FCM Assay Validation in the Era of ISO 15189 and CLSI-H62

I. Marinov
Institute of Haematology and Blood Transfusion, Prague, Czech Republic

Disclosure

I have no potential conflict of interest

ESCCA
European Society
for Clinical Cell Analysis

EU Regulations and Directives

IVD-D
EU Directive (98/79/EC) for in vitro diagnostic medical devices (IVDs) - **self-declared performance claims**
Valid from 07.12.1998 to 25.05.2022

IVD-R
EU Regulation for In Vitro Diagnostic Medical Devices 2017/746 (IVD-R)
Valid from 26.05.2022

REGULATION OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL
Regulation(EU) 2022/112 amending Regulation (EU) 2017/746 as regards transitional provisions for certain in vitro diagnostic medical devices and deferred application of requirements for in-house devices
Valid from 28.01.2022

ESCCA
European Society
for Clinical Cell Analysis

Regulation EU 2017/746- IVDR: IVDMD - classification

IVDMD: reagent, reagent product, calibrator, control material, kit, instrument, apparatus, software, sample receptacles

Class	A	B	C	D
Individual risk	Low	Medium	High	High
Risk for public health	Low	Low	Medium	High
Notified body	Notified body - sterile	Notified body	Notified body	Notified body
Examples	• instruments • buffer solutions • washing solutions • culture media • specimen receptacles	• not classified as class A,C,D • self testing (except for pregnancy, fertility, chole-sterol, glucose, RBC) • controls without quanti-tative or qualitative value	• blood grouping (except for ABO, Rh, Kell, Duffy) • tissue typing • companion diagnostic screening, • diagnostics and staging of cancer • genetic testing	• transmissible agents • infectious load • blood grouping ABO, Rh, Kell, Kidd, Duffy

ESCCA
European Society
for Clinical Cell Analysis

Regulation EU 2017/746- IVDR: European database on medical devices

Key aspects in fulfilling the objectives of Regulation EU2017/746:

- **European database on medical devices (Eudamed)** that should integrate:
  different electronic systems, information regarding devices on the market, relevant economic operators, certain aspects of conformity assessment, notified bodies, certificates, performance studies, vigilance market surveillance to be fully functional from 2023

- **Notified bodies:**
  assessment of quality management system and technical documentation of at least one product of corresponding category: actually 7 but need of conformity assessment for 90% of all IVDMD

- **EU reference laboratories:**
  to verify the performance claimed by the manufacturer and the compliance of class D devices, to carry out appropriate tests on samples of manufactured class D devices, to provide scientific and technical assistance, scientific opinions in response to consultations by notified bodies: no official list of EU reference laboratories so far

ESCCA
European Society
for Clinical Cell Analysis

Regulation EU 2022/112- amending Regulation (EU) 2017/746

Transitional provisions for certain in vitro diagnostic medical devices

- Class A nonsterile devices: 26 May 2022 (no deferred application of requirements)
- Class D devices: 26 May 2025
- **Class C devices: 26 May 2026**
- Class B devices: 26 May 2027
- Class A sterile devices: 26 May 2027
- All certificates issued under IVD-D before the IVD-R fully applies remain valid until 26 May 2025

ESCCA
European Society
for Clinical Cell Analysis

Regulation EU 2017/746- IVDR in-house devices

- Health institutions should have the possibility of manufacturing, modifying and using **devices in-house:**
  - address specific needs of target patient groups
  - non-industrial scale
  - no equivalent device (to address the specific needs of target patient group in an appropriate way) available on the market

- Conditions to qualify for exemption of regulation rules (Article 5.5):
  - no transfer of devices
  - compliance with QMS and ISO 15189 or national provisions regarding accreditation
  - justification of use (no commercially available alternative)
  - info to authorities
  - public declaration regarding general safety and performance requirements
  - extra requirements for class D devices (almost to the level of CE-IVD product)
  - evaluation of use (vigilance, surveillance)

IVDR 2017/76/EU

ESCCA
European Society
for Clinical Cell Analysis

ISO 15189:2012 Medical Laboratories
CLSI H62

- ISO 15189 is the only global standard for the accreditation of medical laboratories
- Specifies international requirements for quality and competence for medical laboratories
- Not mandatory for medical laboratories, however with concern to the EU IVD-R 2017/746 regulation, accreditation becomes a requirement for all clinical laboratories within EU that perform LDTs
- ISO/FDIS 15189 with emphasis on LDTs under development (stage 50.00)
- CSLI H62: provides validation strategies for cell-based tests performed by flow cytometry

  - recommendations for instrument qualification and standardization and rest optimization

  - recommended practices for the examination and post examination phases

  - unique requirements for the analytical validation of cell- based tests

ISO 15189:2012 Medical Laboratories

- **Verification: confirmation of the performance claims through obtaining objective evidence (in the form of performance characteristics)**
  - validated examination procedures used without modification

- **Validation: confirmation through the provision of objective evidence (in the form of performance characteristics), that the specific requirements for the intended use of the examination have been fulfilled**
  - non-standard methods
  - laboratory designed or developed methods
  - methods used outside their intended scope
  - validated methods subsequently modified
  - validated methods at other clinical decision levels than the ones that are of interest

ISO 15189:2012 Medical Laboratories
CSLI H62

Validation shall be "as extensive as necessary" and should include consideration concerning the pre-analytical, analytical and post-analytical processes:

a. pre-analytical processes:
- sample collection and handling
- sample transportation
- sample reception
- pre-examination handling, preparation and storage
- large number of clinical studies, guidelines, expert statements

b. analytical processes:
- instrument performance check: signal linearity, accuracy, resolution, tracking and standardization
- scatter TV optimization
- PMTv optimization
- PMT (MFI TV) standardization
- spectral overlap compensation
- EuroFlow approach, Harmonemia, individual approach

ISO 15189:2012 Medical Laboratories
CSLI H62

Validation shall be "as extensive as necessary" and should include consideration of:

c. post-analytical processes:
- measurement trueness/accuracy
- measurement precision including measurement repeatability and measurement intermediate precision
- measurement uncertainty
- analytical specificity, including interfering substances
- analytical sensitivity (LoB, LoD)
- functional sensitivity (LLoQ)
- clinical specificity
- clinical sensitivity
- positive predictive value
- negative predictive value
- assay carryover
- reportable range
- stability of specimen and processed sample

Regulation(EU) 2022/112 amending Regulation (EU) 2017/746

Deferred application of requirements for in-house devices

- **Valid from 26.5.2022**
  - use within one entity
  - transfer of devices only within one entity
  - no commercial scale

- **Valid from 26.5.2024**
  - info to authorities
  - compliance with QMS/ISO 15189
  - justification of use
  - extra requirements for “class D”
  - post market surveillance

- **Valid from 26.5.2028**
  - need do prove absence of commercially available alternative

ESCCA
European Society
for Clinical Cell Analysis

ISO 15189:2012 Medical Laboratories
CSLI H62

- **Qualitative assays**
  - lack proportionality to the amount of analyte
  - **categorical data is reported**
  - example: leukemia / lymphoma / MDS immunophenotyping, AML MRD

- **Semi quantitative assays**
  - does not use calibration standard to determine the absolute quantitative values for unknown samples
  - **numeric data is reported**
  - use of cut-off or threshold for disease detection
  - example: PNH, double platform CD34 stem cell assay, B-ALL MRD, CLL MRD, MM MRD, TCR Cß1…

- **Quantitative (relative)**
  - **use of calibration standard** to determine the absolute quantitative values for unknown samples
  - example: PMN CD64 assay, CD34 stem cell assay, lymphocyte subset assessment, fetal red cell enumeration…

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Qualitative (clinical) sensitivity, specificity, positive and negative predictive value

	Disease ≥ 5 specimens	No disease ≥ 5 specimens
Positive test	True positive (TP)	False positive (FN)
Negative test	False negative (FN)	True negative (TN)

Positive predictive value:
TP / TP+FP x 100

Negative predictive value:
TN / TN+FN x 100

Qualitative sensitivity:
TP / TP+FN x 100

Qualitative specificity:
TN / TN+FP x 100

acceptance: 100% concordance between new and previously validated (flow or molecular) assays

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Qualitative and quasi-quantitative trueness/accuracy:

- The closeness of agreement between the average value obtained from a large series of test results (trueness) / a test result (accuracy) and the accepted reference value
- Usually, there is no reference standard available for the assay: trueness/accuracy is not directly applicable
- **Interlaboratory comparison (ISO 15189:2012)**

  a. accredited interlaboratory comparison programs: UK NEQAS, CAP's Proficiency Testing Program

  b. alternative approaches:
  - certified reference materials
  - samples previously examined
  - exchange of samples with other laboratories

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ESCCA
European Society
for Clinical Cell Analysis

ESSCA Interlaboratory Comparison Program– EILCP

- EILCP kick off: March, 2019
- Number of cycles: 8
- Frequency: 1-2x per year
- **Availability: ESCCA members**
- Participants: 39
- Countries: 16

EILCP cycle	Frequency
AML MRD	1-2 x per year
B-ALL MRD	1-2 x per year
CLL MRD	1-2 x per year
MM MRD	1-2 x per year
PNH HR	1-2 x per year
Diagnostics	1-2 x per year
T-ALL MRD	1-2 x per year
HCL MRD	1-2 x per year
CML LSC	In preparation

- Aadvantages:
  - result from accredited centre
  - representative dot-plots
  - reporting abs. and rel. values
  - reporting analytical and functional sensitivity
  - analysis of background normal populations
- Disadvantages:
  - not accredited
  - does not reflect pre analytical phase

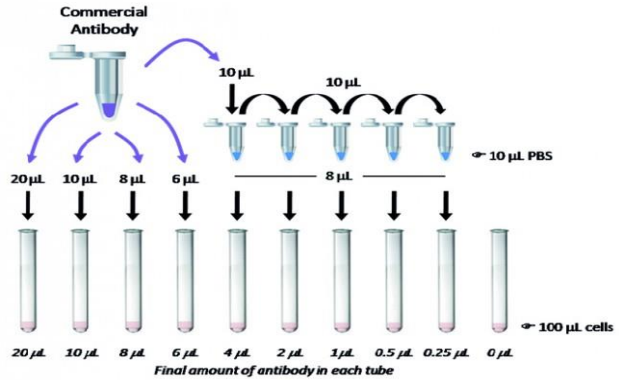
The aim is to offer an interlaboratory comparison of immunophenotypic results for an educational purpose but also as a tool to meet validation requirements following ISO-15189.

ESCCA
European Society
for Clinical Cell Analysis

ISO 15189:2012 Medical Laboratories
CSLI H62

Qualitative and quasi-quantitative (analytical) specificity: clone / fluorochrome / antibody titration

- Reagent validation:
  - clone, fluorochrome validation
  - analytical specificity (compared to validated conjugates)
  - stain index and/or signal:noise ration
  - optimal titration
- Use of previously validated antibody:
  - EuroFlow immunostaining protocols
  - ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in PNH and related disorders
  - International guidelines for flow cytometric evaluation of SS and MF
  - ELN AML MRD 2021 guidelines
  - ELN MDS guidelines



Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
J.J.M. van Dongen et al. Leukemia 2012
M. Heuser et al. Blood 2021
Westers et al., Haematologica 2017
Noemi Munoz-Garcia et al. Cancers 2021
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Quasi-quantitative (analytical + functional) sensitivity:

a. quasi-quantitative (analytical) sensitivity

- limit of blank (LoB), defined by the highest apparent signal detected in replicates of a sample containing no measurand
- limit of detection (LoD) defined by the lowest level of measurand, that can be reliably distinguished from the LoB

b. quasi-quantitative (functional) sensitivity

- lower limit of quantitation (LLoQ), defined by the lowest level of measurand that can be reliably detected at predefined levels of bias and imprecision

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Limit of blank (LoB):

evaluate 5 negative samples in 5 replicates run over a few separate days and calculate the mean and standard deviation of the events falling outside the expected distribution of target cells:

LoB = mean of blank + 1.645 SD of blank assuming, that 95% of negative values will be below this limit

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

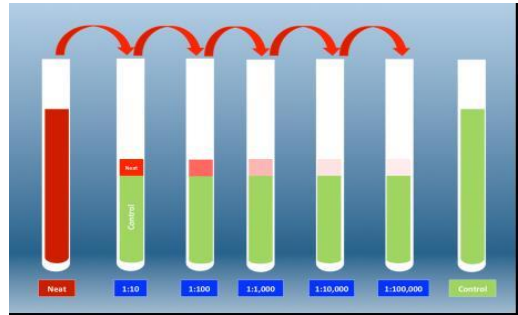
ISO 15189:2012 Medical Laboratories
CSLI H62

Limit of detection (LoD):

- evaluate 3-5 replicates of neat sample, control sample and 5 serial dilutions (1:10, 1:100, 1:1000, 1:10 000, 1:100 000) run over a few separate days (spiking test) and calculate the lowest level of target cells, that can be reliably distinguished from LOB with acceptable CV (<20%)

- evaluate a few replicates of a few negative specimens run over a few separate days and calculate the mean and SD according to: LoD = mean of blank + 2SD (3SD) of blank

Acceptability: CV < 20% between replicates



Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Lower limit of quantification (LLoQ):

- evaluate 3-5 replicates of neat sample, control sample and 5 serial dilutions (1:10, 1:100, 1:1000, 1:10 000, 1:100 000) run over a few separate days (spiking test) and calculate the inter-assay imprecision for a few positives near the expected LLoQ expressed as CV%

Acceptability: < 20% deviation

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

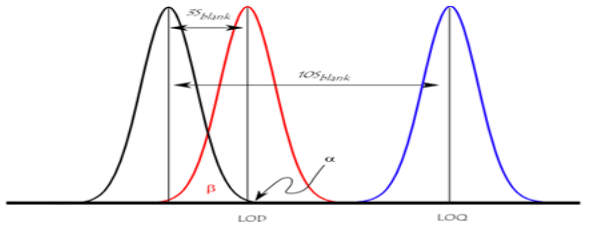
ESCCA
European Society
for Clinical Cell Analysis

ISO 15189:2012 Medical Laboratories
CSLI H62

Analytical sensitivity (LoD = 20)	Functional sensitivity (LLoQ = 50)	Cells required for flow cytometry	Cells required for molecular analysis
0.1 % (10^{-3})	0.25 % (10^{-3})	> 20 000	> 2.5 000 (0.015 µg DNA)
0.01 % (10^{-4})	0.025 % (10^{-4})	> 200 000	> 25 000 (0.15 µg DNA)
0.001 % (10^{-5})	0.0025 % (10^{-5})	> 2 000 000	> 250 000 (1.5 µg DNA)
0.0001 % (10^{-6})	0.00025 % (10^{-6})	> 20 000 000	> 2 500 000 (15 µg DNA)
0.00001 % (10^{-7})	0.000025 % (10^{-7})	> 200 000 000	> 25 000 000 (25 µg DNA)

LoD: the smallest number of events required to reproducibly detect a target population-**20 events**
LLoQ: smallest number of events required to reproducibly quantify a target population-**50 events**

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021



ISO 15189:2012 Medical Laboratories
CSLI H62

Qualitative and quasi quantitative precision:

- intra-assay imprecision (repeatability): 3 replicates from at least 5 samples including low, medium and high levels of disease) assayed within a single analytical run

- inter-assay imprecision (intermediate precision): 3 replicates from at least 5 samples including low, medium and high levels of disease) assayed in separate analytical runs (instrument power-down and recalibrated)

Acceptability: < 20% deviation

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

Uncertainty of measured quantity values: the expression of the statistical dispersion of the values attributed to a measured quantity, when all sources of error have been considered

i. No reference material available: only one uncertainty - intermediate measurement precision (u_{Rw})

- standard uncertainty of measurement*: $u_{Rw} = SD_{IMP}$
- relative uncertainty of measurement*: $u_{Rw,rel} = CV_{IMP}$
- expanded standard uncertainty of measurement*: $U = u \times 2 = SD_{Rw} \times 2$ (95% confidence level)
- expanded relative uncertainty of measurement*: $U_{rel} = u_{rel} \times 2 = CV_{Rw} \times 2$ (95% confidence level)

ii. Reference material available: intermediate measurement precision (u_{Rw}) + uncertainty of the reference material (u_{ref})

- combined relative uncertainty of measurement**: $u_{c,rel} = \sqrt{u_{Rw,rel}^2 + u_{ref,rel}^2}$
- expanded combined relative uncertainty of measurement**: $U'_{c,rel} = 2 \times u'_c$ (95% confidence level)

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Quasi quantitative linearity:

- not possible given the lack of reference standard

Assay carryover:

- run one negative sample, followed by one high positive specimen in triplicate, followed by the negative specimen in triplicate and assess the latter events carried over from the positive specimen

Acceptability: qualitative concordance 100%

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Quasi quantitative reportable range:

- abnormal cells not detected < LOD
- abnormal cells detectable but not quantifiable >LOD but <LLOQ
- abnormal cells quantifiable: >LLOQ

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Quasi quantitative reference range:

- set of values obtained from the analysis of a cohort of healthy (normal) adult and/or pediatric individuals for the purpose of interpretation of results

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ISO 15189:2012 Medical Laboratories
CSLI H62

Stability of specimen and processed sample:

- specimen stability: ≥ 3 specimens run within 2 h and at 24, 48 and 72 h time points.

  Acceptability: 100% concordance

- processed sample stability: ≥ 3 specimens with no delay in acquisition, compared to samples acquired at 2, 4 and 8 h time points.

  Acceptability: < 20% deviation

Barnett D et al.; Cytometry 2013;84B;309-314
Wood B et al.; Cytometry 2013;84B;315-323
Validation of Cell-Based Fluorescence Assays-ICSH/ICCS Practice Guidelines, Cytometry 2013
CSLI-H62 Validation of Assays Performed by Flow Cytometry, 2021

ESCCA
European Society
for Clinical Cell Analysis

Take-home messages

- EU IVDR 2017/746 intends to guarantee high standards of quality and safety for IVDMD- **valid from 26 May 2022**
- Key aspects in fulfilling the objectives of Regulation EU2017/746 (Eudamed, Notified Bodies, EU Reference Laboratories) **still pending**
- **Transitional provisions** for certain IVDRMD (26 May 2026 for Class C devices, valid IVD-D certificates until 26 May 2025
- **LDTs will still represent the majority of laboratory performed tests**
- Regulation EU2017/746 requires compliance with appropriate **quality management systems** and **ISO 15189** or national provisions regarding accreditation for devices in-house

ESCCA
European Society
for Clinical Cell Analysis

Take-home messages

- ISO 15189 is not mandatory for medical laboratories, however with concern to the EU IVD-R 2017/746 regulation, accreditation becomes a **requirement for all clinical laboratories within EU that perform LDTs**
- ISO 15189 requires **independent verification** in the form of performance characteristics of the performance claims for previously validated assays
- ISO 15189 requires **validation** in the form of performance characteristics, that specific requirements have been fulfilled for non-standard assays, laboratory designed tests, validated tests subsequently modified and off-label use of validated assays
- **ISO 15189** and **CLSI-H62** provide guidelines for in-house device validation assuring conformity with EU IVD-R 2017/746
- The EU IVD-R requirements of compliance with ISO 15189 for EU labs will be mandatory from **26 May 2024**

THANK YOU FOR THE ATTENTION