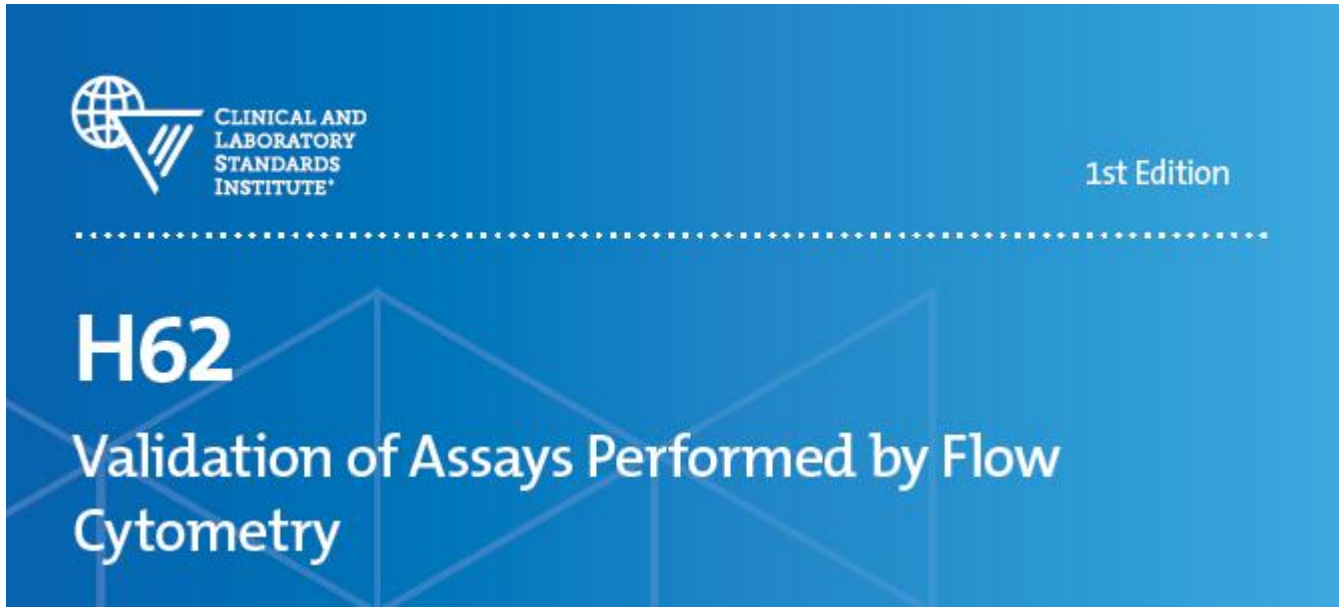
Assay modification and ad hoc antibody modification – recommendations and safeguards

APR. BIOL. ELENI LINSKENS – UZ BRUSSEL

CLSI H62

- Clinical and Laboratory Standards Institute
- H62: Validation of assays performed by flow cytometry



Chapter 1: Introduction

Chapter 2: Path of WorkFlow and Quality System Essentials

Chapter 3: Fit-for-Purpose Approach to Analytical Method Validation of Flowcytometric Methods

Chapter 4: Instrument Qualifications, Setup and Standardization

Chapter 5: Assay Development and Optimization

Chapter 6: Analytical Method Validation

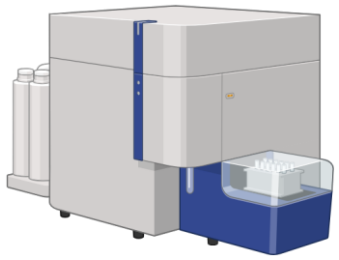
Chapter 7: Examination and postexamination Phase

Qualitative FC assay (Leukemia/Lymphoma panel)	Semi-quantitative FC assay (TBNK analysis, PNH, MRD)
Accuracy/diagnostic concordance	Accuracy/Truness
Selectivity (assay-development)	Selectivity (assay-development)
Detection Capability (LLOD e.g. if abnormal population reported in %)	Detection Capability (LOB/LLOD/LLOQ)
Precision	Precision / MU / Linearity
Stability (specimen, AB cocktail)	Stability (specimen, AB cocktail)
Reference intervals (depending on the intended use of the FC panel)	Reference intervals
Carryover	Carryover
Clinical specificity/sensitivity	Clinical specificity/sensitivity

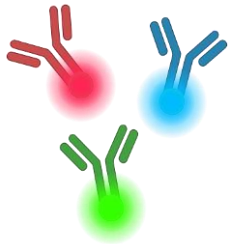
CLSI H62

- Clinical and Laboratory Standards Institute
- H62: Validation of assays performed by flow cytometry

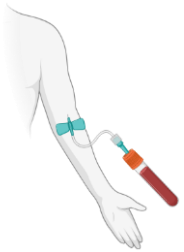
Assay Modifications



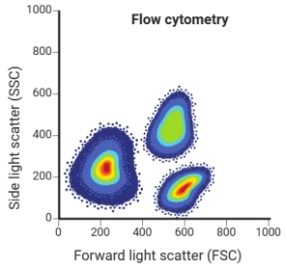
INSTRUMENT OR PLATFORM TECHNOLOGY



ANTIBODIES OR FLUOROPHORES



SAMPLE COLLECTION



GATING STRATEGY

126 cells/µL or above

100 -125 cells/µL

99 cells/µL or below

REFERENCE RANGES

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ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Flow cytometry assay modifications: Recommendations for method validation based on CLSI H62 guidelines

Monaghan, S. A., Eck, S. et al. *Cytometry part B: Clinical Cytometry 108*(3), 252-266.

Received: 1 May 2025 | Revised: 19 August 2025 | Accepted: 2 September 2025
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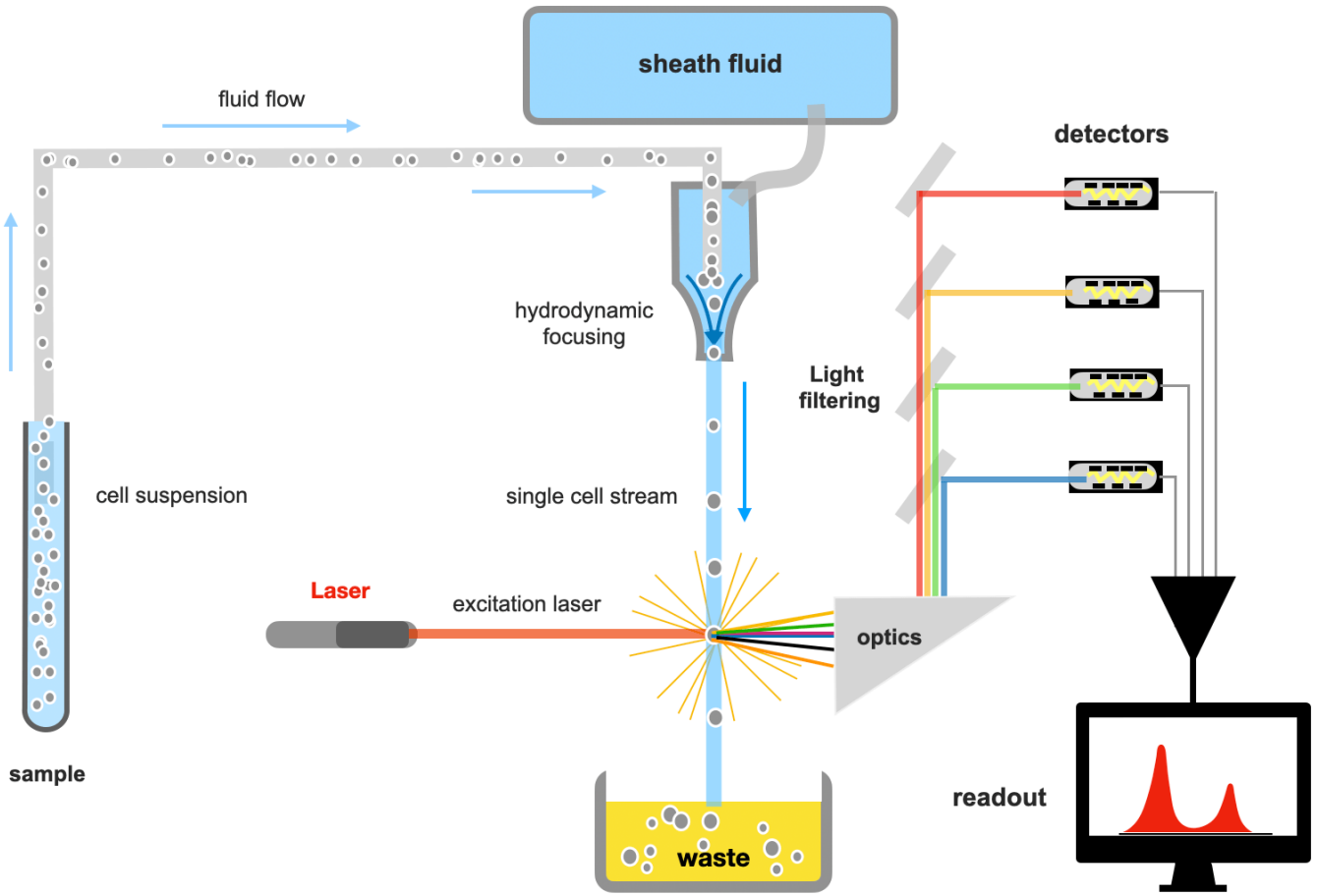
BEST PRACTICE

CLINICAL CYTOMETRY WILEY

Ad hoc antibody modification of a validated flow cytometric immunophenotyping panel—recommendations and safeguards for clinical laboratories

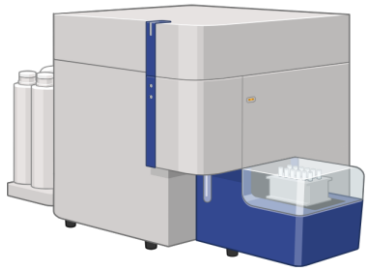
Groves, C. J., Linden, M. A. et al. *Cytometry part B: Clinical Cytometry, 110*(1), 11-21.



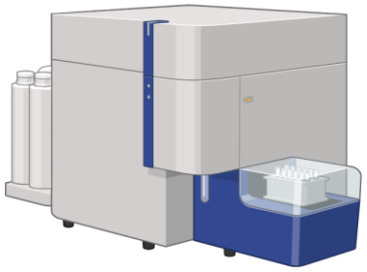
Instrument and Platform changes

- Considerations
  - Type of instrument (conventional vs. spectral)
  - Type of lasers
  - Optical filter configuration
  - Fluidics system
  - Detector sensitivity
  - Acquisition software

Instrument and Platform changes

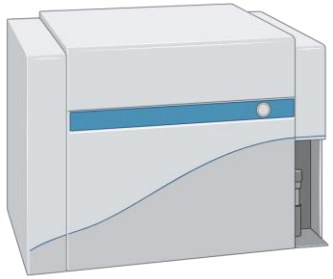


BD FACSLyric



BD FACSLyric

Navios



Cytoflex

TABLE 1 Validation studies suggested for flow instrument/platform assay modifications.

	Qualitative assay	Quasi/semiquantitative assays
Flow cytometer of the same model (e.g., BD FACSCanto™ II to BD FACSCanto™ II; or Navios to Navios)	Accuracy ($n = 3$)	Accuracy ($n = 5$) including low-positive samples around LLoQ
Flow cytometer of a different model but similar performance parameters (e.g., BD FACSCanto™ II to BD FACSLyric™; or Navios to Cytoflex)	Accuracy ($n = 5$)	Accuracy ($n = 5$) Precision ($n = 2$) LLoD/LLoQ ($n = 2$) Carryover ($n = 1$)

Abbreviations: LLoD, lower limit of detection; LLoQ, lower limit of quantitation.

Monaghan, S. A. et al. *Cytometry part B*

Panel modifications:
antibody or fluorophore changes

- CLSI H62 Table A6. Laboratory-intended Assay Revision
  - Minor changes: changes in non-critical reagents
  - Moderate changes: antibody or fluorophore changes

Table A6. Laboratory-Initiated Assay Revision

Extent of Change	Category of Change	Revision	Reason for Change	Required QA Process
Minor	Operational	Source of buffer or other simple reagents should be changed.	Item no longer available, or a less-expensive alternative is available.	• Reagent qualification • $N = 5$
Minor	Operational	≥ 1 markers should be omitted from a validated panel.	Simplification of assay when a marker is not adding to the clinical sensitivity/specificity of assay	None

Table A6. Laboratory-Initiated Assay Revision

Extent of Change	Category of Change	Revision	Reason for Change	Required QA Process
Moderate	Operational	An antibody of the same specificity but representing a different clone conjugated to the same fluorophore should be used as a substitute.	Item no longer available, or a less-expensive alternative is available.	• Verification that the population identified and the strength of the fluorescent signal are the same is required • *N* = 10
Moderate	Operational/clinical	An antibody of a different specificity with the same fluorophore without requiring any additional changes in the antibody panel (other than leaving out an antibody, eg, changing an antibody on a fluorophore with no compensation issues in the panel).	Better identification of disease subcategories, eg, substituting CD200 for CMC-7 in a B-cell panel	• Verification that there is no difference in the sensitivity and specificity for disease is required • *N* = 20 (5 is normal, and 15 is abnormal)
Moderate	Operational/clinical	An antibody of the same specificity, conjugated to a different fluorophore without requiring any additional changes in the antibody panel (other than leaving out an antibody), should be used as a substitute.	Better identification of disease	• Verification that there is no difference in the sensitivity and specificity for disease is required. • *N* = 20 (5 is normal, and 15 is abnormal)
Moderate	Operational/clinical	An antibody of the same or different specificity, conjugated to a different fluorophore but necessitating additional changes in the antibody panel (because of potential compensation issues), should be used as a substitute.	Better identification of disease	• CLIA/IMDR validation is required. • *N* = 20 for sensitivity/specificity and experiments for precision, reproducibility, LoB, LLoD, and if semiquantitative, LLoQ and linearity
Moderate	Operational/clinical	≥ 1 antibodies of novel specificity and fluorophore should be added to an existing panel (eg, going from 8 to 10 color).	Better identification of disease	• CLIA/IMDR validation is required. • *N* = 20 for sensitivity/specificity and experiments for precision, reproducibility, LoB, LLoD, and if semiquantitative, LLoQ and linearity

Abbreviations: CLIA, Clinical Laboratory Improvements Amendments of 1988; IMDRF, International Medical Device Regulators Forum; LLoQ, lower limit of quantitation; LoB, limit of blank; LLoD, lower limit of detection; QA, quality assurance.

Panel modifications:
antibody or fluorophore changes

- CLSI H62 Table A6. Laboratory-intended Assay Revision
  - Moderate changes: antibody or fluorophore changes
  - Vary in technical complexity and potential impact

→ Full validation

Antibody or fluorophore changes

	Moderate change level 1	Moderate change level 2			Moderate change level 3	
General modification	Single antibody substitution	Single antibody substitution	Antibody of same antigen specificity conjugated to a different fluorophore	Sample collection changes (different specimen/ matrix type, or different anticoagulant)	Any antibody or fluorophore modifications otherwise considered moderate change level 1 or 2 but found to require changes related to instrument setup (e.g., compensation, voltage)	Adding 1–2 antibody(ies) to open channel(s)
Fluorophore	Identical	Identical	Different: Antibody conjugated to a fluorophore previously used in the panel with the prior antibody left out	Different: Antibody conjugated to a fluorophore to be evaluated in a previously open channel	Identical	Different (i.e., as stipulated—new for the panel)
Antigen specificity	Identical (different clone)	Different (e.g., CD56 PE to replace CD7 PE)	Identical (e.g., CD20 FITC changed to CD20 APC, dropping FMC7 APC)	Identical (e.g., replacing CD10 FITC with CD10 BV421)	Identical	Different (i.e., as stipulated—new for the panel)

Monaghan, S. A. et al. *Cytometry part B*

Antibody or fluorophore changes

- To improve resolution of cell population ✓
- To improve assay for situations in which otherwise may fail ✓
- Impact reportable results or need to characterize a new cell population

  ↳ Validation as new assay needed

	Moderate change level 1	Moderate change level 2			Moderate change level 3	
					y antibody or orophore modifications herwise considered derate change level 1 or ut found to require anges related to trument setup (e.g., mpensation, voltage)	Adding 1–2 antibody(ies) to open channel(s)
						Different (i.e., as stipulated—new for the panel)
			the prior antibody left out	previously open channel		
Antigen specificity	Identical (different clone)	Different (e.g., CD56 PE to replace CD7 PE)	Identical (e.g., CD20 FITC changed to CD20 APC, dropping FMC7 APC)	Identical (e.g., replacing CD10 FITC with CD10 BV421)	Identical	Different (i.e., as stipulated—new for the panel)

Panel modifications:
antibody or fluorophore changes

- Validation parameters recommended also depend on the intended use of the FC assay
  - Low clinical risk (e.g. basic research, drug discovery, clinical trials with exploratory endpoints)
  - Moderate clinical risk (LDT's used as an aid to diagnosis in a medical laboratory)
  - High clinical risk (e.g. measurable redisual disease (MRD) LDT's)

Antibody or fluorophore changes: Moderate change level 1

e.g. same antigen, same fluorophore, different clone

Validation	Moderate clinical risk	High clinical risk
Accuracy/diagnostic concordance	Verify pertinent populations are identified, strength of fluorescent signals	
	≥ 10 samples (including normal and abnormal)	≥ 10 samples (including normal and abnormal) Samples span the expected reportable range
Selectivity	Document any differences in specificity when using different clone Use accuracy dataset to demonstrate the intended selectivity	
Detection Capability (LLOD/LLOQ)	NA	Recommended (abbreviated approach)
Precision intra-assay inter-assay	Recommended (abbreviated approach) Not required	Recommended (abbreviated approach) Not required
Linearity	NA	Recommended

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Antibody or fluorophore changes: Moderate change level 1

e.g. same antigen, same fluorophore, different clone

Validation	Moderate clinical risk	High clinical risk
Stability	Not required	Recommended (abbreviated approach)
Reference intervals	Not required	NA
Carryover	To be managed during QA	Not required, if no intent to increase sensitivity
Clinical specificity/sensitivity	Not required	Not required

Antibody or fluorophore changes: Moderate change level 2/3

Level 2: e.g. same antigen/different fluorophore, different antigen/same fluorophore
Level 3: e.g. adding 1-2 antibodies in an open channel

Validation	Moderate clinical risk	High clinical risk
Accuracy/diagnostic concordance	Verify pertinent populations are identified, strength of fluorescent signals	
	≥ 10 samples (including normal and abnormal)	≥ 10 samples (including normal and abnormal) Samples span the expected reportable range
Selectivity	Document any differences in specificity when using different clone Use accuracy dataset to demonstrate the intended selectivity	
Detection Capability (LLOD/LLOQ)	NA	Recommended (abbreviated approach)
Precision intra-assay inter-assay	Recommended (abbreviated approach) Recommended (abbreviated / full)	Recommended (abbreviated approach) Recommended (abbreviated / full)
Linearity	NA	Recommended

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Antibody or fluorophore changes: Moderate change level 2/3

e.g. same antigen/different fluorophore, different antigen/same fluorophore
e.g. adding 1-2 antibodies in an open channel

Validation	Moderate clinical risk	High clinical risk
Stability	Recommended (abbreviated approach)	Recommended (abbreviated approach)
Reference intervals	Depends on intended use	NA
Carryover	To be managed during QA	Not required, if no intent to increase sensitivity
Clinical specificity/sensitivity	Using the accuracy dataset	

Ad hoc antibody modifications

WHEN

- Rare cases/circumstances
  - Aid in phenotypic characterization of hematologic neoplasms
  - Typically no more than five patients a year
- Short-term supply chain problems

OUT OF SCOPE – NOT RECOMMENDED

- Modifications altering intended use
- > 2 modifications
- Tandem-fluorochromes
- Uncharacterized antibody-fluorochrome
- Method alteration

Groves, C. J., Linden, M. A. et al. *Cytometry part B: Clinical Cytometry, 110*(1), 11-21.

Ad hoc antibody modifications

- Characterized antibody-fluorochrome
  - Direct hands-on experience with the antibody-fluorochrome introduced
  - Experience in staining pattern, intensity
  - Pre-established titer
  - Appropriate compensation/unmixing controls

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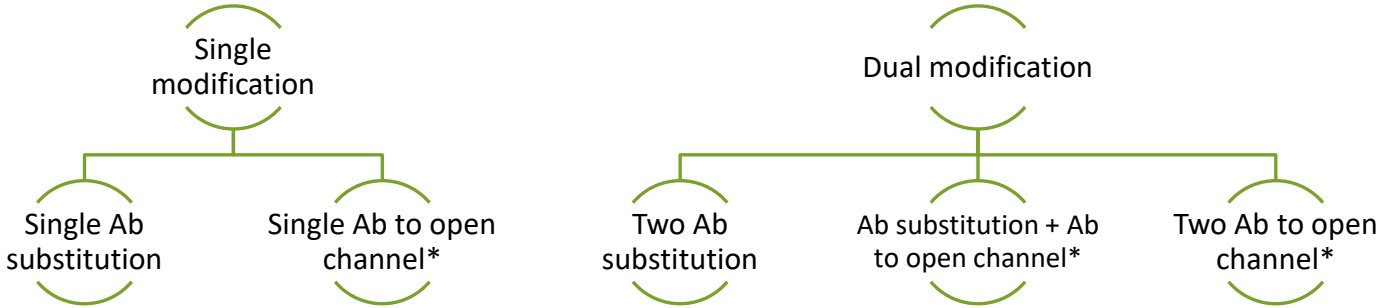
Ad hoc antibody modifications



Fluorochrome	Original	1	2	3	4
FITC	TRBC1	TRBC1	TRBC1	TRBC1	TRBC1
PE	TRBC2	TRBC2	TRBC2	**CD7**	TRBC2
PECy7	CD5	CD5	CD5	CD5	CD5
PerCpCy5.5	CD3	CD3	CD3	CD3	CD3
APC	CD7	**CD2**	CD7	**CD2**	**CD2**
APC-R718					
APCH7	CD8	CD8	CD8	CD8	CD8
V450			**CD2**		**CD7**
V500	CD45	CD45	CD45	**CD45**	CD45
BV605	CD4	CD4	CD4	CD4	CD4
BV786	CD3	CD3	CD3	CD3	CD3
BV711					

Groves, C. J., Linden, M. A. et al. *Cytometry part B: Clinical Cytometry, 110*(1), 11-21.

Ad hoc antibody modifications

Steps and troubleshooting

Steps to demonstrate no adverse effect(s) of the ad hoc modification	• Evaluate the performance by examining NxN plots of all the fluorescent channels to compare each antibody-fluorochrome in the modified tube against each other. Determine if there is any adverse impact that may affect the interpretation. • Compare performance to the original unmodified set of antibodies in the tube (whenever feasible) • Ensure that the key population(s) are interpretable safely and confidently, document the change, and review by the medical director (or designee). • If the staining patterns appear to be inappropriate or concerning/uncertain for adverse effects: A. Troubleshooting may involve one or more of the following: 1. If enough specimen is available: A. Compare performance to the original unmodified set of antibodies in the tube B. FMO studies can be performed C. If the data appears to be under- or over-compensated, in a way that affects the interpretation, this can be managed based on the experience of the pathologist (e.g., post-acquisition adjustment of SOV, or reestablishing SOVs/ compensation matrix) 2. If no specimen remains, the above troubleshooting studies could be conducted on a similar specimen to determine if the concern can be resolved, and the findings can be reported or not. 3. If the pathologist can justify reporting the findings without additional work, the results can be reported; however, such interpretation must be made with caution. It is highly recommended to consult an experienced pathologist before signing out the report. • Document all the steps taken. • The medical director (or designee) of the flow cytometry laboratory should decide to either approve or disapprove the use of the ad hoc modification for reporting. • Add a disclaimer to the report to indicate the deviation from the standard assay protocol.

Note: If the substitution of one of two antibodies into a used channel is performed, then comparison with the original panel may be challenging and the appropriate use of internal controls must be made to accurately determine if any effects to the perform have occurred.

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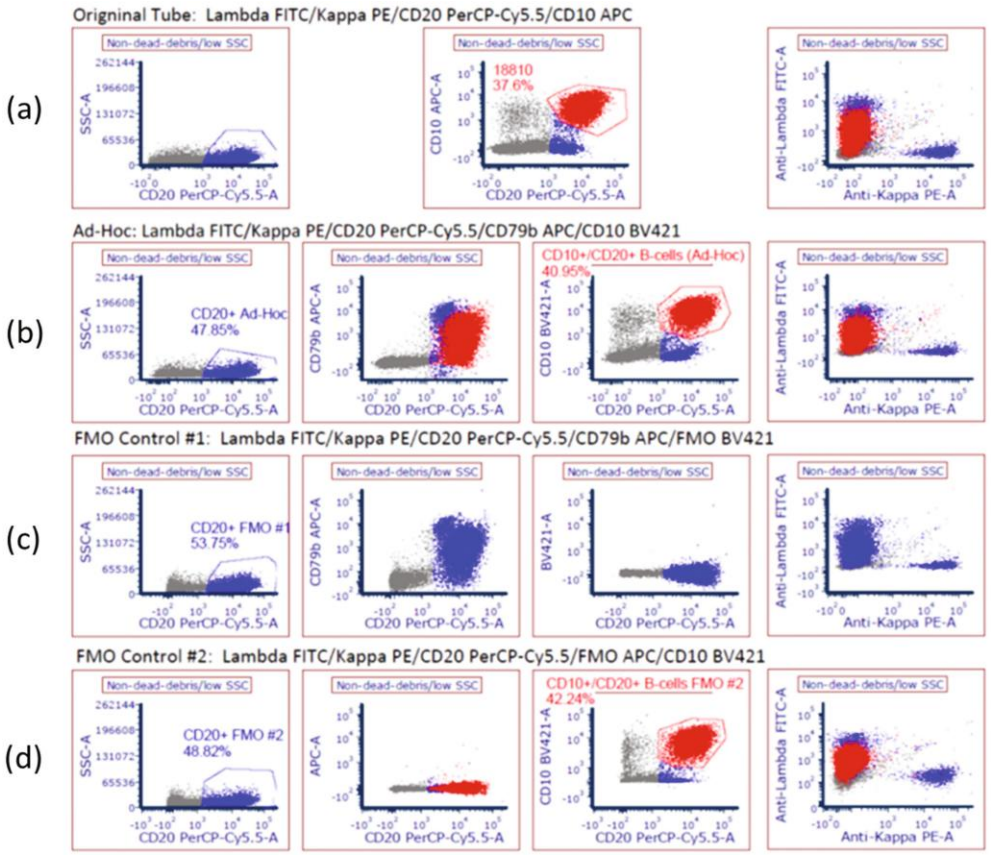
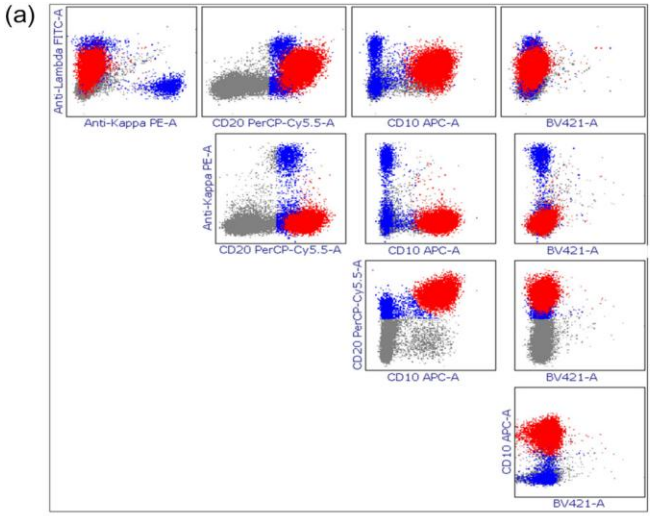
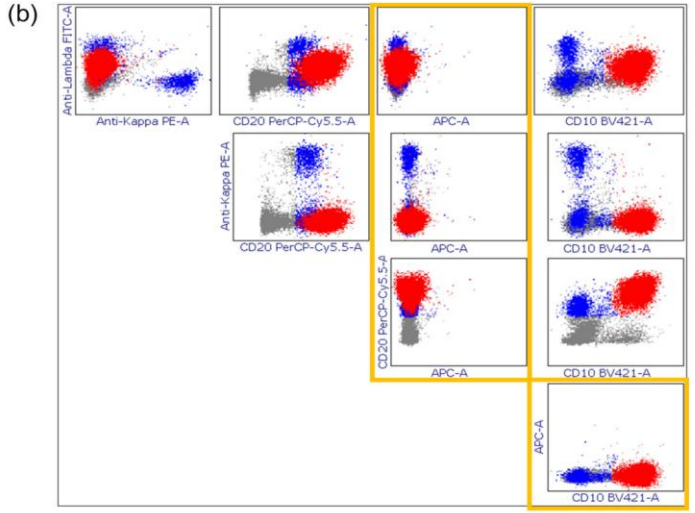
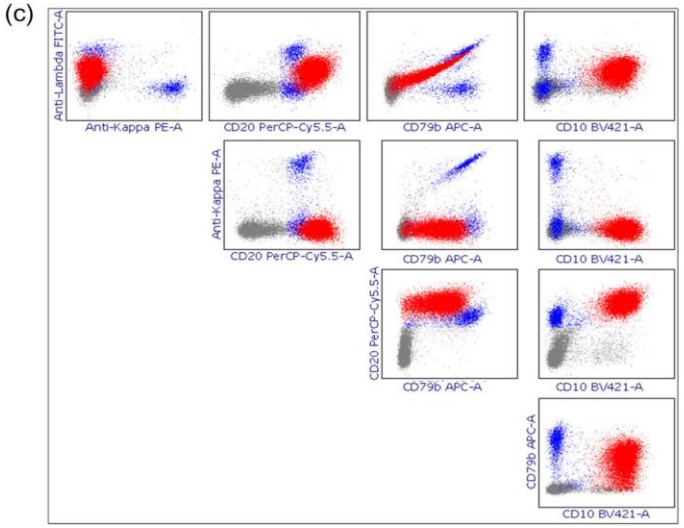

(a)
Origninal Tube: Lambda FITC/Kappa PE/CD20 PerCP-Cy5.5/CD10 APC
Non-dead-debris/low SSC
SSC-A
CD20 PerCP-Cy5.5-A
CD10 APC-A
18810
37.6%
Anti-Lambda FITC-A
Anti-Kappa PE-A
(b)
Ad-Hoc: Lambda FITC/Kappa PE/CD20 PerCP-Cy5.5/CD79b APC/CD10 BV421
CD20+ Ad-Hoc
47.85%
CD79b APC-A
CD10 BV421-A
CD10+/CD20+ B-cells (Ad-Hoc)
40.95%
(c)
FMO Control #1: Lambda FITC/Kappa PE/CD20 PerCP-Cy5.5/CD79b APC/FMO BV421
CD20+ FMO #1
53.75%
(d)
FMO Control #2: Lambda FITC/Kappa PE/CD20 PerCP-Cy5.5/FMO APC/CD10 BV421
CD20+ FMO #2
48.82%
CD10+/CD20+ B-cells FMO #2
42.24%

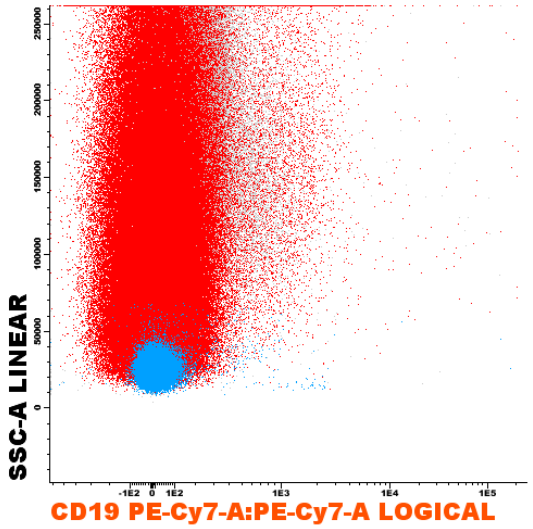

(a)
Anti-Lambda FITC-A
Anti-Kappa PE-A
CD20 PerCP-Cy5.5-A
CD10 APC-A
BV421-A

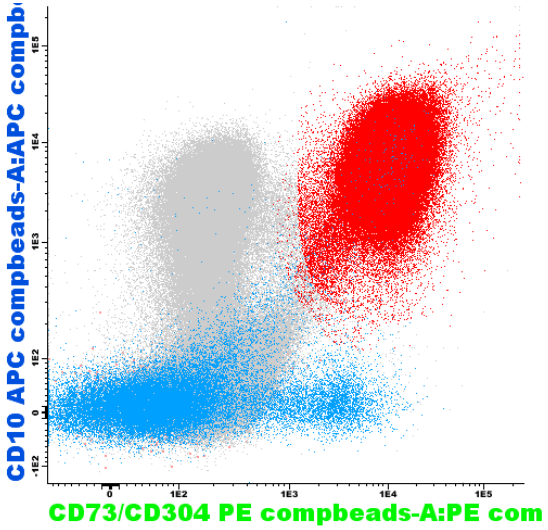

(b)
Anti-Lambda FITC-A
Anti-Kappa PE-A
CD20 PerCP-Cy5.5-A
APC-A
CD10 BV421-A

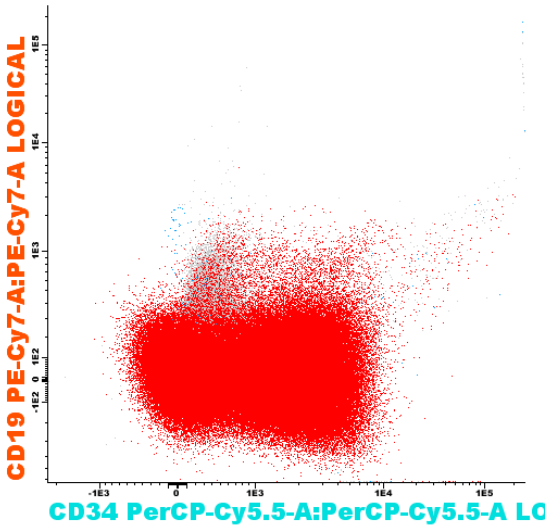

(c)
Anti-Lambda FITC-A
Anti-Kappa PE-A
CD20 PerCP-Cy5.5-A
CD79b APC-A
CD10 BV421-A

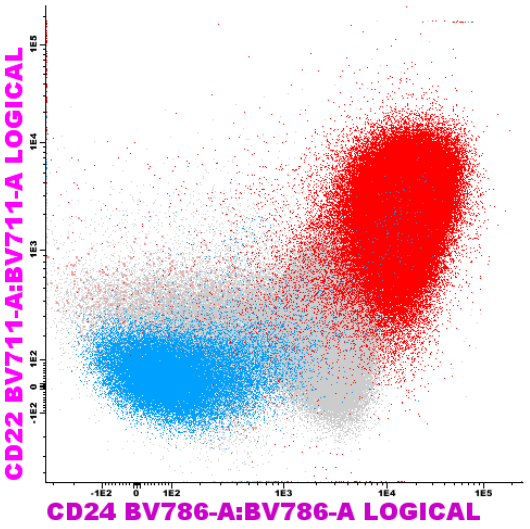
Change in gating strategy

- B-ALL with CD19 negative relapse after Blinatumumab (anti-CD19 immunotherapy)









Change in gating strategy

LDTs in response to immune targeted therapy (e.g. anti-CD19 or anti-CD33 therapy)

- Dry-lab part:
  - Using existing data obtained with the original gating strategy by unaltered FCS files
  - Compare to new gating strategy with altered FCS files (fluorescence data of therapy target ellectronically removed)

- Wet lab part:
  - Comparison of the two different analysis strategies using patient samples including a subset treated with the targeted therapy.

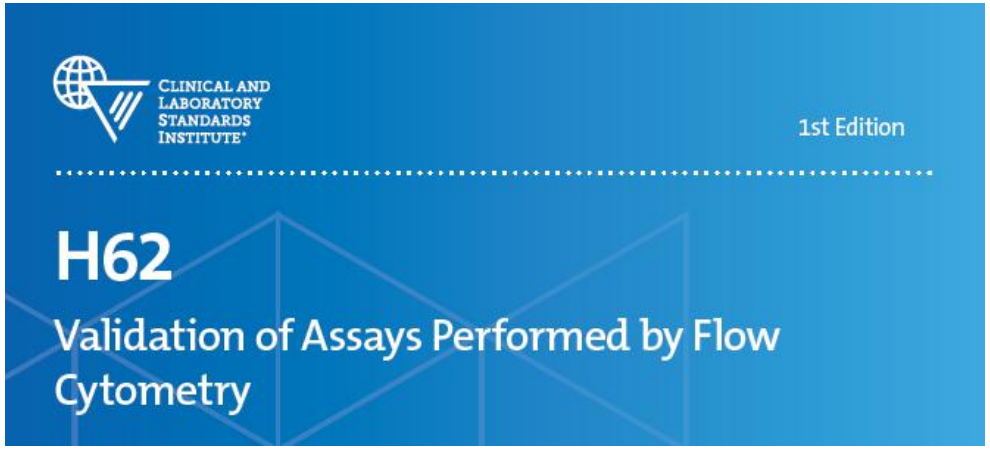
Monaghan, S. A. et al. *Cytometry part B;* Verbeek et al. *Br. J. Haematol*. 2022

Change in gating strategy
Including additional reportable results (potential new biomarkers)

- Potential new population(s) are present in initial validation cohort
  - Reanalyze generated FCS-files using the new gating strategy
  - Demonstrate performance characteristics of the newly defined population
  - Fit-for-purpose validation approach according to CLSI H62
- Potential new population(s) are not present in initial validation cohort
  - Additional wet-lab work is needed to validate the parameters.
  - Using a cohort of samples with presence of the potential new population
  - Fit-for-purpose validation approach according to CLSI H62

Take-Home message

- Validation of assay modifications
  - Change of the assay should be evaluated
  - Often an abbreviated validation is possible
  - Choice of validation plan should be well documented
- Ad hoc antibody modifications
  - In rare cases / circumstances
  - Check for adverse effects on the panel interpretation due to modification



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CLINICAL CYTOMETRY WILEY

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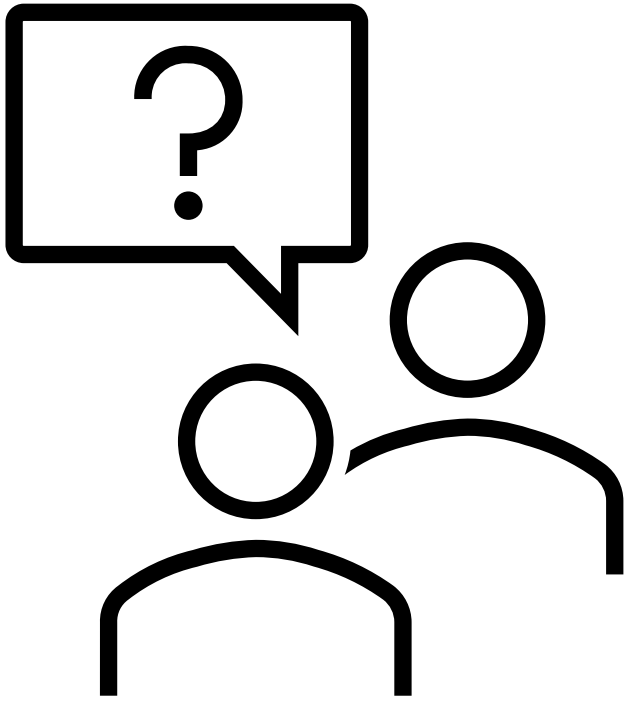
MB&C COURSE 2026 WORKSHOP OVERVIEW

Topic/Level	WORKSHOP SESSION 1 5 February 2026 14.00u -15.15u	Speaker	Room
Flow/Basic	Validation of flowcytometry tests	Malicorne Buysse (UZ Gent)	001
Molec/Basic	Nanopore sequencing	Jozef Dingemans (Jessa, Hasselt)	002
Molec/Advanced	IGH somatic hypermutation analysis	Sabine Franke (CHU Liège)	003
Molec/Advanced	Molecular MRD applications in hemato-oncology	Marleen Bakkus, Jona Van Ver Straeten, Emma Kabongo Kanjinga (UZ Brussel)	004
Molec/Flow/Advanced	From Variant to Visualization: Mutation Detection Meets Flow Cytometry with superRCA® technology	Sara Bodbin (Rarity Bioscience AB/Analis)	013



Questions?