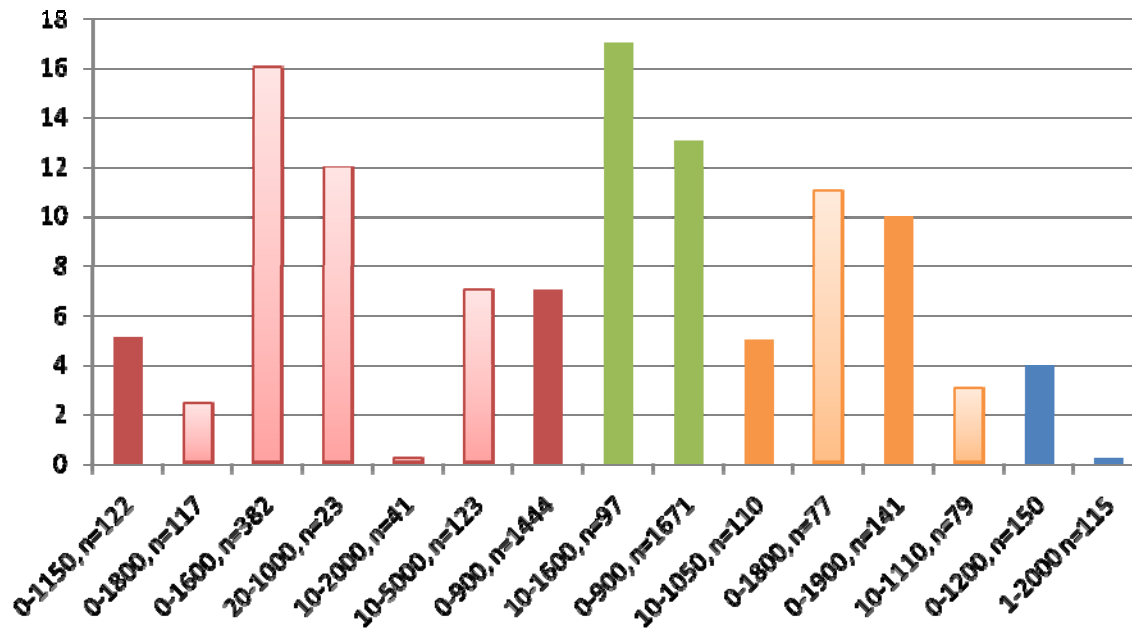


Does a particular method have ‘acceptably low’ misclassification probabilities? i.e. can I be confident that >350 is really >350

- 31 studies, 15 gave data from which can calculate misclassification either side of 200, and only 5 provided data which allowed calculation of misclassification either side of 350



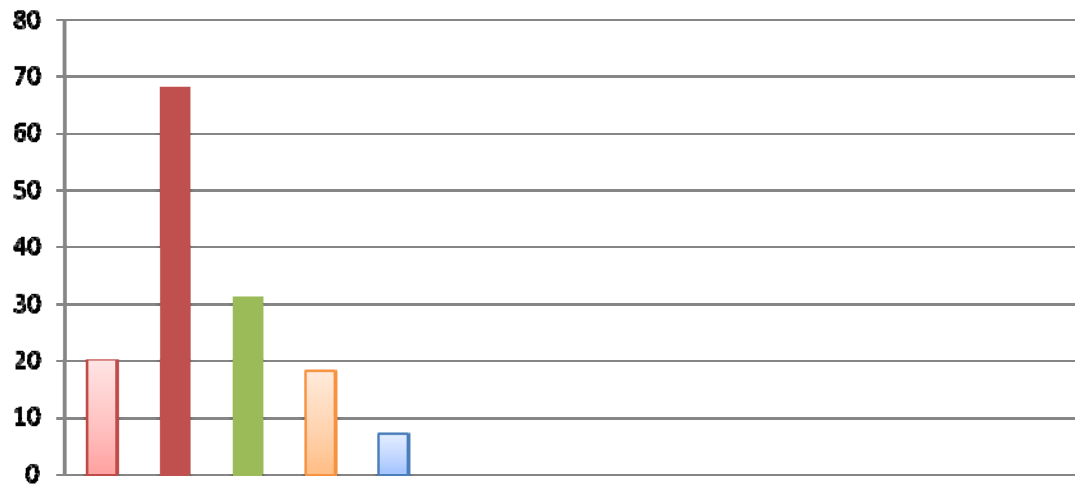
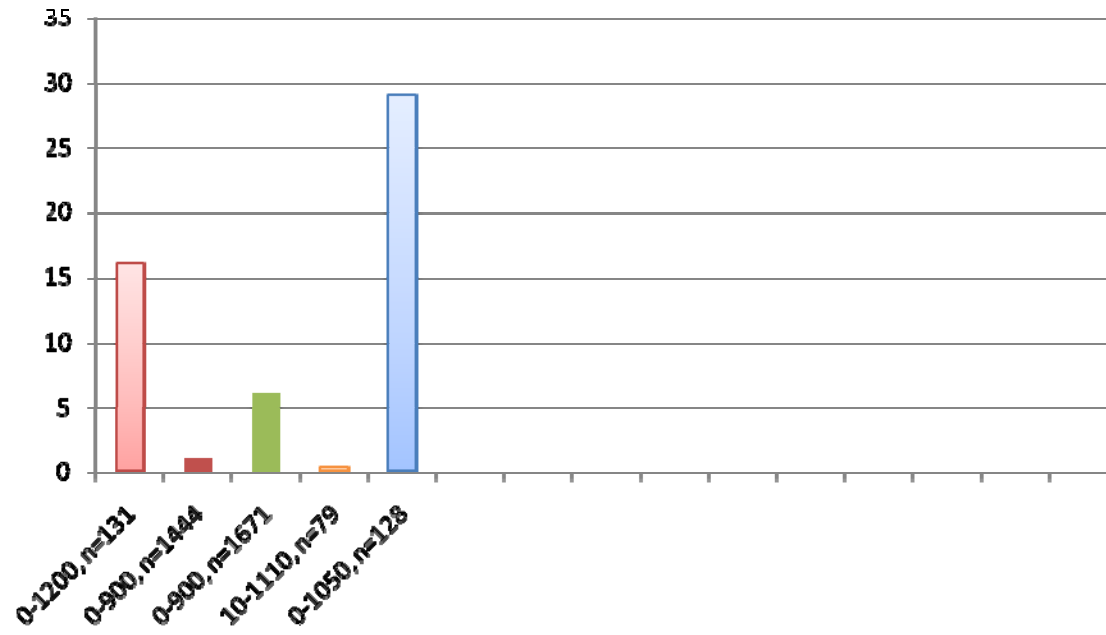
- Cytospheres
- Dynabeads
- Guava
- CyFlow



Misclassification up (upper figure) and down (lower figure), using a threshold of 200 cells/ μ l



- Cytospheres
- Dynabeads
- Guava
- CyFlow



Misclassification up (upper figure) and down (lower figure), using a threshold of 350 cells/ μ l.



- Both misclassification up and down are likely to be underestimates (particularly down) as none of the studies are restricted to the most clinically relevant range
- We cannot tell from the published papers the magnitude of the misclassification: are they mostly barely away from the threshold (e.g. 10 CD4 cells) or are they mostly far away (e.g. 100 CD4 cells)?
- A more pertinent question might be how many samples in the 150 - 250 range are being misclassified as having $CD4 > 350$, or how many samples in the 450-550 range are being misclassified as < 350
- But this is impossible to answer from the published literature (although authors likely to have primary data from which these could be calculated)



Precision

- Reproducibility on repeat testing of same sample by same method
- Important if following a patient's serial measurements
- Probability of misclassification is worse if precision is worse, although bias of 10% has more of an effect on misclassification than CV (measure of precision) of 10%



Method	Study	CD4 if given	No. of replicates per donor	Within-lab whole-process reproducibility on whole blood (CV%)	
				Index test	Flow cytometry
Cytospheres	1	200	?10	11	2.5
	2	Not given	?2	58	6
Dynabeads	3	Not given	2	8	8
	4	350	10	8	2



Given the limitations of available data, what can we say with any degree of confidence?

- There is variability associated with CD4 measurement, both physiological and technology-related, whichever technology is used
- Different technologies are associated with different performance characteristics, both in terms of misclassification and precision
- These characteristics, particularly misclassification, should be considered before choosing to implement a technology, but the data are not always available
- Given the potential for error, participation in EQA programmes and access to QC reagents is essential



- Hierarchy of technologies based on performance levels:

Single platform flow cytometry > dual platform

--doesn't rely on hematology analyzer, so less variability, especially with older blood specimens

Dual platform >> Manual methods

--lower misclassification probabilities, better precision, availability of EQA materials and programmes

Difficult to place Guava and Cyflow in hierarchy

--limited data on misclassification. Widely varying results with CyFlow in different papers, and wide variety of instruments and reagents make papers difficult to compare



Essential operational characteristics for CD4 and viral load technologies at different levels of the health care system

Desirable characteristics for viral load technologies in the clinical management of patients with HIV			
	At tertiary/reference lab level	At district level	At primary level
Investigations	Measure RNA HIV 1 (2 if relevant to location), RT	Measure RT or send plasma or DBS away for RNA testing	Collect DBS
Performance criteria	Reliably 1000 or less copies/ml or RT equivalence	Reliably 1000 or less copies/ml or RT equivalence	
Method	PCR or RT-ELISA	RT-ELISA	Send DBS or POC when available
Specimen type	Whole blood (EDTA not heparin), plasma or dried blood spot	Whole blood (EDTA not heparin), plasma or dried blood spot	Whole blood finger or heel prick
Specimen volume: adult	Up to 1.2ml plasma depending on method	1ml	50-75 ul
Specimen volume: paediatric	Preferably <0.5ml	Up to 1ml	50-75 ul
Time to result	Within 2 weeks*	Within 2 weeks*	Within 3-6 weeks
Further analysis of data required? Eg multiplication	Maybe required	No	No
Throughput	Medium or high (at least 30 per two weeks)	Medium or high (at least 30 per two weeks)	Variable
Complexity	May be high	Medium	Low
Training required	Dedicated training essential	Dedicated training essential	Training essential
Environmental/energy issues	Requires uninterrupted mains electricity, may require climate control	Requires uninterrupted mains electricity, may require climate control	None
Storage requirements of reagents	Requires freezer and fridge	Requires freezer and fridge	None
Storage requirements of specimens	Requires freezer and fridge	Requires freezer and fridge	Requires dessicant
Maintenance/machine calibration	Essential	Yes	No
Service after the sale	Essential	Yes	No
Additional equipment/facilities required	Requires sufficient space for PCR suite	ELISA reader, incubator	No
Availability of QC reagents	Must be available but not necessarily from manufacturer	Must be available but not necessarily from manufacturer	No
Participation in manufacturer-independent QA programme	Essential for all platforms	Essential for all platforms	Regular auditing
How does the test perform on aged specimens and what is upper limit of storage time before testing	Specimens should be processed within 24 hours then plasma may be frozen	Specimens should be processed within 24 hours then plasma may be frozen	Thoroughly dried then stored with desiccant prior to shipping

* except in PMTCT services where more urgent results might be required

Desirable characteristics for CD4 technologies in the clinical management of patients with HIV			
	At tertiary/reference laboratory level	At district level	At primary level
Instrumentation	Flow cytometry	Flow cytometry or microscope	POC - variable
Parameters measured	Both absolute count and CD4%	Both absolute count and CD4%	Absolute CD4 as a minimum (qualitative or quantitative)
Performance criteria	?	?	?
Method	Single (preferable) or dual platform flow cytometer	Single (preferable) or dual platform flow cytometer or manual method	POC when available
Automation	Yes	Not essential	No
Specimen type	Whole blood	Whole blood	Whole blood
Specimen volume: adult	Volume should equal at least the minimum volume of EDTA blood collection tube	Volume should equal at least the minimum volume of EDTA blood collection tube	Finger prick
Specimen volume: paediatric	Volume should equal at least the minimum volume of EDTA blood collection tube	Volume should equal at least the minimum volume of EDTA blood collection tube	Finger prick/heel prick
Time to result	Same day	Same day	Less than 1 hour
Further analysis of data required? Eg multiplication	Optional	Optional	None
Throughput	High: >50 per day	Medium: 10-50 per day	Low-medium: 1-20 per day
Complexity	High	Medium	Low
Ease of use/training required	May be sophisticated, with supervision required and significant training. May require dedicated laboratory and technical expertise	Moderate, with minimal supervision required after training. Does require laboratory and technical expertise	Simple, with no supervision required after minimal training (<4 hours). Does not require laboratory or technical expertise
Environmental/energy issues	Requires uninterrupted mains electricity, climate control recommended	Runs on battery, climate control recommended	Runs on battery or solar power or no need for electricity
Storage requirements of reagents	May require fridge and freezer	May require fridge	No cold chain requirements
Storage requirements of specimens	Ambient temperature 10-35C	Ambient temperature 10-30C	Samples should be run immediately
Machine maintenance	Essential 6 monthly	Essential 6 monthly for flow, microscopes should be maintained	Variable
Machine calibration	Daily	Preferably daily	
Service after the sale	Essential	Essential for flow	Required if instrumentation involved
Equipment/facilities required	Standard laboratory equipment including computer	Standard laboratory equipment including computer	Variable
Availability of QC reagents	Essential	Essential	Essential
Participation in manufacturer-independent QA programme	Essential	Essential	Essential
Upper limit of storage before testing	Within 24 hours (possibly up to 5 days with PLG)	Within 24 hours	Immediate
Transport requirements if samples transferred to another location for testing	Ambient temperature less than 35C or with stabilising agent	Ambient temperature less than 35C or with stabilising agent where assay compatible	NA
Can fixed specimens be used?	Yes	Yes depending on technology	Not determined



What is 'acceptable performance'?

- What constitutes 'acceptable' performance remains undefined— how much under- or over-treatment is acceptable, and is this different at different levels of the healthcare system?
- It is easy for a site to decide which method they prefer based on operational characteristics (cost, stated training time, desired throughput, etc. - information provided by the companies)
- It is harder for sites to decide what performance levels they desire, and even harder to obtain data on performance features such as misclassification probabilities
- What misclassification probabilities are acceptable to a site may depend on the characteristics of asymptomatic, therapy naive patients who need a therapeutic decision at that site, as well as what other decisions are being made on the basis CD4 testing at that site