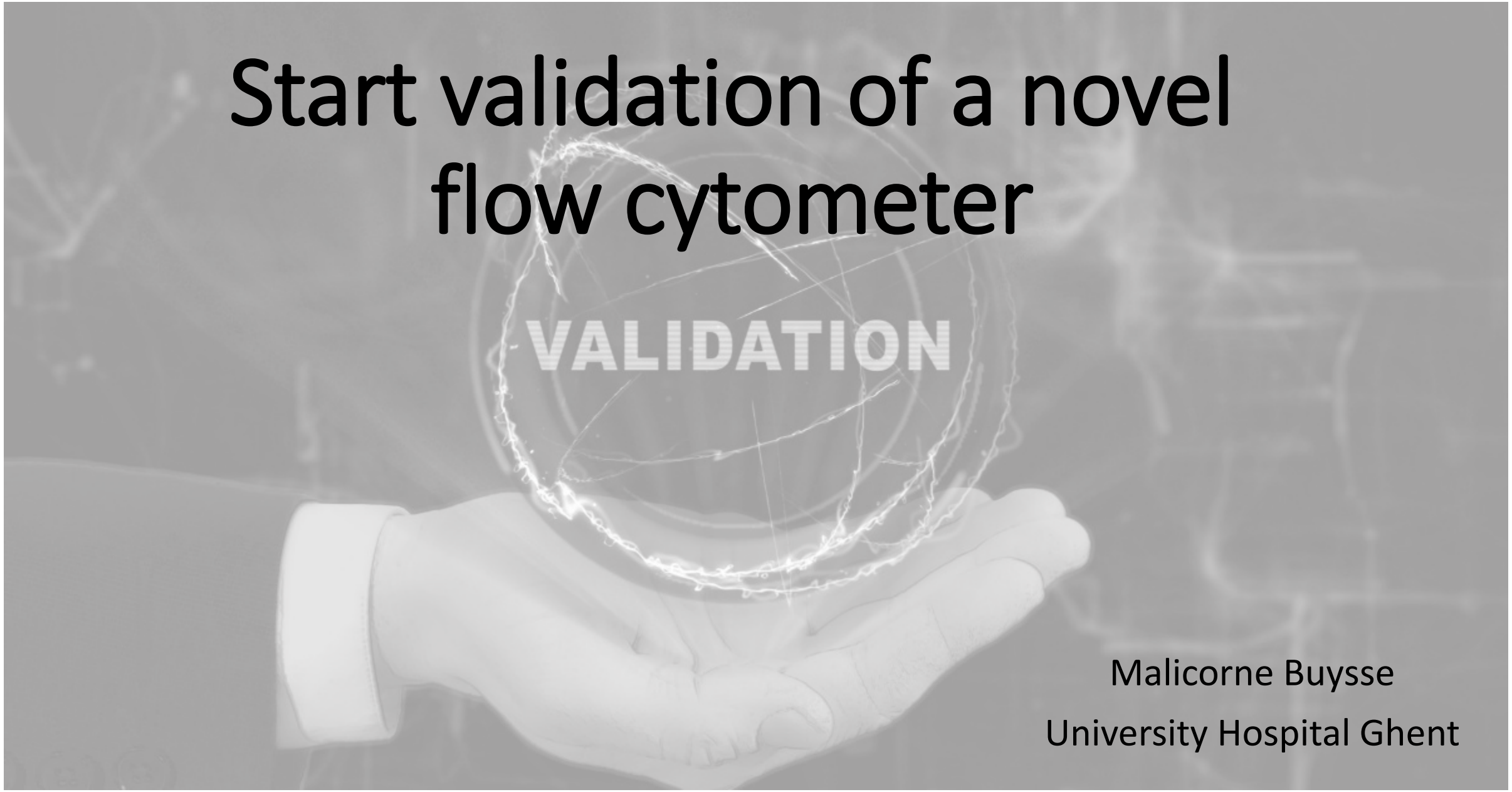
Start validation of a novel flow cytometer



Malicorne Buysse

University Hospital Ghent

How to participate?

Copy participation link

1 Go to wooclap.com

2 Enter the event code in the top banner

Event code
JCCAYN

Enable answers by SMS

- Introduction
  - Validation-related challenges
  - References, publications
  - CLSI H62 guideline 2021
  - Get to know your instrument
  - Acceptance criteria

- Instument validation plan for a novel flow cytometer (for example: FACS LyricTM)
  - Instrument validation parameters
  - Extended PQ parameters
  - Method validation parameters
  - (Addenda)

- Q&A

- Introduction
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- Q&A

Validation-related challenges for flowcytometry

“Flow cytometers are complex, flexible instruments, with unique validation needs”

Green et al. 2011

https://app.wooclap.com/events/JCCAYN/questions/65c3c5b21c4abb96ee44640f

Validation-related challenges for flowcytometry

- **Almost no reference materials /standards** available (data is **not derived from a calibration curve)**
- Data is **often qualitative and semi-quantitative:**
  - e.g. most populations are expressed as percentages in relation to a reference population
  - Quantitive analysis is possible by the use of calibrated beads (cfr. internal standard)

    in combination with the results of the cell counters
  - Challenging to assess linearity and accuracy
- Stability of the samples is an important parameter of **variability**
- Difficulty in obtaining **samples with variation in expression levels → creative thinking!**
- Limited sample volume available for testing
- Cellular measurands → existing guidance for quantifying soluble analytes is not fully applicable

References

Selliah, N., Eck, S., Green, C., Oldaker, T., Stewart, J., Vitaliti, A., & Litwin, V. (2019). Flow cytometry method validation protocols. *Current Protocols in Cytometry*, *87*, e53. doi: 10.1002/cpcy.53

Cytometry Part B (Clinical Cytometry) 84B:315–323 (2013)

Validation of Cell-based Fluorescence Assays: Practice Guidelines from the ICSH and ICCS – Part V – Assay Performance Criteria

Brent Wood,[1] Dragan Jevremovic,[2] Marie C. Béné,[3] Ming Yan,[4] Patrick Jacobs,[5] Virginia Litwin[6]*; on behalf of ICSH/ICCS Working Group[1]

Best practices in performing flow cytometry in a regulated environment: feedback from experience within the European Bioanalysis Forum

Barry van der Strate*,[1], Robin Longdin[2], Marie Geerlings[1], Nora Bachmayer[3], Maria Cavallin[4], Virginia Litwin[5], Minesh Patel[6], Wilfried Passe-Coutrin[7], Corinna Schoelch[8], Arjen Companjen[3] & Marianne Scheel Fjording[9]

Characterization of Flow Cytometer Instrument Sensitivity

Contributed by Robert A. Hoffman and James C.S. Wood

Current Protocols in Cytometry (2007) 1.20.1-1.20.18

The evolution of guidelines for the validation of flow cytometric methods

L. DU*, A. GROVER†, S. RAMANAN*, V. LITWIN†

Evaluation and validation of a novel 10-color flow cytometer

Lin Sun[1] | Hui Wu[1] | Baishen Pan[1] | Beili Wang[1] | Wei Guo[1,2,3]

Validation of a flow cytometry-based method to quantify viable lymphocyte subtypes in fresh and cryopreserved hematopoietic cellular products

Bechara Mfarrej*, Julie Gaude, Jerome Couquiaud, Boris Calmels, Christian Chabannon, Claude Lemarie

Validation and quality control of immunophenotyping in clinical flow cytometry

Marilyn A. Owens[a,*], Horacio G. Vall[a], Anne A. Hurley[b], Susan B. Wormsley[c]

Determination of Optimal Replicate Number for Validation of Imprecision Using Fluorescence Cell-Based Assays: Proposed Practical Method

Bruce H. Davis,[1*] Christine E. McLaren,[2] Anthony J. Carcio,[1] Linda Wong,[1] Benjamin D. Hedley,[3] Mike Keeney,[3] Adam Curtis,[1] and Naomi B. Culp[1]

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

Guideline on bioanalytical method validation

Accurate and reproducible enumeration of T-, B-, and NK lymphocytes using the BD FACSLyric 10-color system: A multisite clinical evaluation

Imelda Omana-Zapata[1☯*], Caren Mutschmann[2☯], John Schmitz[3☯], Sarah Gibson[4☯], Kevin Judge[1☯], Monika Aruda Indig[5☯], Beverly Lu[1☯], Doreen Taufman[2‡], Alan M. Sanfilippo[3¤‡], Wendy Shallenberger[4‡], Sharon Graminske[5‡], Rachel McLean[6‡], Rubal I. Hsen[6‡], Nicole d'Empaire[7‡], Kimberly Dean[1‡], Maurice O'Gorman[6☯]

High-sensitivity flow cytometric assays: Considerations for design control and analytical validation for identification of Rare events

Ulrike Sommer[1] | Steven Eck[2] | Laura Marszalek[3] | Jennifer J. Stewart[4] | Jolene Bradford[5] | Thomas W. McCloskey[6] | Cherie Green[7] | Alessandra Vitaliti[1] | Teri Oldaker[8] | Virginia Litwin[9]

Expanding NIST Calibration of Fluorescent Microspheres for Flow Cytometry to More Fluorescence Channels and Smaller Particles

Paul DeRose [1,*], Linhua Tian [1], Elzafir Elsheikh [1], Aaron Urbas [2], Yu-Zhong Zhang [3] and Lili Wang [1]

2020 White Paper on Recent Issues in Bioanalysis: BAV Guidance, CLSI H62, Biotherapeutics Stability, Parallelism Testing, CyTOF and Regulatory Feedback (Part 2A – Recommendations on Biotherapeutics Stability, PK LBA Regulated Bioanalysis, Biomarkers Assays, Cytometry Validation & Innovation Part 2B – Regulatory Agencies' Inputs on Bioanalysis, Biomarkers, Immunogenicity, Gene & Cell Therapy and Vaccine)

Susan Spitz[†,1], Yan Zhang[†,2], Sally Fischer[†,3], Kristina McGuire[†12], Ulrike Sommer[†,¶,4],

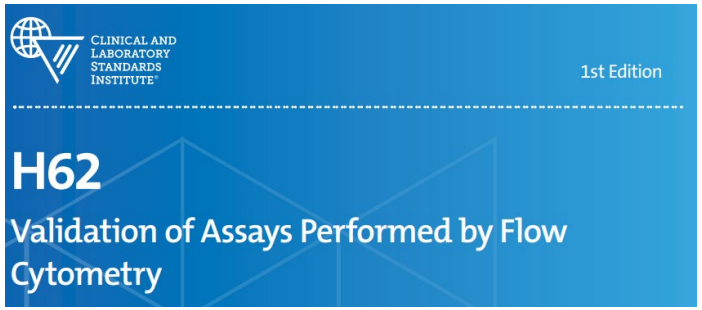
Recommendations for the validation of flow cytometric testing during drug development: I instrumentation

Cherie L. Green [a,*], Lynette Brown [b], Jennifer J. Stewart [b], Yuanxin Xu [c], Virginia Litwin [d], Thomas W. Mc Closkey [e]

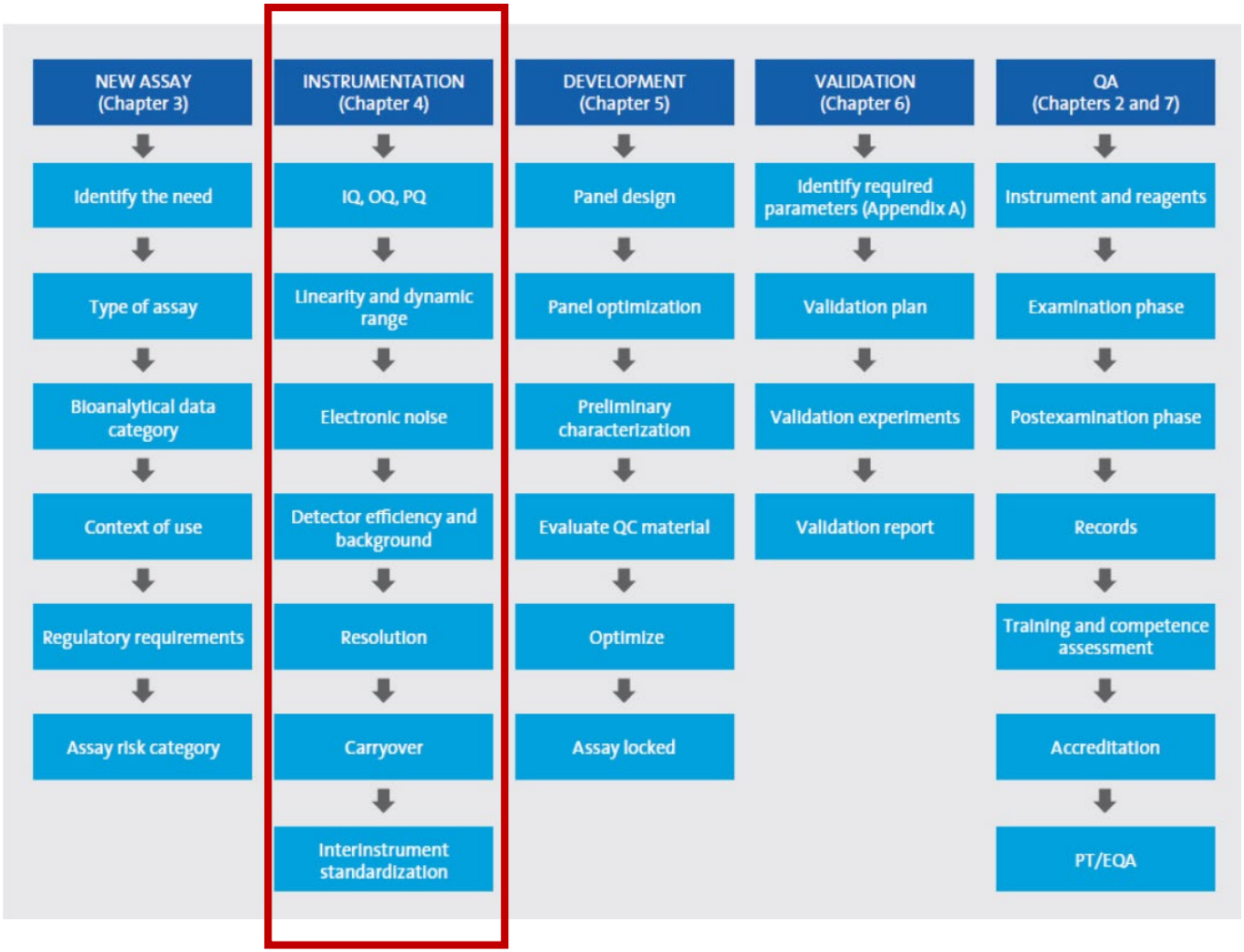
Flow Cytometry Method Validation Protocols

Nithianandan Selliah,[1] Veronica Nash,[1] Steven Eck,[2] Cherie Green,[3] Teri Oldaker,[4] Jennifer Stewart,[5] Alessandra Vitaliti,[6] and Virginia Litwin[7,8]

CLSI H62 Guideline 1st edition 2021

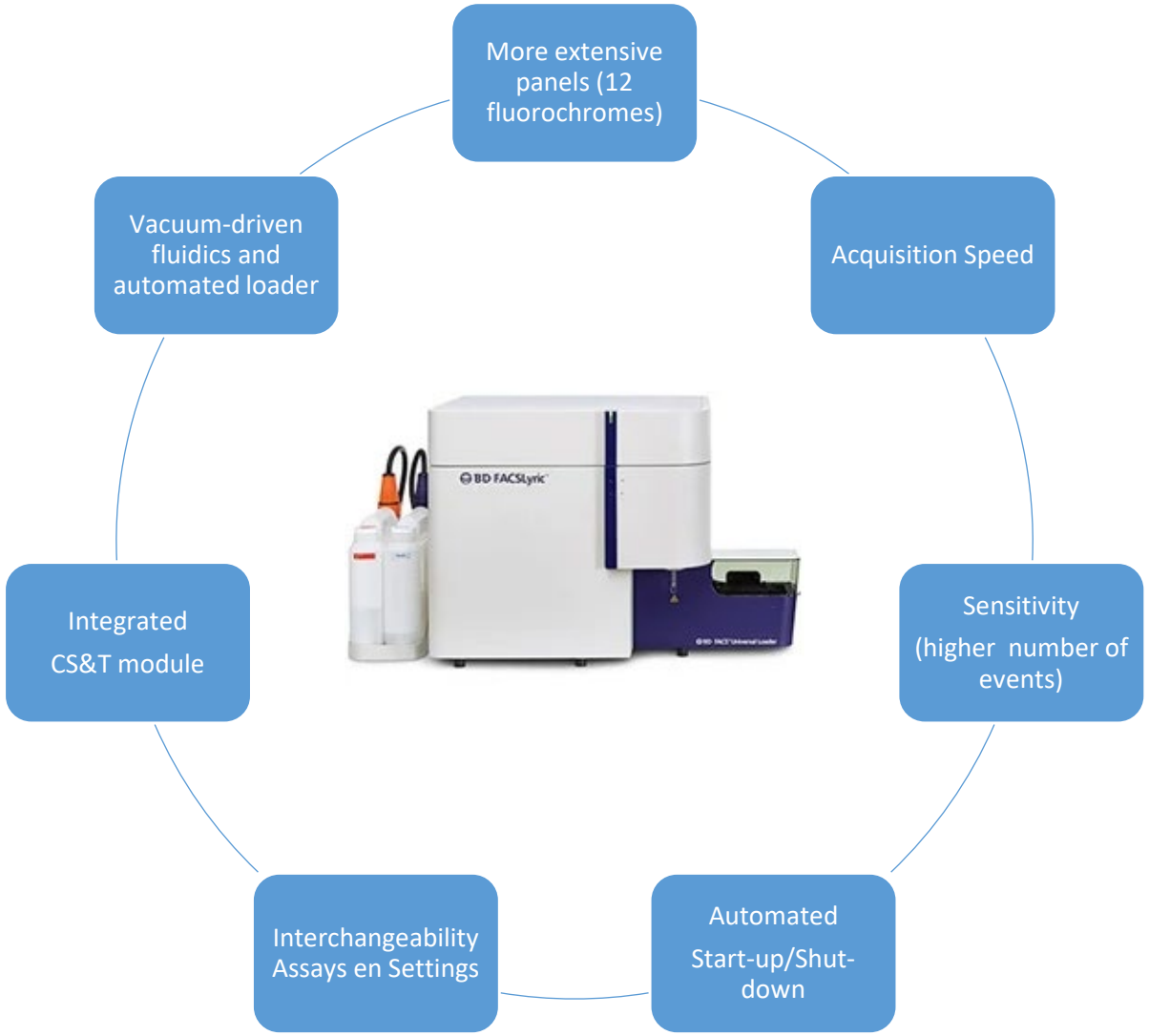


Practical recommendations for:
- research facilities
- manufacturers
- biopharmaceutical companies
- clinical/medical laboratories



Chapter 4 Instrument qualification

Get to know your instrument



"Instrument validation starts with adequate knowledge of the instrument and its intended use"

Du et al. 2015

Acceptance criteria

Based on:

1) Literature

Instrument validation:

Flow Cytometer Performance Characterization, Standardization, and Control

Lili Wang and Robert A. Hoffman

+

Cytometry Part B (Clinical Cytometry) 84B:291–308 (2013)

Validation of Cell-based Fluorescence Assays: Practice Guidelines from the ICSH and ICCS – Part III – Analytical Issues

Shabnam Tangri,[1*] Horacio Vall[2] David Kaplan,[3] Bob Hoffman,[4] Norman Purvis,[5] Anna Porwit,[6] Ben Hunsberger,[7] T. Vincent Shankey[8]; on behalf of ICSH/ICCS Working Group[1]

+

CLSI H62 Guideline → Chapter 4

Method validation:

Flow Cytometry Method Validation Protocols

Nithianandan Selliah,[1] Veronica Nash,[1] Steven Eck,[2] Cherie Green,[3] Teri Oldaker,[4] Jennifer Stewart,[5] Alessandra Vitaliti,[6] and Virginia Litwin[7,8]

+

CLSI H62 Guideline → Chapter 6 + Appendix A

Framework, based on H62/White papers
Protocol 3 addresses the type of validation performed in ***clinical laboratories*** *for moderate-risk tests* ***developed in house***

Minimal requirements on:
When to validate, How to validate, How to make calculations?
Type of validation Samples, time points, replicate number, n° of instruments, replicates, runs, operators, statistics…
and **clear acceptance criteria/templates**

Acceptance criteria

Based on:

2) Vendor specifications :

- device
- CS&T beads
- calibration beads
- reference standards (MultiCheck/Stem cell control/ Immuno-TROL..)

3) Expert opinion

→ Only as an additional criteria, or in case no other specifications exist

→ Justify the reason!

4) No acceptence criteria possible

→ Validation parameter is informative only (for ex. LOB)

BD CS&T Beads

50 Tests—Catalog No. 656504
150 Tests—Catalog No. 656505

Table 2 Accuracy of cytometer setup using BD® CS&T Beads

Parameter	Bright bead MFI		% Difference
	Target	Actual	
FSC	17,997	17,998	0.01
SSC	126,535	126,107	–0.34
FITC	5,477	5,479	0.04
PE	12,877	12,873	–0.03
PerCP-Cy5.5	15,574	15,622	0.31
PE-Cy7	11,883	11,867	–0.13
APC	41,354	41,370	0.04
APC-R700	28,455	28,486	0.11
APC-Cy7	60,190	60,386	0.33
V450	6,140	6,158	0.29
V500-C	24,442	24,356	–0.35
BV605	6,457	6,465	0.12
BV711	38,812	39,014	0.52
BV786	75,550	75,556	0.01

Performance

Acquisition rate
Up to 35,000 events per second
No limit on number of events acquired in a single FCS file

Carryover
<0.10% with default SIT flush
<0.05% with 3 or more SIT flushes

Sensitivity
FITC: <85 MESF
PE: <20 MESF

Channel Qr (x1,000)

FITC	20
PE	133
PerCP-Cy™5.5	13
PE-Cy™7	17
APC	10
BD Horizon™ APC-R700	8
APC-Cy7	7
BD Horizon™ V450	47
BD Horizon™ V500	17
BD Horizon™ BV605	133
BD Horizon™ BV711	43
BD Horizon™ BV786	16

Fluorescence precision
<3% CV for chicken erythrocyte nuclei (CEN)

Fluorescence linearity
2 ±0.05% for CEN

Data resolution
Uncompensated data has a range of 0–262,143

SSC and FSC resolution
Enables separation of 0.2-µm beads from noise

System throughput
Absolute count, multicolor assay example: ≤50 minutes for a 40-tube rack

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  - Method validation parameters
  - (Addenda)

- Q&A

Instrument validation plan

Installation Qualification (IQ)

= Verification of correct

- Space
- Temperat
- Electrical
- Hardware/software

Operational Qualification (OQ)

= Verification that the system

- Optical precision
- Precision of automated loader

Performance Qualification (PQ)

= Verification that the

- Carry-over

(+ extended PQ)

For IVD instrument systems: the vendor dictates the IQ, OQ, and PQ:

*"Performance monitoring for **IVD instruments** should be conducted according to vendor specifications using either bead-based or stabilized cellular control material."*

CLSI H62 guideline

Certificate of conformance

Checklists/Screenshots

Using multi-intensity beads/cells
Part of integrated software (CS&T)

Validation Plan: example

Instrument Validation (PQ)	Extended PQ (~ intended use)	Method validation	Addenda
Linearity	Acquisition speed	Bias	Software calculations
Dynamic Range	Storage capacity	Imprecision	Workflow manager
(Light scatter) Sensitivity	Light scatter resolution	Total error	Automatic export raw data
Electric Noise	Small particle resolution	Method comparison	Changes in panels
Carry over	Cross-instrument/lab standardization	Other parameters	New panels (12 colors)

Linearity/Dynamic Range/Sensitivity

Linearity

Detectors/amps functioning properly?

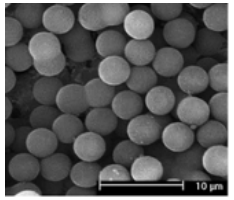
Dynamic Range

The limit within which all data is reproducible and linear

Sensitivity

The ability to detect events above background and to resolve dim events

With beads!



	Stained	Advantage	Disadvantage	Fluorescence intensity is assigned in…	Examples
Antibody captured beads	On the surface	Spectrally matched, same ex/em properties as your samples	Unstable, sensitive to buffer (PH, salt conc)	**MESF** values (molecule of equivalent **soluble** fluorochrome)	Quantum 24 beads (Bangs Lab) QuantiBrite (BD)
Hard dyed beads	Internally, multiple peaks	Stable	May not be fully excited by some lasers May resolve fewer peaks	**MEF** values (molecule of equivalent fluorochrome)	Rainbow beads (Sperotech) Cyto-Cal beads

Linearity/Dynamic Range/Sensitivity

Expanding NIST Calibration of Fluorescent Microspheres for Flow Cytometry to More Fluorescence Channels and Smaller Particles

Paul DeRose [1,*], Linhua Tian [1], Elzafir Elsheikh [1], Aaron Urbas [2], Yu-Zhong Zhang [3] and Lili Wang [1]

National Institute of Standards and Technology (NIST):

- Assigns **equivalent reference fluorophore (ERF) values** to calibration beads
- How: Fluorophore solutions of known concentration (such as Standard Reference Material (SRM) 1934; comprised of 4 reference fluorophores) AND bead suspension are measured with a CCD-fluorescence spectrometer
- Goal: accurately quantify the number of 'antibodies bound per cell' (ABC)
- Advantage: **standardization** *(H62: "they provide a common fluorescence intensity scale that is consistent across flow cytometers with identical optical configurations")*

→NIST has assigned calibration beads for more than 50 fluorescence channels using five different laser colors and continues to develop additional reference fluorophores for fluorescence channels not yet covered

Commercially available product from SpheroTech with certified ERF values **with SRM 1934 certificate:**
→ Ultra Rainbow Quantitative Particle Kit: 6 intensities (fluorescent channels FITC/PE/APC/PacBlue)
→ Supra Rainbow Quantitative Particle Kit: 4 intensities (fluorescent channels with emission ranges 425 - 810nm)

Linearity/Dynamic Range/Sensitivity

CLSI H62 Guidelines:

"A set of good-quality multi-intensity beads that includes both unstained beads and beads with defined fluorescence intensity units is adequate for characterizing cytometer performance."

→ Calibration curves can be obtained with **multi-intensity beads** with assigned fluorescence intensity units (gain-independent fluorescence units: **MEF/MESF/ERF**)

would be suitable calibrators. Indeed, clinical assays that require mean fluorescence measurements with 10% accuracy use fluorophore-specific surface-stained beads for calibration. Hard-dyed beads can be a good standard to set up the fluorescence scale and verify linearity and dynamic range of the instrument among a group of study instruments. If a factor-of-2 variation in the mean fluorescence from cells can be tolerated, hard-dyed beads can be used as a standard.

Wooclap question 2

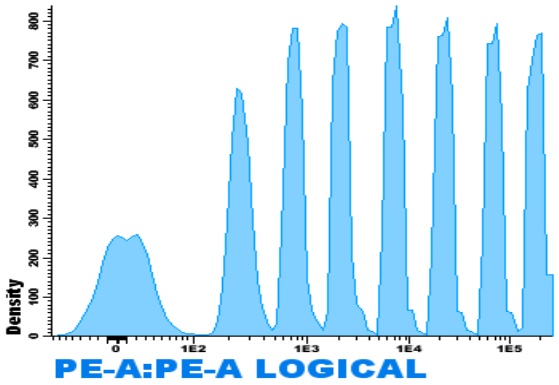
https://app.wooclap.com/events/JCCAYN/questions/65c3c601b7c0977e49946eb2

Which statement is correct?

- The Operational qualification provides documented evidence that the system performs consistently over the period of time for the intended purpose
- Ultra Rainbow Particles show poorer performance in the far-red region compared to Rainbow Particles
- The fluorescence emission of hard dyed beads may be affected by the pH, salt concentration and other factors present in the buffer they are exposed to
- An MESF value of 1000 for the FITC channel is equivalent to the fluorescence of a solution containing 1000 FITC molecules
- The advantage of ERF assigned beads is the traceability and standardization across instruments with different optical configuration

Linearity/Dynamic Range: Rainbow beads

Optimize voltages
Record 5000 events



MedFI

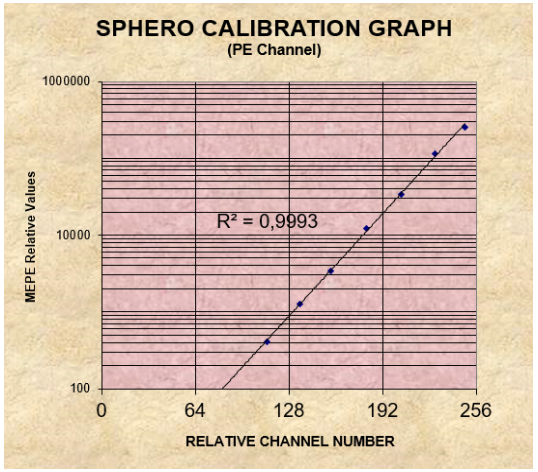
Convert into relative channel numbers

TABLE NO. 4	
10^5 MEAN CH# to 256 REL. CH# CONVERSION	
10^5 CH#	256 CH#
8,87	44,78
244,68	112,85
744,39	135,68
2069,96	156,66
6772,62	180,98
21463,39	204,65
67256,93	228,09
179593,9	248,24

MEF values assigned by Spherotech

PEAK #	CH #	MEPE	MEPE LOG	CALC.	RESIDUAL	CALC. MEPE
1	44,78			1,210		16
2	112,85	409	2,612	2,627	0,59%	424
3	135,68	1250	3,097	3,103	0,18%	1266
4	156,66	3428	3,535	3,540	0,13%	3464
5	180,98	12229	4,087	4,046	1,02%	11120
6	204,65	34294	4,535	4,539	0,08%	34594
7	228,09	113118	5,054	5,027	0,53%	106431
8	248,24	256134	5,408	5,447	0,70%	279746
			Ave Residual		0,46%	
					Slope: 0,0208	
					Intercept: 0,2770	
					Rsq: 0,9993	

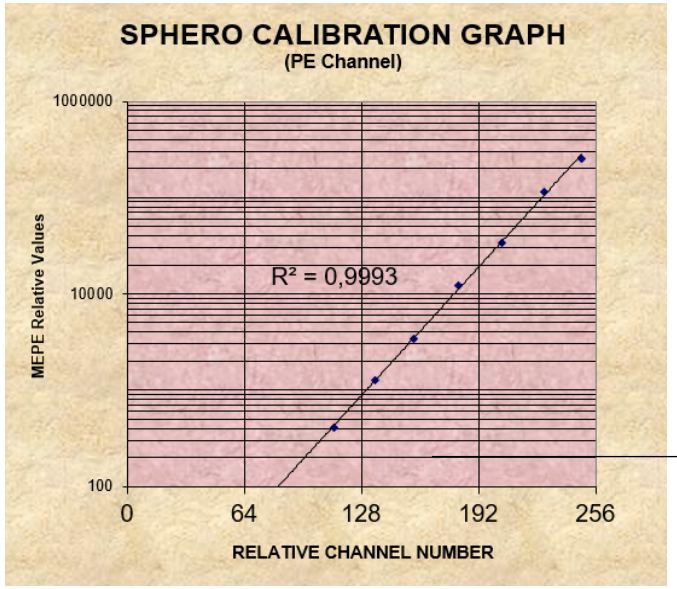
Linear relationship between observed and expected fluorescence signal



Hard-dyed beads (3,4 µM)
Peak 1 =blanco,
Peak 2- 8: increasing amount of fluorochrome

Technical notes STN-14 (linearity) and STN-17 (sensitivity Q en B): https://www.spherotech.com/tech.htm
Supportive templates with calculations (Rainbow + Ultra Rainbow beads): Technical Page - Templates – Spherotech
Webinar: https://youtu.be/w9iCYwUX0Hk

Linearity/Dynamic Range: Rainbow beads



Regression Equation:
Y= ax+b
A= slope
B= intercept

PEAK #	CH #	MEPE	MEPE LOG	CALC.	RESIDUAL	CALC. MEPE
1	44,78			1,210		16
2	112,85	409	2,612	2,627	0,59%	424
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8	248,24	256134	5,408	5,447	0,70%	279746
				Ave Residual	0,46%	
					Slope: 0,0208	
					Intercept: 0,2770	
					Rsq: 0,9993	

These beads allow us to judge linearity, dynamic range and detection simultaneously

Dynamic range		
Channel 0	(0-Intercept)/Slope	0,3
Channel 2^18	(((2^18)-1)-Intercept)/Slope	5,2
Dynamic Range (log decades)	Channel 2^18- channel 0	4,9

Specifications

Linearity

Average Residual	< 5%	Leaflet Spherotech
Correlation coefficiënt (R^2)	> 0,99%	Leaflet Spherotech

Dynamic Range

Log Amp decade	> 5	Van Bockstaele et al.

Sensitivity

a higher Q and a lower B increases the ability to resolve a dim population from the background noise

Sensitivity: Rainbow beads

Light scatter sensitivity is often expressed as the MESF value of the smallest detectable bead or intercept

WRONG: only specifies the **detection threshold** provides **no information about the ability to resolve dim populations!**

→ Sensitivity should be measured in terms of **Q and B** (take into account the broadness of the unstained bead and compare is to the MFI of a stained antibody-captured bead)

→ Q and B can also be calculated with templates provided by Sperotech

(Hard died beads: unstained + dim1/dim2/dim3 beads +

Antibody-captured beads: bright bead (CV))

PEAK #	CH #	MEPE	MEPE LOG	CALC.	RESIDUAL	CALC. MEPE
1	44,78			1,210		16
2	112,85	409	2,612	2,627	0,59%	424
3	135,68	1250	3,097	3,103	0,18%	1266
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			Ave Residual		0,46%	
					Slope:	0,0208
					Intercept:	0,2770
					Rsq:	0,9993

≠ sensitivity!
WRONG

$$Q = \frac{1}{slope}$$

$$B = \frac{intercept}{slope}$$

Hofmann and Wood 2007

Specifications

Linearity		Dynamic Range			
Average Residual	< 5%	Leaflet Spherotech			
Correlation coefficiënt (R^2)	> 0,99%	Leaflet Spherotech	Log Amp decade	> 5	Van Bockstaele et al.

Sensitivity

a higher Q and a lower B increases the ability to resolve a dim population from the background noise

Linearity/Dynamic Range: ERF Ultra Rainbow beads

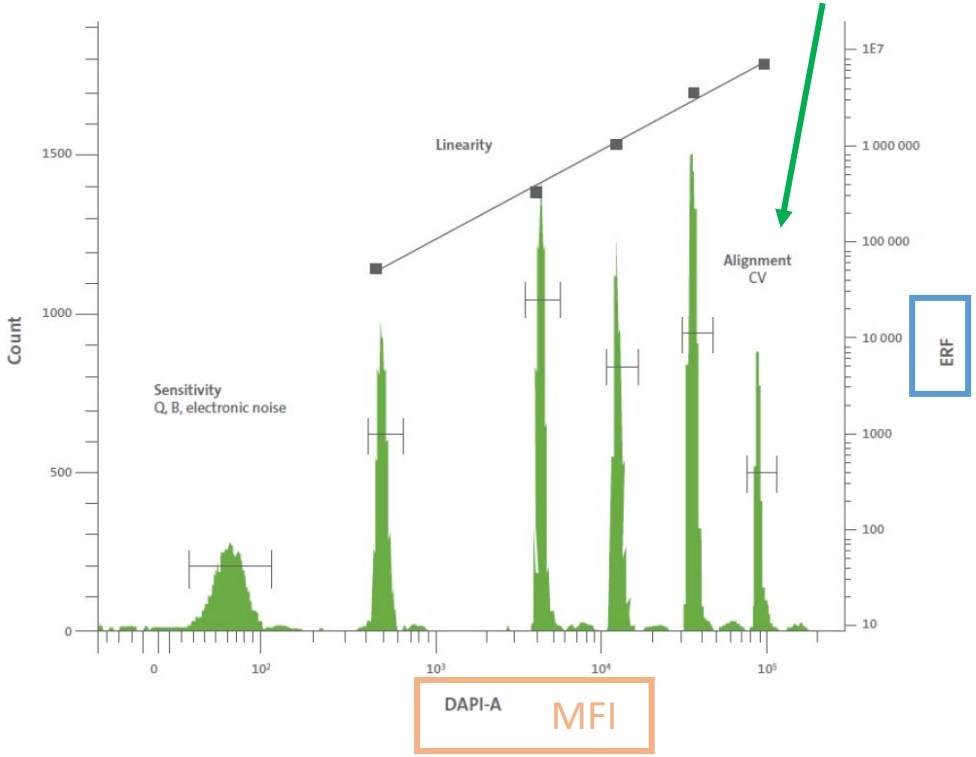
Example: **URQP beads** for Pacific Blue Channel (DAPI)

Bead Population	MFI	SD	% CV	ERF
Peak 1 (blank)	62	16	25.1	N/A
Peak 2	487	32	6.5	0.524×10^5
Peak 3	4250	196	4.6	3.23×10^5
Peak 4	12 385	520	4.2	9.95×10^5
Peak 5	35 060	1472	4.2	38.3×10^5
Peak 6	88 138	3790	4.3	71.7×10^5

Plot on x-as

Plot on y-as

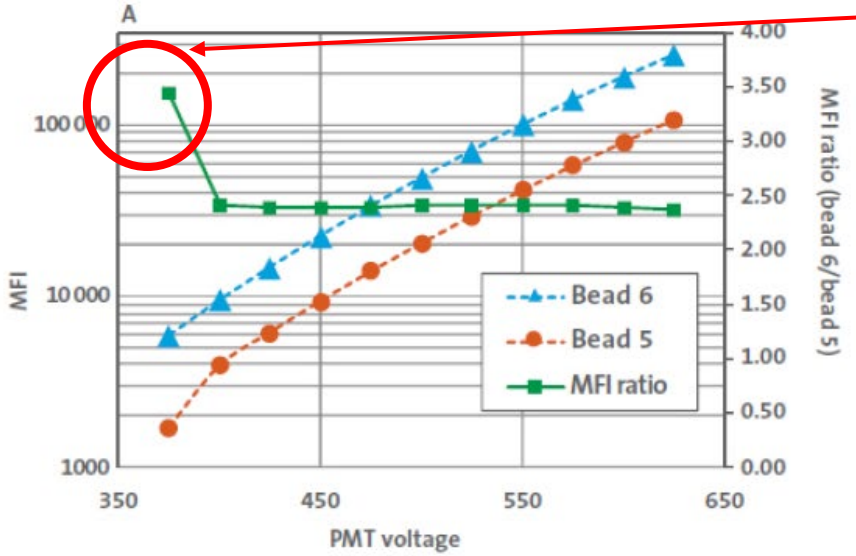
Brightest peak CV assesses optical alignment



Linearity/Dynamic Range: ERF Ultra Rainbow beads

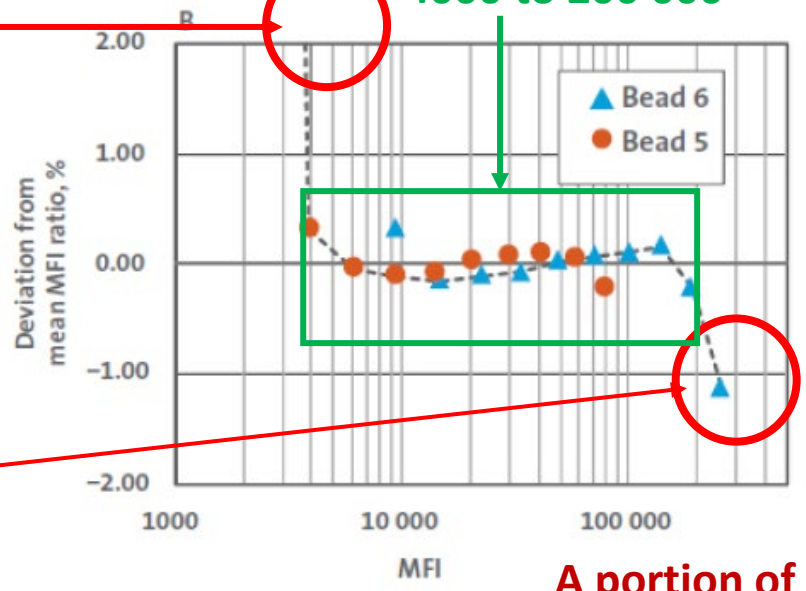
Alternative method: Ratiometric method with URQP beads

non-linear response



Voltage	MFI Bead 6	MFI Bead 5	Ratio (6/5)	Deviation from mean, %
375	5920	1715	3.45	43.70
400	9512	3947	2.41	0.32
425	14854	6193	2.40	−0.15
450	22636	9433	2.40	−0.11
475	33787	14076	2.40	−0.08
500	49398	20558	2.40	0.03
525	71270	29647	2.40	0.07
550	100811	41922	2.40	0.10
575	140363	58405	2.40	0.04
600	191911	80070	2.40	−0.23
625	257771	108545	2.37	−1.14
	Mean (400–600 volts)		2.40	

Excellent linearity over dynamic range from 4000 to 200 000



A portion of Bead 6 fell OFF SCALE

→ Detector voltage ↓ in 25V steps to move the brightest peak from the upper end to the lower end
→ Measures MedFIs of bead 5 and 6 and MFI ratio is plotted vs PMT Voltage
→ Both bead populations must be fully on scale for all voltages tested
→ Advantage: **more accurate** assessment
easy to perform (no assignment of fluorescent intensity units needed), takes only a short time

Electronic Noise

The contribution of electronic noise becomes more significant at the lowest end of the measurement scale.

→ Contributes to the broadening of signals in negative or very dim populations

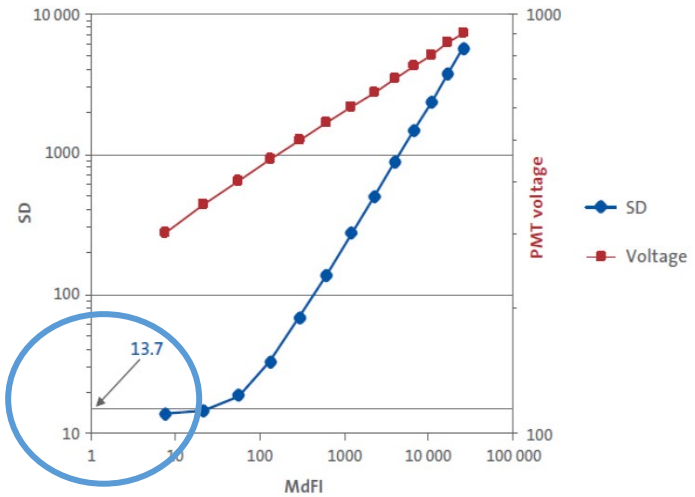
Turn the voltage to ZERO and measure the rSD of the resulting signal

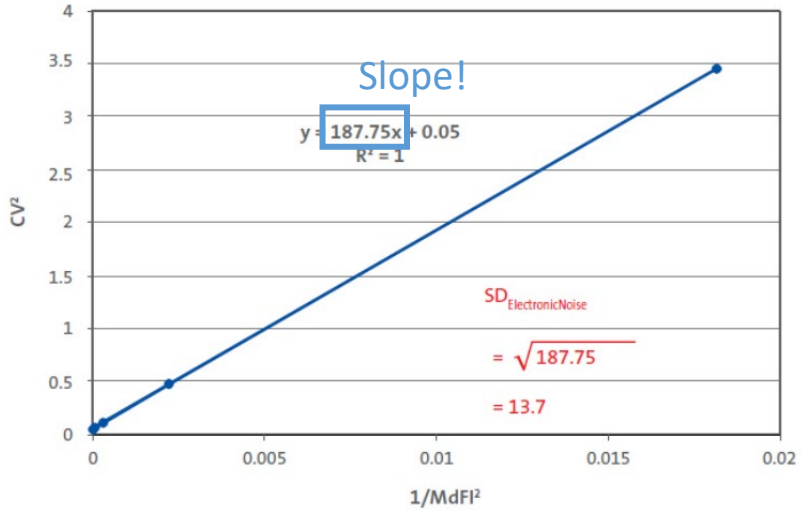
OR: measure **SD, CV and MedFI** of **a Dim/Moderate bead** when **reducing the detector voltage to lower values**

→ The rSD will tend toward a **stable** number

→ rSD can be determined by plotting CV^2 vs 1/MdFI2 → rSD^2 = slope → obtain rSD= $\sqrt{(rSD^2)}$

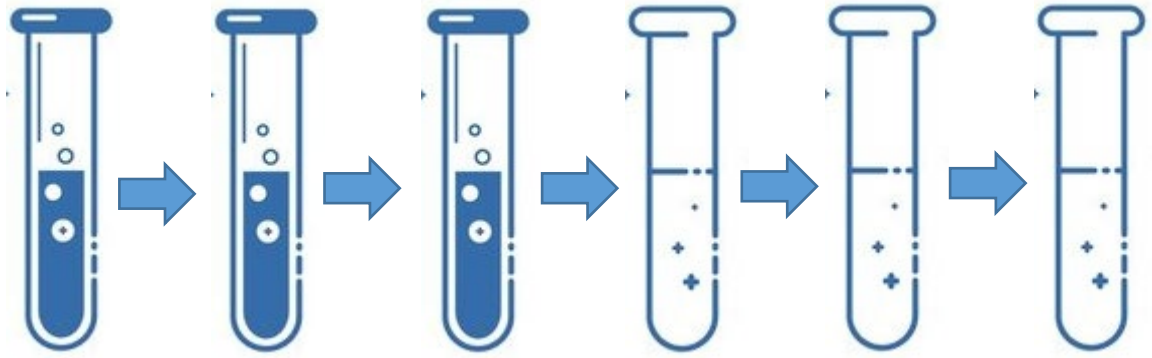
For the best resolution of dim signals: set the voltage where CV of unstained population is not significant broadened by the electronic noise





Carry-over

Check whether cells from one sample can be transferred via the device to the next sample: how many cells are measured in the blanco?



Cell-rich sample stained (CD45+)	Cell-rich sample stained (CD45+)	Cell-rich sample stained (CD45+)	Buffer or low-level sample	Buffer or low-level sample	Buffer or low-level sample
A1	A2	A3	B1	B2	B3

→ Determination of this parameter is crucial for rare event determinations!
→ Higher risk on carry-over with automated sample loaders

Carry-over (Q%) = ((B1-B3)/(A3-B3))*100

Specifications

1 SIT flush	< 0,1%	Spec company
3 SIT flushes	< 0,05%	Spec company

Carry-over

When it is difficult to obtain cell-rich samples:

Alternative: beads in Trucount tubes

BD Calibrate APC Beads mixed with PBS in BD Trucount™ Tubes were collected three times containing at least 100,000 standard particles each time. The carry-over contamination rates of the cycles were calculated as 0.17% (blanks result: 302, 183, and 133; beads result: 100019, 100020, and 100175), 0.13% (blanks result: 204, 320, and 74; beads result: 100188, 100166, and 100169), and 0.14% (blanks result: 227, 91, and 83; beads result: 100145, 100186, and 100175).

Sun et al. 2021

Preventive actions in case of out-of-spec:

- Standard 3 SIT flushes between samples
- Sensitive assays (MRD, LF,..): 60''cleaning sample before each sample + clean sample port with tissue

Wooclap question 3

https://app.wooclap.com/events/JCCAYN/questions/65c3c6501c3f14438923fed7

Which statement is correct?

- The Ratiometric approach is more accurate than the linear fitting approach for determining linearity as it relies on assigned fluorescence units
- The detection threshold as a measure of sensitivity, gives information about the ability to resolve dim or rare from bright populations
- The dimmest peak of the Ultra Rainbow particle kit is used to assess optical alignment: the smaller the CV, the better the alignment of the sample stream to the laser beam
- The contribution of electronic noise is dependent on the PMT voltage

  and becomes more significant at the lowest end of the measurement scale
- To determine the carry-over, it is important to run every condition for the same amount

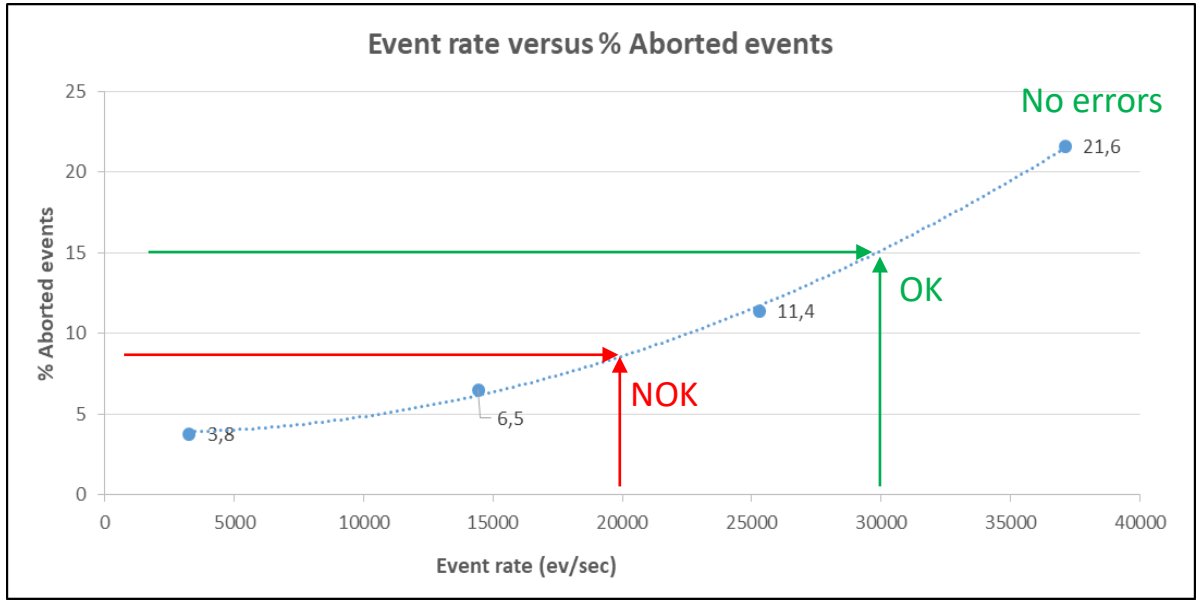
  of time

Validation Plan: example

Instrument Validation (PQ)	Extended PQ (~ intended use)	Method validation	Addenda
Linearity	Acquisition speed	Bias	Software calculations
Dynamic Range	Storage capacity	Imprecision	Workflow manager
(Light scatter) Sensitivity	Light scatter resolution	Total error	Automatic export raw data
Electric Noise	Small particle resolution	Method comparison	Changes in panels
Carry over	Cross-instrument/lab standardization	Other parameters	New panels (12 colors)

Acquisition speed

At what speed can you measure with an acceptable abort rate and free of errors?



→ Cell-rich sample (bulk lysed) stained for subset panel
→ Prepare a dilution series
→ 3 replicates, 3 different speeds (low/medium/high)
Same time per condition
→ Register the abort rate

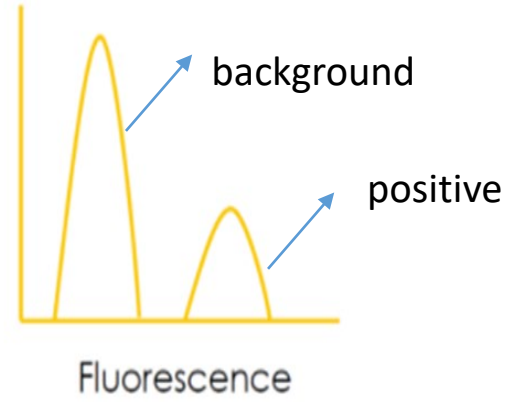
Specifications

Abort rate at 20 000/sec	< 6%	Vendor specifications
Abort rate at 35 000/sec	< 15%	Expert opinion
35 000/sec feasible	without alarms	Expert opinion

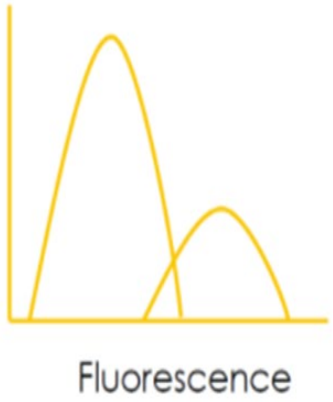
Acquisition speed

At what speed can you measure with an acceptable resolution?

Low speed



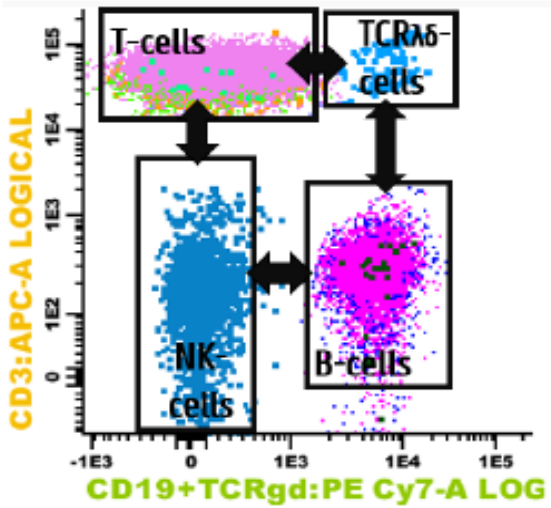
High speed



Impact of acquisition speed on resolution can be evaluated using the Staining Index or Resolution Index (for ex. for the major cell population within subset analysis)

$$\text{Stain Index} = \frac{(MFIpos \;\; MFI_{neg})}{2 \, x \, SDneg}$$

$$Resolution\ index\ (RI) = \frac{MFI_{pos} - MFI_{neg}}{\sqrt{SD_{pos}^2 + SD_{neg}^2}}$$



Specifications

Difference in staining index (SI) between HIGH and LOW speed	<10% difference	ICCS/ICSH guidelines

Storage capacity

Is there a limitation on the number of events saved in the FCS.file? Control the effect of big data files on the performance of the software

Cell-rich sample (CLL) stained for CLL panel
Test 2 different stopping criteria (2 FCS.files)
Is there a delay when adjusting gates?
Analysis preferable done in external software on a workstation with sufficient RAM

Cell populations	File 1: 1 x 10^6 events	File 2: 10 x 10^6 events	% Bias
B cells	98.2%	98.6%	0.04%
CLL+ cells (on lymfocytes)	97.6%	98.1%	0.5%
CLL+ cells (on WBC)	88.5%	94.4%	6.7%

Specifications

Comparable results between 1 x 106 and 10 x 106 events	%bias <10% Same clinical interpretation	Expert opinion
Analysis of data file	Without blockage No limits of events	Vendor specifications (BD)

Light scatter resolution

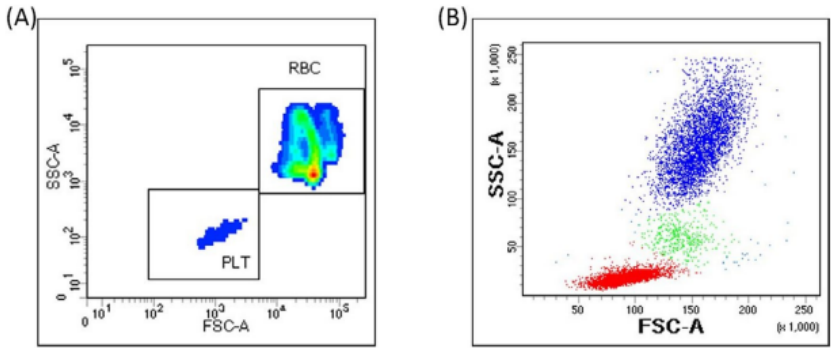
Based on FSC and SSC, is there a clear separation between the cell populations?

Recommended to verify with **cells** of interest instead of beads

As light scatter is affected by:

- Size: controllable
- refractive index: > RI of polymer beads is higher then RI of cells
  > even silica beads are not analogue
  > different devices measure different angles of scatter
  > cells are not homogeneous (RI of nucleus ≠ cytoplasma)

Newer materials (HYDROGELS) are being produced as light-scatter standards



Specifications

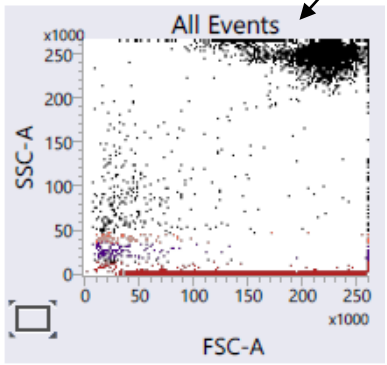
Clear separation between platelets and red blood cells, and between lymfocytes, monocytes and neutrophils

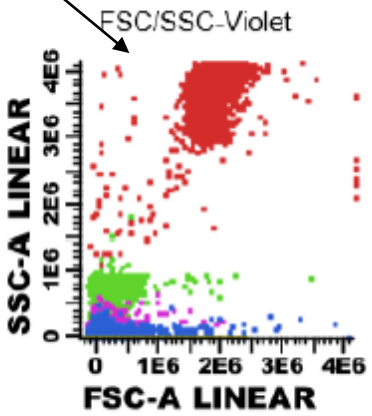
Du et al.
un et al. 2021

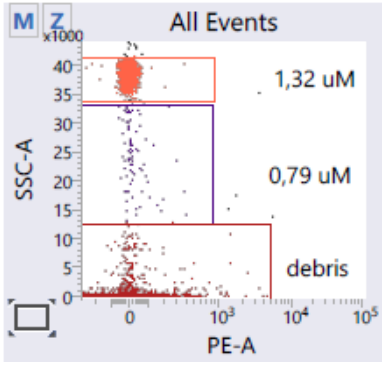
Small particles

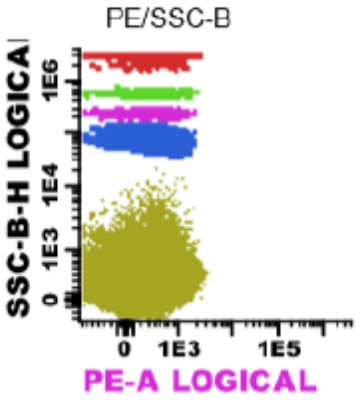
What is the smallest size of particles that can still be measured with an acceptable resolution?

FACSLyric™ Rainbow beads Aurora™









Capacity to discriminate small particles from background

SPHERO™ Flow Cytometry Nano Fluorescent Size Standard Kit (4 diameters)

Other options: Apogee beads

BioCytex Megamix-Plux

Optimize TRESHOLD and VOLTAGES/GAINS

Specifications

Distinguish beads up to a size of 0,25 µM Erdbrugger et al.

Cross-instrument standardisation

Can you obtain the same experimental result regardless of where, when and by whom the sample is analysed?

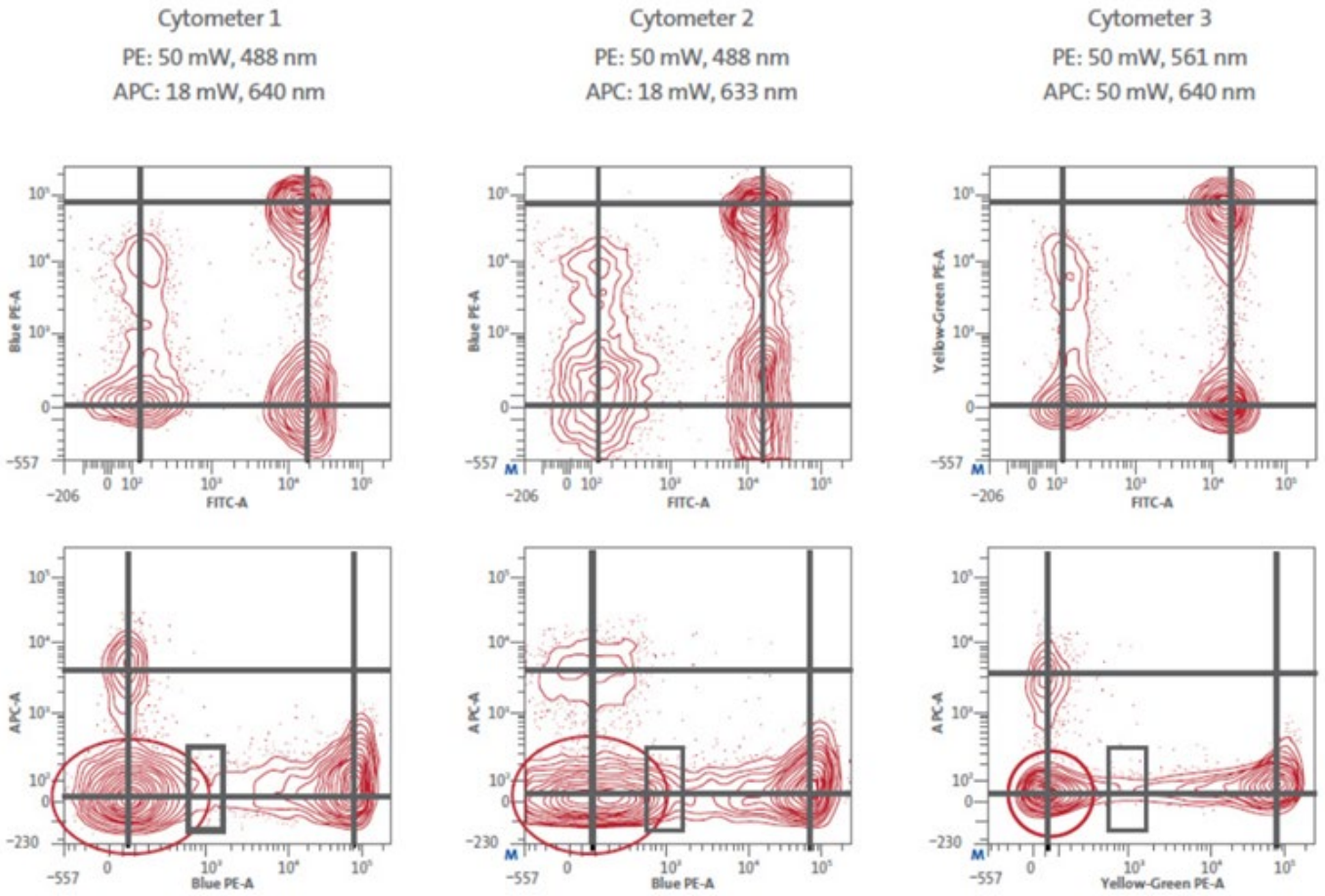
- To ensure consistency
- The sensitivity of the instruments must be as close as possible
- With spectrally matched (antibody-binding) beads OR cells of interest: equivalent intensity values (MESF or MedFI) should be obtained by adjusting voltages
- After cross-standardisation monitor each cytometer daily with hard dyed beads (CS&T)!

Specifications

Moderate- to high intensity beads	Similar MedFI +/- 7% on all instruments	CLSI H62
Cells of interest	Similar dot plots with +/- 15% for positive staining	CLSI H62

WooClap excercise 4

https://app.wooclap.com/events/JCCAYN/questions/65c3c6a9b7c0977e49949feb



Validation Plan: example

Instrument Validation (PQ)	Extended PQ (~ intended use)	Method validation	Addenda
Linearity	Crossinstrument standardization	Bias	Software calculations
Dynamic Range	Acquisition speed	Imprecision	Workflow manager
(Light scatter) Sensitivity	Storage capacity	Total error	Automatic export raw data
Electric Noise	Light scatter resolution	Method comparison	Changes in panels
Carry over	Small particle resolution	Other parameters	New panels (12 colors)

Fit-for-purpose approach

""A validation strategy appropriate for the intended use should be applied"

CLSI H62

Regulatory Setting	Intended Use of Data	Assay Type	Recommended Validation Strategy
Nonregulated	Basic research	Novel assay	FFP validation type 1
Nonregulated	Drug discovery	Novel assay	FFP validation type 1
Nonregulated	Exploratory end points in clinical trials	Novel assay	FFP validation type 1
Nonregulated (GCLP recommended)	Secondary end points in clinical trials	Novel assay	FFP validation type 2
Medical laboratory (CAP, CLIA, or ISO[34])	Patient care and/or treatment	IVD	Verification[a]
Medical laboratory (CAP, CLIA, or ISO[34])	Patient care and/or treatment	Qualitative LDT assay	CLIA/IMDRF qualitative validation
Medical laboratory (CAP, CLIA, or ISO[34])	Patient care and/or treatment	Quantitative LDT assay	CLIA/IMDRF quantitative validation
Medical laboratory (CAP, CLIA, or ISO[34])	Patient care and/or treatment	Laboratory-initiated assay revision	Laboratory-initiated assay revision validation
GLP, GCLP[b]	Primary end point in clinical development	Novel assay	Analytical validation type 1
Manufacturing (GMP, ISO[64])	Regulatory submission for new diagnostic test	Novel assay	Analytical validation type 2
Manufacturing (GMP, ISO[64])	CDx	Novel assay	Analytical validation type 2

Table 22: lists all validation scenario's
+ Appendix A: minimal requirements and criteria

CE-IVD assays: verify precision/stability/reference intervals, NO LOB/LOD/LLOQ!

Qualitative LDT assays: comparison with a confirmed diagnosis or comparative method (clinical validation), calculate concordance!, determine also carry-over

Quantitative LDT assays: also LOB/LOD/LLOQ! Most validation parameters for this scenario

Revision: changes afterwards in reagents, moabs clones..

Bias/Imprecision/Total error

Bias

Degree of agreement between the measured data and the reference values

Imprecision

<u>Within-run imprecision:</u>
Repeatability of samples on same day, under same conditions

<u>Between-run imprecision:</u>
Samples tested on different moments

<u>Inter-operator variability:</u>
Evaluates the preanalytic steps

Total Error

Determines the uncertainty of the measured values

Test with certified reference material

Bias/Imprecision/Total error

Phenotype	% Total Lymphocytes (Range)	Absolute Number/µL (Range)
$CD3^+$	72.9 (62.9 – 82.9)	1165.7 (932.5 – 1398.8)
$CD3^+CD4^+$	46.9 (40.4 – 53.4)	749.9 (599.9 – 899.9)
$CD3^+CD8^+$	23.6 (16.6 – 30.6)	377.4 (272.1 – 482.6)
$CD19^+$	13.8 (9.8 – 17.8)	220.7 (152.7 – 288.6)
$CD3^-/CD16+CD56^+$	11.4 (4.4 – 18.4)	182.3 (96.6 – 268.0)
$CD3^+HLA\text{-}DR^+$	6.1 (3.1 – 9.1)	NA
$CD45^+/CD14^-$	95.0 (95.0 – 100.0)	NA

Population	Within-run/Between-run imprecisie + inter-operator variability (SUBSETS)																	
Day	DAY1						DAY2						DAY3					
Sample type specification	Multicheck						Multicheck						Multicheck					
Timepoint	VM		MIDDAG		NM		VM		MIDDAG		NM		VM		MIDDAG		NM	
Operator	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Replicate 1 (%)																		
Replicate 2 (%)																		
Replicate 3 (%)																		
Mean (over replicates)																		
SD (over replicates)																		
% CV (over replicates)																		
Satisfy criteria? <10 %																		
<25% (below 5%)																		
<35% (below 1%)																		
Mean %CV (all samples)																		
Satisfy criteria?																		

Calculate the MEAN over all days/replicates

Bias = (MEAN – target)/target

CV = (SD/Mean)*100

ANOVA

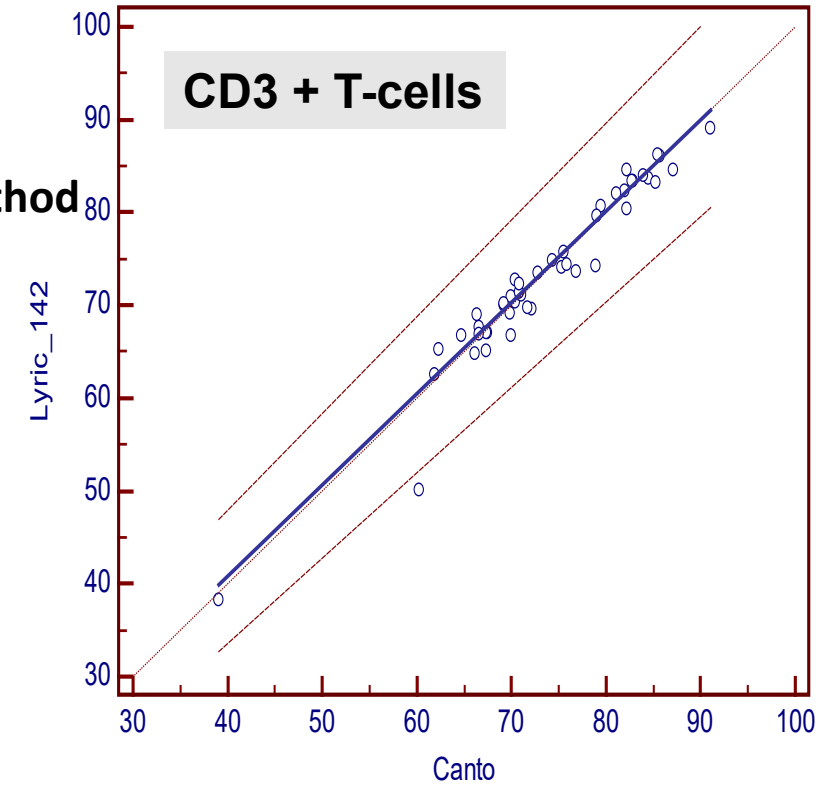
Specifications

Total error = bias + 1.65 x between-run CV

Bias			Imprecision			Total Error		
MultiCheck control	<10% (T and NK) <15% (B)	Omana-Zapata	Imprecision	%CV<10% %CV<25% %CV<35%	Selliah et al.	Acceptable error	<25%	Expert opinion
Stem Cell Control	<15% (for % and abs)	Expert opinion						
Range company	Comply with range	Specs BD	Inter-operator	Similar %CV	Selliah et al.			

Method comparison (accuracy)

- Optimally 30 samples (spread over entire measuring range)
- Passing bablok regression



Populatie	Regressie vergelijking	Intercept A	95% CI	Slope B	95% CI	Conclusie
T-cells	y = 1.299601 + 0.985890 x	1.2996	-3.1895 to 6.1047	0.9859	0.9196 to 1.0442	No difference
		Contains 0 → no systematic error		Contains 1 → No proportional error		

Specifications

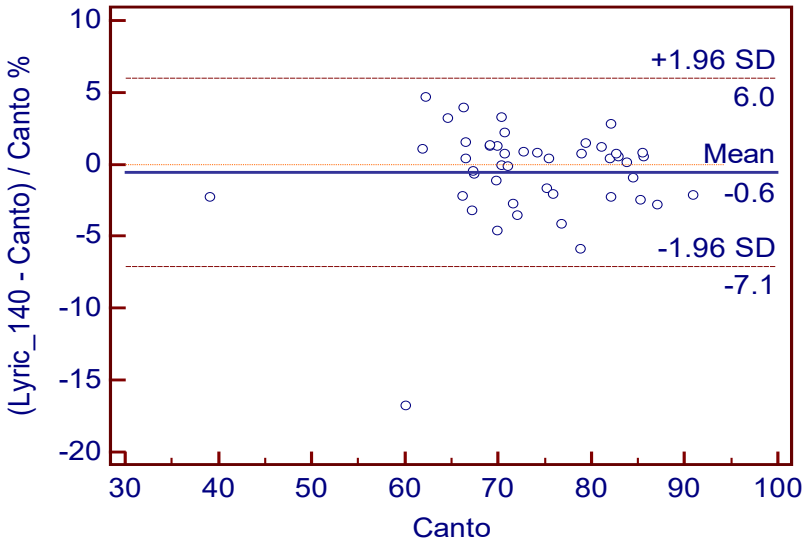
The intercept	should not differ significantly from 0	
The slope	should not differ significantly from 1	Omana-Zapata

Method comparison (accuracy)

- Bland-Altman analysis

=graphical method in which the **differences** between the two techniques are plotted against the **averages** of the two techniques.

Horizontal lines are drawn at the mean difference, and at the limits of agreement (which are defined as the mean difference ±1.96 times the standard deviation of the differences)



Usefull method:

- To look for relationship between the differences and the magnitude of measurements
- To illustrate systematic bias
- To identify outliers

Specifications

	<20%	
	<25% (below 5%)	
Mean %bias	<35% (below 1%)	Selliah et al.

Method comparison (accuracy)

Semi-quantitative assays

Percentage blasts		Matrix	Lyric 140	Lyric 142	Canto	% Bias Lyric 140	% Bias Lyric 142
1	Sample 1	PB	56.48	56.57	52.55	7.5%	7.6%
2	Sample 2	BM	15	14.79	13.66	9.8%	8.3%
					Mean % bias	8.6%	8.0%

Qualitative assays

	Sample 1		Sample 2			Sample 3		
Marker	Lyric 142	Canto II	Lyric 140	Lyric 142	Canto II	Lyric 140	Lyric 142	Canto II
CD11b	neg	neg	neg	neg	neg	neg	neg	neg
CD7	neg	var	neg	neg	neg	var	var	var
CD22	neg	neg	weak pos	weak pos	pos	neg	neg	neg

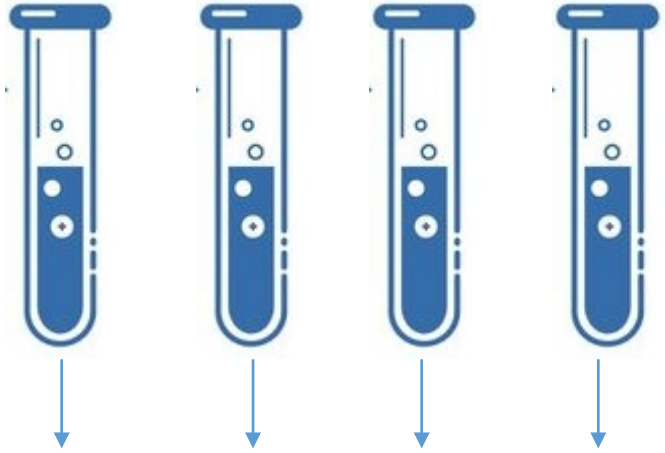
Specifications

Semi-quantitative results	%Bias <20% <25% (below 5%) <35% (below 1%) No impact on clinical interpretation	Selliah et al. Expert opinion
Qualitative assays	Same expression pattern No impact on clinical interpretation **>95% concordance between results**	Omana-Zapata CLSI H62/CLSI EP12

Other validation parameters

Measures the robustness of the test for older samples (antigen expression and viability may change over time during sample storage)

Specimen Stability



Test on min. 3 samples (when possible also disease-state samples) with enough volume **(qualitative assays: min. 5 samples)**

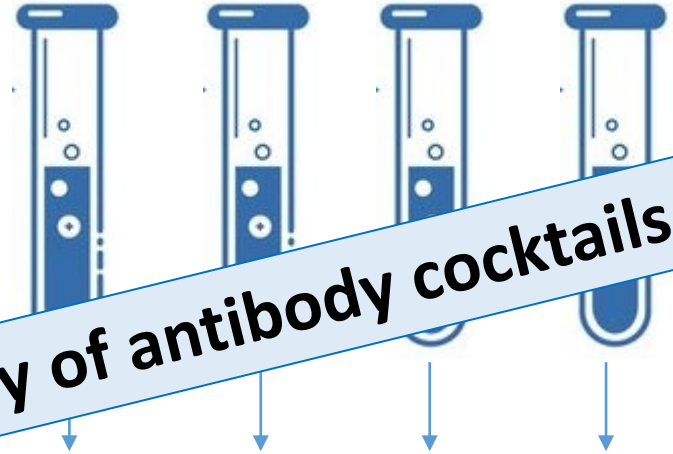
Stain immediately (<2h after collection) **BASELINE**

Stain after 24h

Stain after

timeframe)

Processed Sample Stability



Stain all tubes immediately
For the panel of interest

Acquire immediately **BASELINE**

Acquire after 24h

Acquire after 48h

Acquire after 72h

Same principle for assessing stability of antibody cocktails

Specifications

Quantitative data	%Bias from baseline <20% %CV between 3 replicates <30% >80% of the samples are within interassay precision **(CV%)** And/or are within 20% change of baseline **(% change)**	Selliah et al. CLSI H62
Qualitative data	Same interpretation as baseline for 4 out of 5 tested samples (concordance)	Selliah et al.

Other method validation parameters

- LOB/LOD

→ Blanco samples (repeated measurement o f 10 samples)

→ LLOD in clinical setting: 5 negative + low-positive samples (clinical setting)

→ Samples: preblock with non-labeled moab, HD samples, Pathological control (post-treatment BM), depletion with beads

less recommended: FMO/FMX

LOB = Mean + 1,65 SD

Pre/non-clinical setting:
LLOD = LOB + 1,65 SD (estimation)

Clinical setting:
LLOD obtained from 5 neg+ 5 low-positive samples (empirically)
Qualitative assays: create an LLOD sample by spiking

- LLOQ

→ Important for rare event analysis

→ 3 donors, 5 different levels per donor
OR min. 5 clinical samples near the LLOQ

→ Samples: dilutions series after admixing, non-labeled moab and admixing, partial depletion with beads

less recommended: cell lines

Specifications

LOB: blank replicates (10x)	no more than 5% of the blank replicates > LOB	CLSI H62
LLOD: samples with low levels	95% of low levels of measurand will be > LoB no more than 5% of the replicates < target LLoD Qualitative assays: see 6.1.3.1.2 for specs LLOD CV between 1-20% (table 13) Linearity: R^2 as close to 1 as possible	CLSI H62
LLOQ: dilution series	**N/A for qualitative assays (no LLOQ/linearity)**	CLSI H62

Wooclap question 5

https://app.wooclap.com/events/JCCAYN/questions/65c3c765b133df538880e205

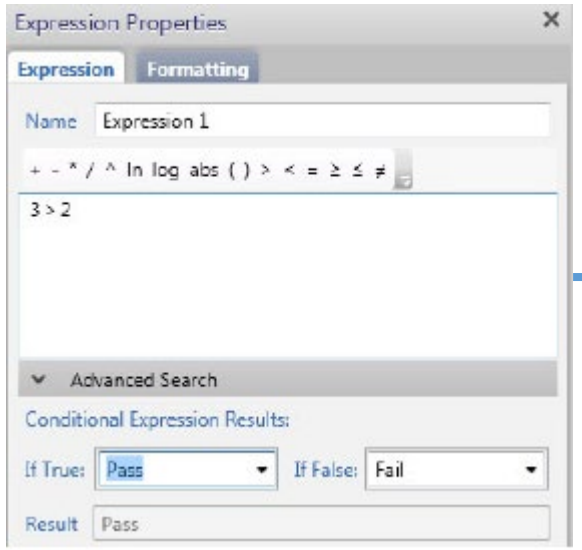
Which statement is correct?

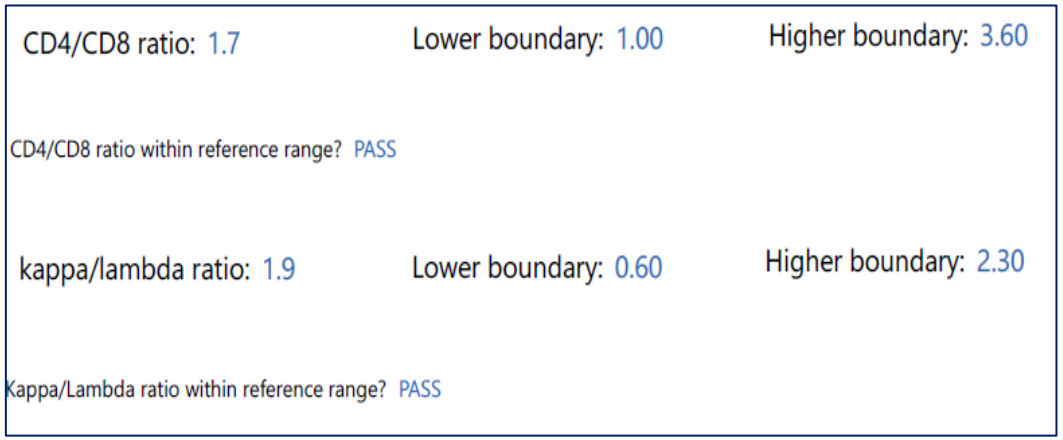
- Method validation of an CE-IVD approved assay requires verification of the precision, sensitivity, stability and reference interval ranges
- To assess inter-operator variability, it is important that every operator **works separately from the start (split the sample)** to be able to evaluate the pre-analytic phase
- The total error can be calculated based on Bias and within-run imprecision
- Between-day intermediate precision can only be assessed with biological samples
- If the confidence interval (obtained with Passing Bablok regression) for the intercept does not contain the value 0 we will confirm a proportional error

Validation Plan: example

Instrument Validation (PQ)	Extended PQ (~ intended use)	Method validation	Addenda
Linearity	Acquisition speed	Bias	Software calculations
Dynamic Range	Storage capacity	Imprecision	Workflow manager
(Light scatter) Sensitivity	Light scatter resolution	Total error	Automatic export raw data
Electric Noise	Small particle resolution	Method comparison	Changes in panels
Carry over	Cross-instrument/lab standardization	Other parameters	New panels (12 colors)

Software calculations





Expressions : program a mathematical formula

Expression range : test againt a reference interval

Conditional expressions (FAIL/PASS) possible

Automatic calculation based on statistics/keywords

Validate every calculation on at least 5 samples:

→compare manual calculation with software calculation

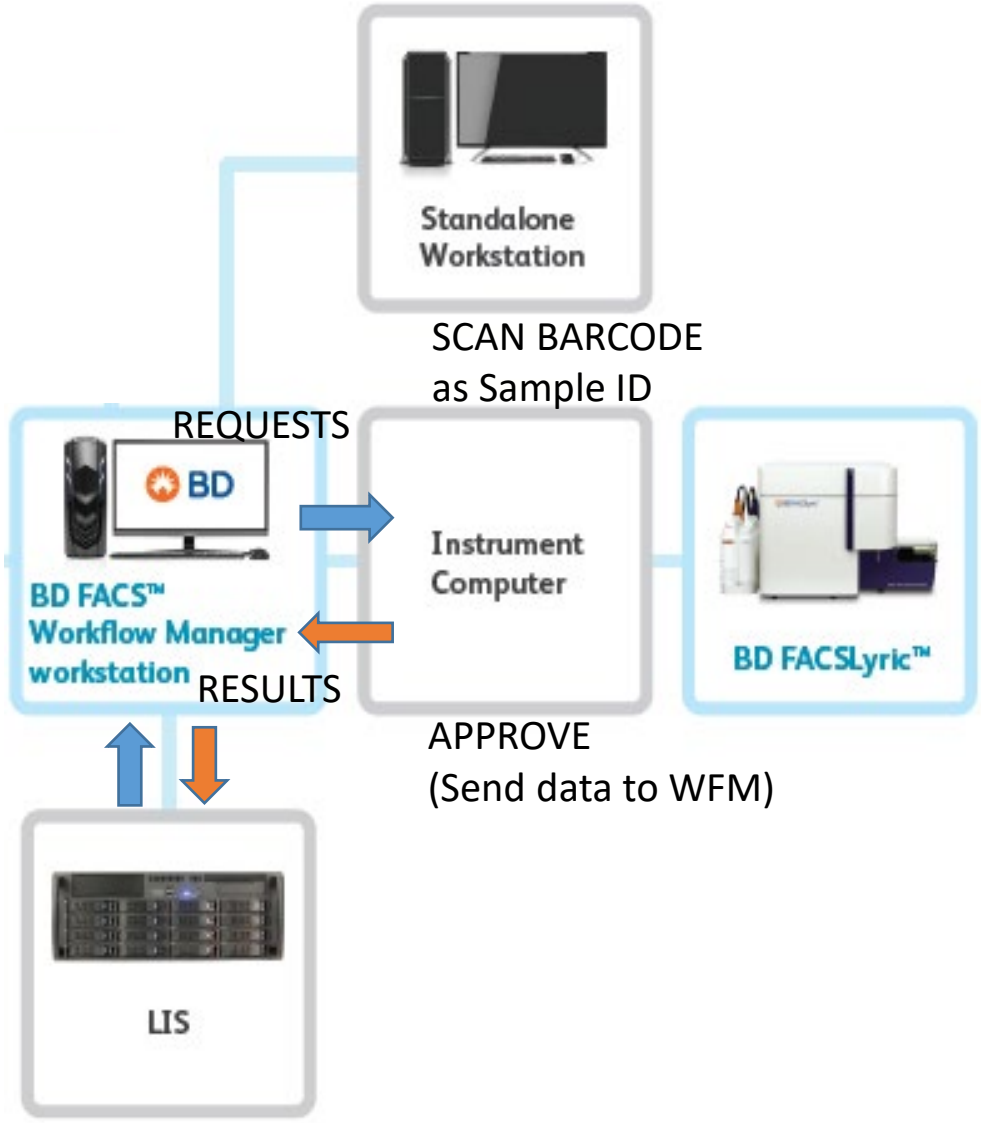
Specifications

Software calulcations (expressions) must correspond to the manual calculation

Up to 2 decimals

BD specifications

Workflowmanager



Middelware; works BI-DIRECTIONALLY with the LIS:

Sample information (REQUESTS) from LIS → Lyric

RESULTS from Lyric → LIS

= **automated data transfer**

\+ Reduces workload

\+ Reduces manual intervention in patiënt data handling

\+ Encrypted data!

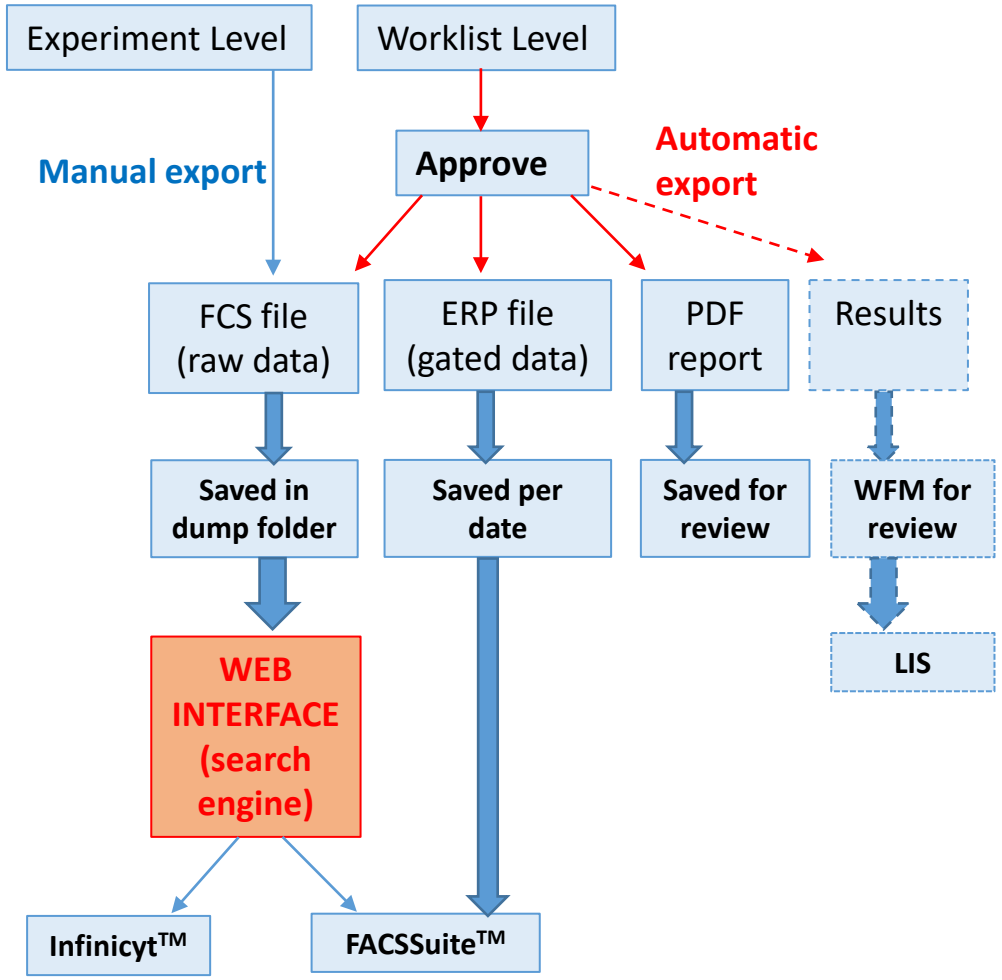
Only for assays acquired on worklist level (CD34, subset,..)

Validate 10 to 30 samples per assay

Specifications

Correct patiënt information is drawn into WFM and FACS Suite™	Same name/ Sample ID	BD specifications
Correct data transfer to WFM/LIS	Up to 2 decimals	BD specifications

Automatic export raw data



We developed an **efficient workflow**

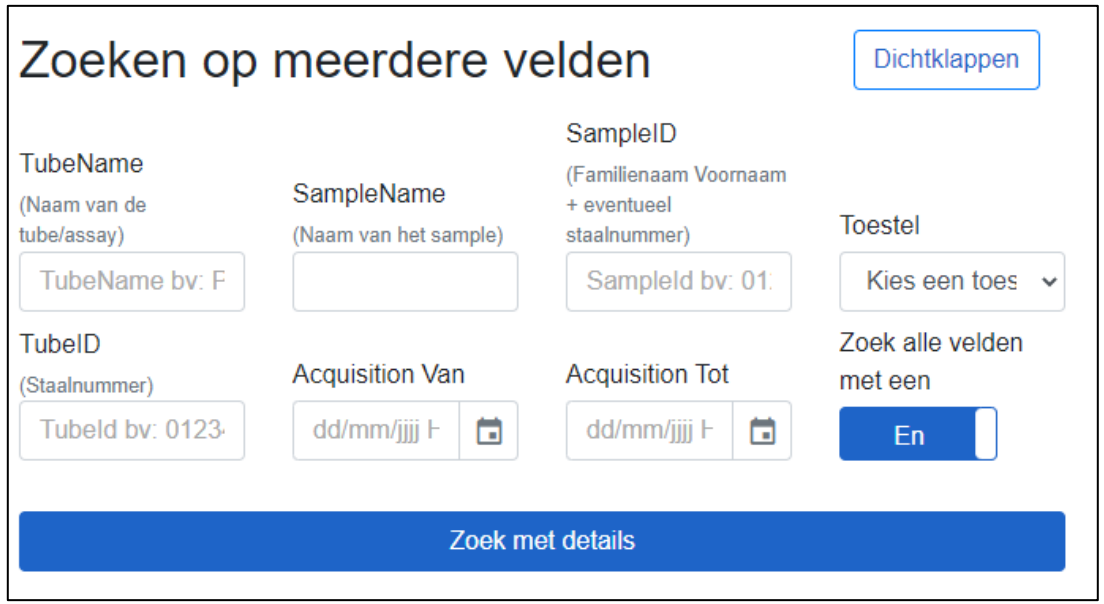
On worklist level: by Approving

→The corresponding FCS/ERP/PDF is automatically exported to the correct folder on the server

On experiment level: still manual export

Web application was developed to retrieve fcs.files easily for further analysis (Infinicyt™/Suite™)

Automatic export raw data



Secured screen in LabPortal that allows us to search and download FCS.files efficiently

Advanced filters also available: specific search

Several validation runs (test phase > production)

All files of a specific patient can be found?
All fcs.files can be retreived? Export exact number of fcs.files and check.
All fcs.files acquired on a specific date can be retreived? (search with data filters)
FCS.files can be downloaded and heavy files can be downloaded within an accaptable timeframe and without errors (MM-MRD)
FCS.files can easily be imported in external software (FACSSuite™ and Infinicyt™)
Try to search files by testing out each filter separate and in combination (device type, Sample ID, Tube id, SampleName,…)
AND-function generates more files than OF-function?
Check if the application still works when it contains many files
All fcs.files linked to the same sample ID can be found?

Specifications

Webapplication

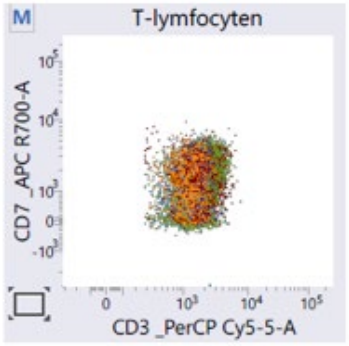
Automatic export of data files (fcs./ERP/PDF)

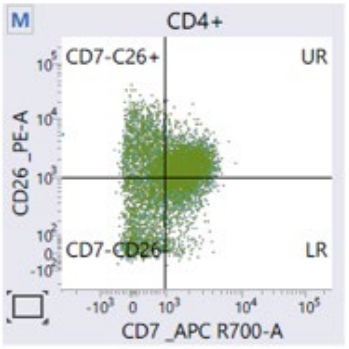
Possible to retrieve FCS. files based on every possible combination of filters

Saved in correct folders

Changes in panels

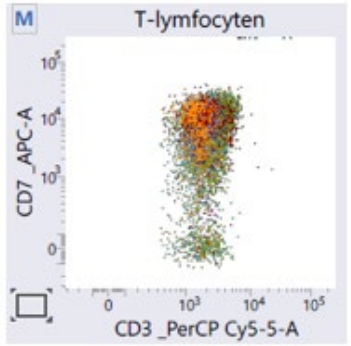
Old TCLPD panel

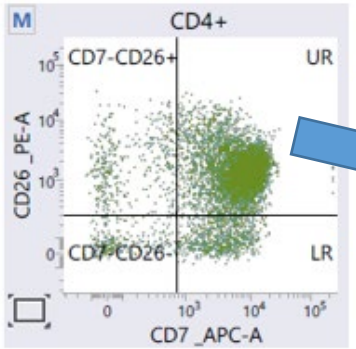




APC	APC-R700
CD2	CD7

New TCLPD panel





APC	R718
CD7	CD2

Expression pattern of abberant populations was compared between the old and new panel on 10 samples

→CD7 in APC-R700: too weak

→Change CD2 and CD7

→Check for an increase in staining index

(on min. 3 samples)

Better resolution

Specifications

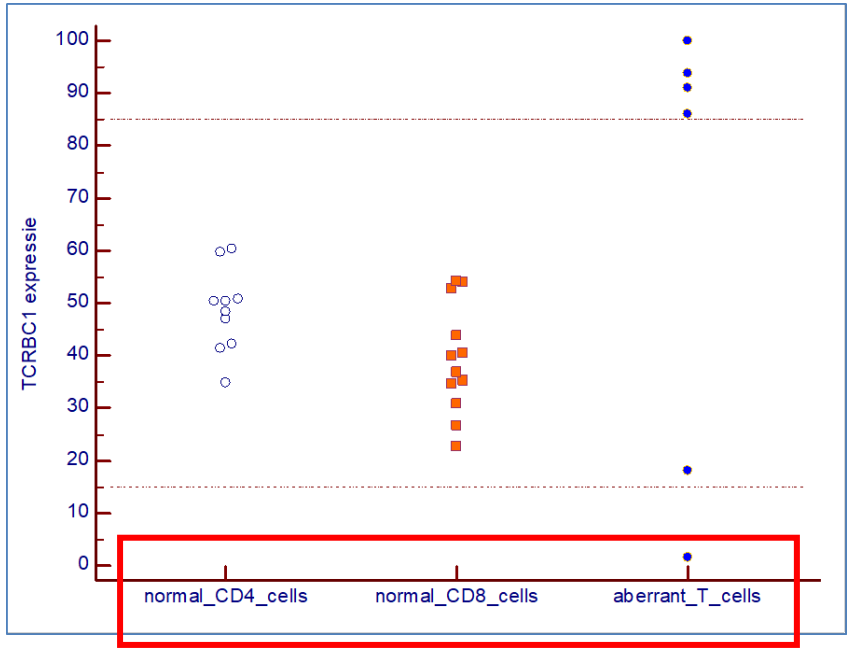
Positive impact of change in panel on resolution	Staining index is increased

New/extended panels

LST panel (8-color) >< Extended LST panel with JOVI-1 (9-color)

We added an extra T cell clonality marker in our LST panel (JOVI-1 in BV786)

Investigate the impact on the interpretation of the lymfocyte subpopulations by testing NORMAL and ABNORMAL samples



Specifications

Method comparison	
Passing-Bablock	No systematic and proportional differences
Bland-Altmann	%bias <20%
Establish reference values for JOVI-1 on CD4+ and CD8+ T cells and compare with literature	Range based on minimum-maximum and 95% CI Literature cut-off: <15 and >85%
Check accuracy	Comparison with TCR gene rearrangement technique Check klonality of HEALTHY/ABBERANT samples

ANY Questions?