
Challenges in MRD analysis in T-ALL by flow cytometry

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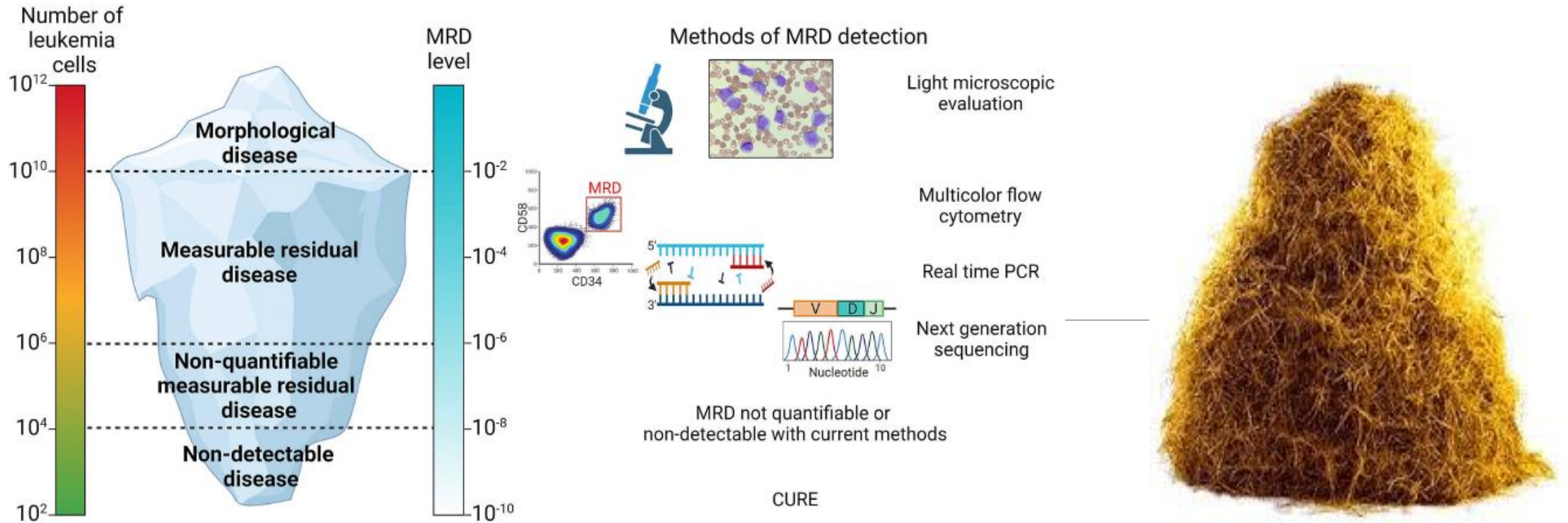
Disclosure commercial conflict of interest

<input checked="" type="checkbox"/>	No, nothing to disclose
<input type="checkbox"/>	Yes, as specified below:



MRD (*Minimal/Measurable Residual Disease*)

→ the remnants of leukemia/lymphoma cells (blasts) among the cells of normal immunophenotype that endure the therapy, detectable in an analysed specimen sample



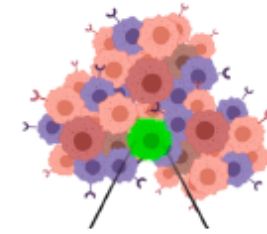
Why MRD monitoring in ALL is important ?

treatment progress evaluation

keystone prognostic factor enabling post-therapy risk stratification and guiding risk-adapted therapeutic approaches in hematological malignancies

MRD level is crucial in most current treatment protocols (e.g., AIEOP-BFM 2017, ALLTOGETHER, EWALL, GMALL, UKALL, PETHEMA, DCOG, GRAALL, NILG...)

- MRD-positivity at particular time points is usually a marker of higher relapse risk
- FC-MRD at day 15. $\geq 10\%$ and no complete remission before day 33. are unfavorable prognostic factors in both BCP-ALL and T-ALL
- MRD values at TP2 used to correct the risk estimate



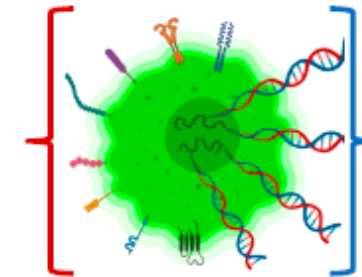
Measurable Residual Disease (MRD)

Detection of rare neoplastic cells (<1%) during post-treatment follow-up, by using complementary approaches:

Multiparametric Flow Cytometry (MFC)

Immunophenotypic analysis to detect abnormal expression of specific antigens

- sensitivity $10^{-3} - 10^{-4}$
- applicability >95%



Molecular diagnostics (PCR, RT-qPCR)

Genetic analysis to detect specific DNA signatures

- sensitivity $10^{-3} - 10^{-6}$
- applicability >90%

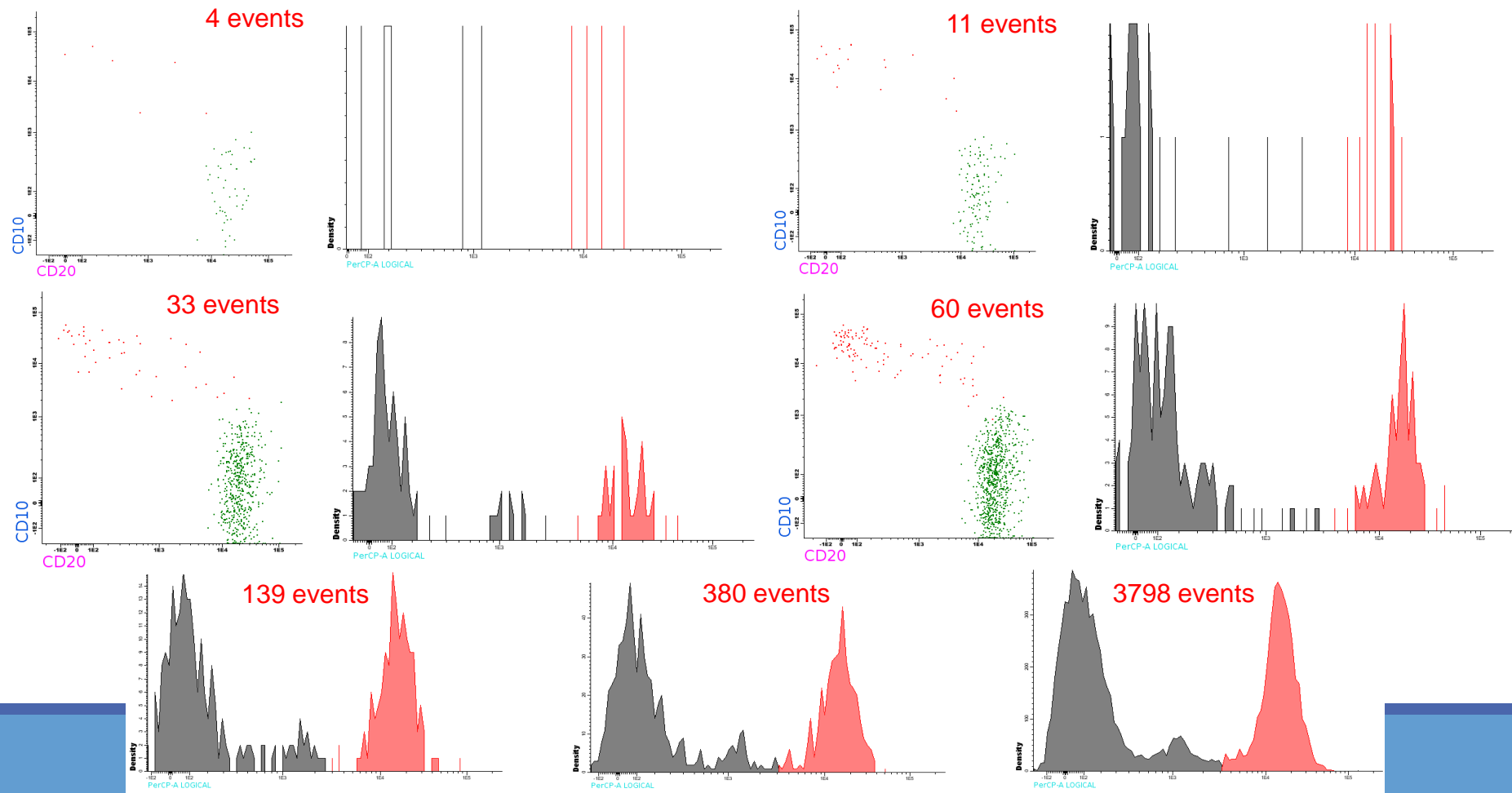
Next Generation Flow (NGF)

- sensitivity $< 10^{-5} - 10^{-6}$
- applicability >99%

Next Generation Sequencing (NGS), Digital PCR (ddPCR)

Sensitivity level of FC – minimal count number per population

- sensitivity of flow cytometric MRD detection can in practice be as low as 10^{-5} , i.e., it enables to detect **one single cell among 100 000** cells
- minimal event count number forming a population should be defined experimentally – how many is enough for a population?



Sensitivity level of FC – minimal count number per population

- **LOB#** (*limit of blank*) – number of events meeting MRD criteria in MRD negative samples (MRD=0)
LOB# = mean evt number of negative samples + 1,645 SD
- **LOD#** (*limit of detection*) – the smallest event number required for reliable **detection** of population of interest
LOD# = LOB# + 1,645xSD(blank)
- Actual LOD is expressed as percentage and depends on the total number of recorded events (N):

$$\text{LOD} = \text{LOD\#/N}$$

- **LLOQ#** (*lower limit of quantification*) or **LOQ#** – the lowest event number required for reliable **quantitative determination** of population of interest:

$$\text{LOQ\#} = 3 \times \text{LOD\#}; \quad \text{LOQ} = 3 \times \text{LOD}$$

Limit of Detection (LOD):	0.0039	Lower Limit of Quantification (LLOQ):	0.0098
COMMENT			
No B-cell precursors (neither normal nor pathological) are detected at a sensitivity level of 0.0039%.			
Add additional comment:			
CONCLUSION			
No abnormal B-cell precursors are detected at a sensitivity level of 0.0039%. Note: The number of analyzed events is lower than the recommended standard.			

Sensitivity level of FC – how to increase it?

- problem of low sample cellularity during treatment, particularly at day 15 and 33
-
- often not possible to acquire more than 100 000 cells from standard sample volume (usually 50µl or 100 µl)
 - sample condensation via centrifugation and discarding the plasma
→ *condensation not always satisfactory (usually not more than 2-3x)*
 - initial erythrocyte lysis, so called „bulk lysis”* in ammonium chloride in high sample volume (1 – 2ml) in 50ml tubes

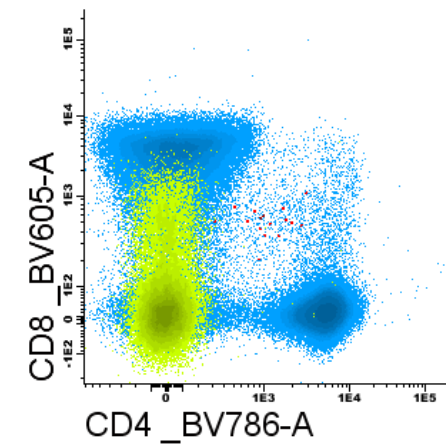
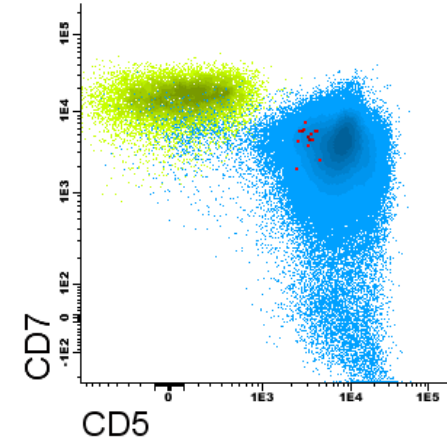
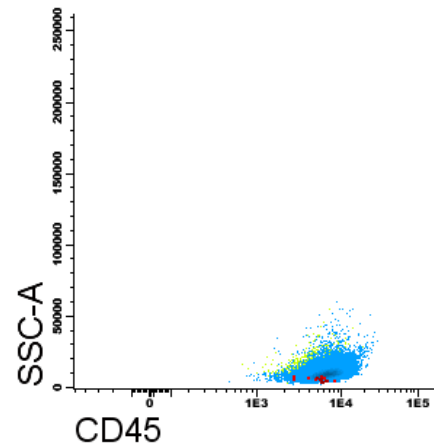
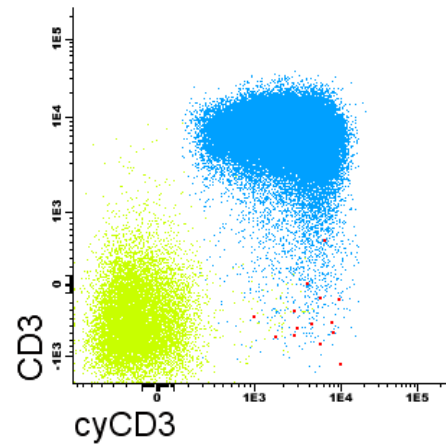
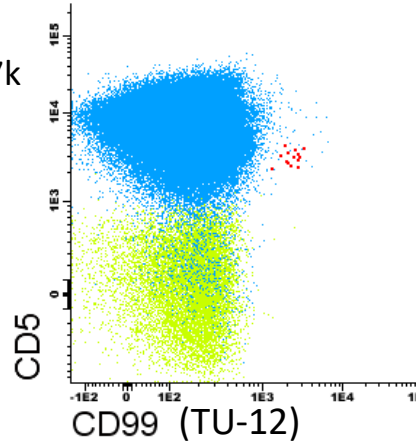


- suspension of „pure” leukocytes
- even 10-20x sample condensation

standard LW protocol

total events: 347k

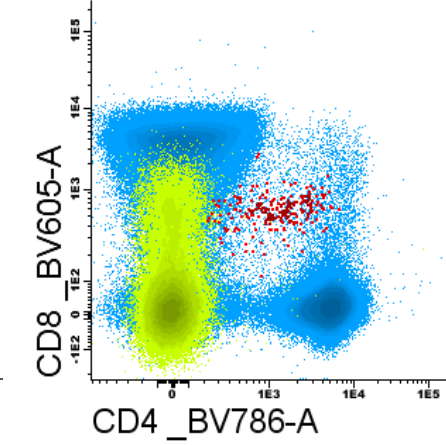
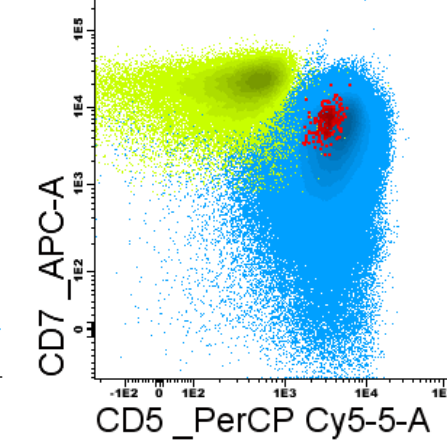
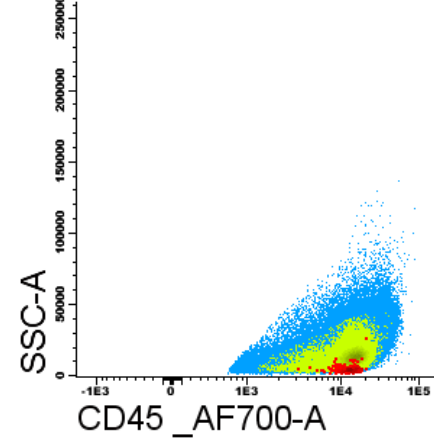
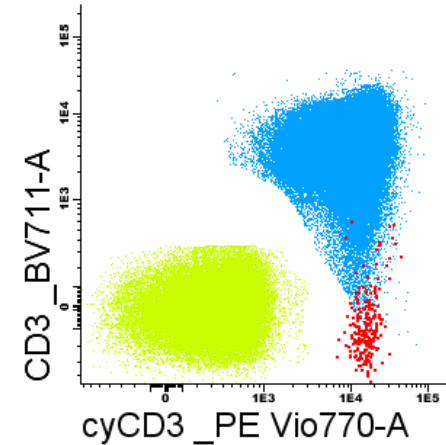
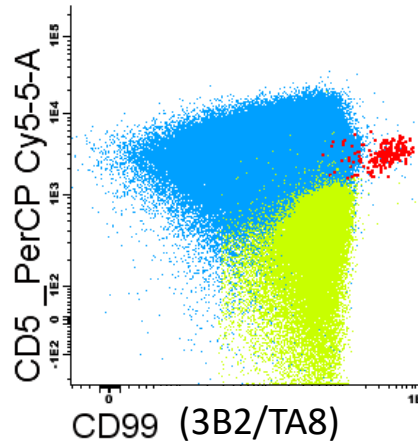
14 evts,
suspected MRD



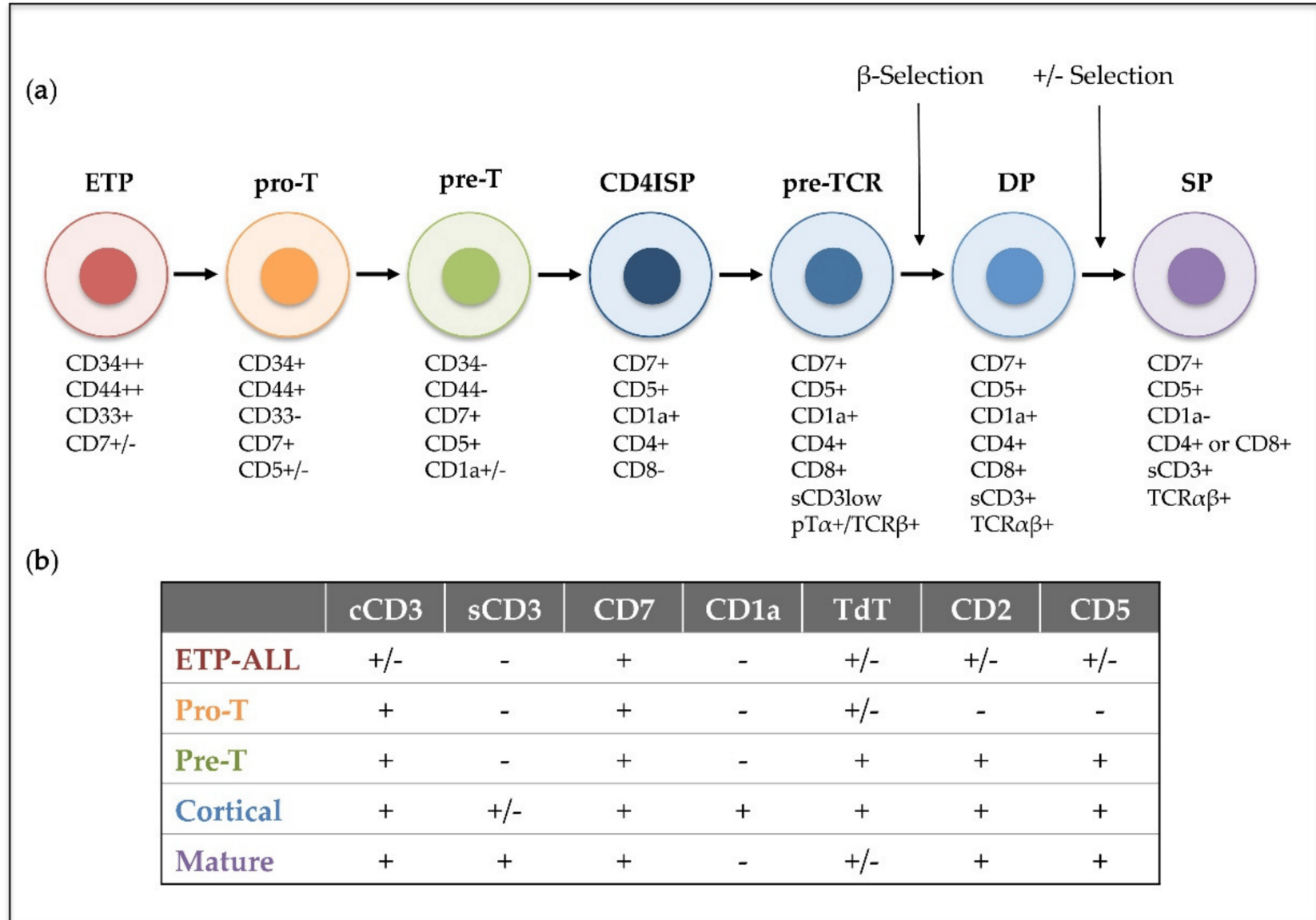
EuroFlow bulk lysis protocol

total events: 4M

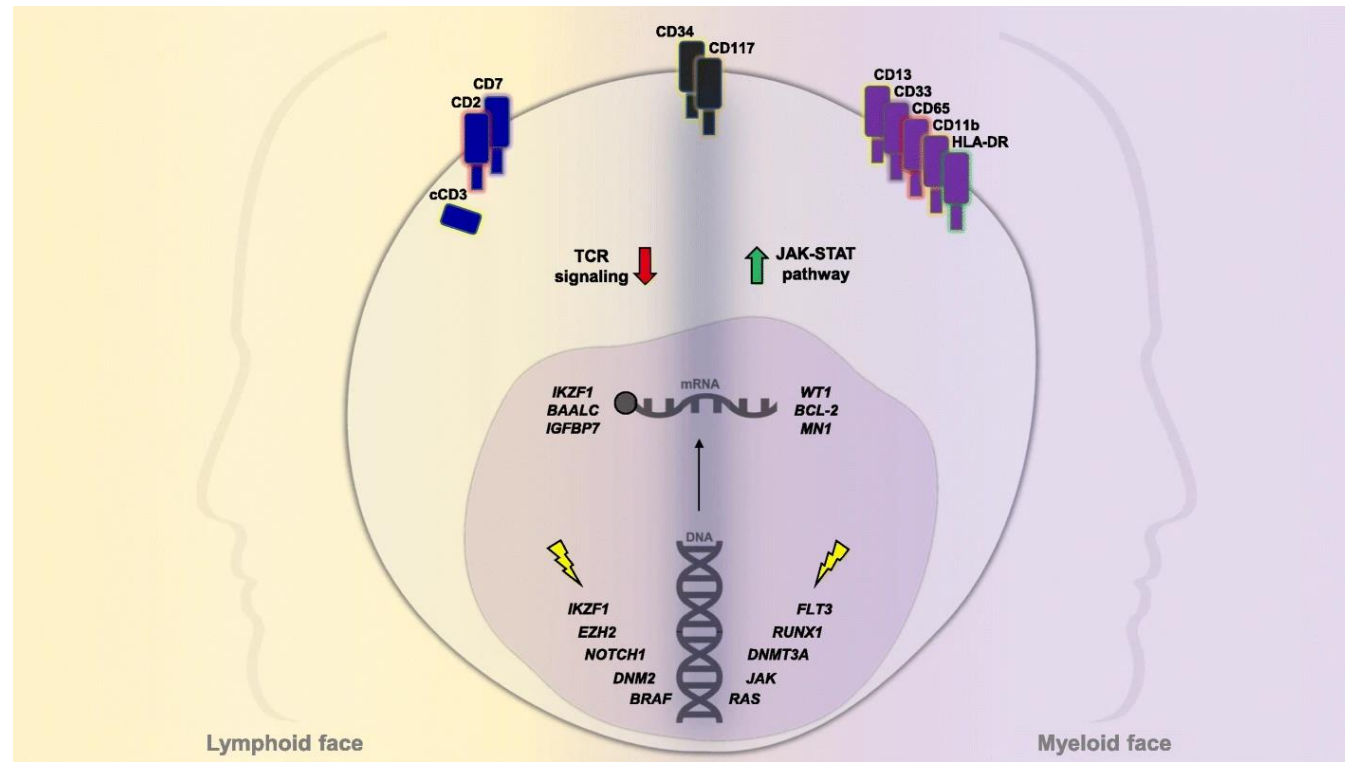
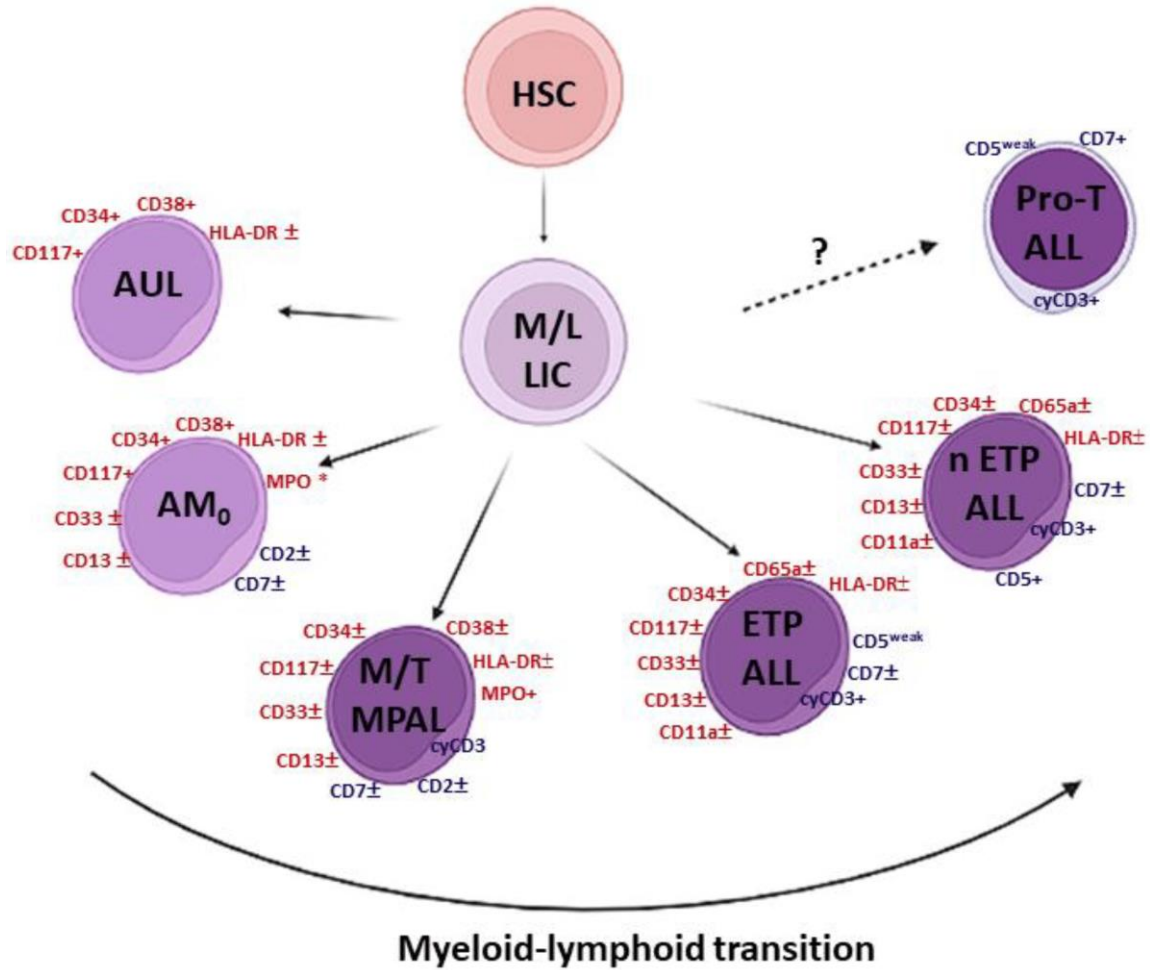
168 evts,
clear MRD



Biology and classification of T-ALL



Biology and classification of T-ALL



MRD monitoring in T-ALL – the Competitors

Subsets of:

- mature T-cells
- **NK-cells**
- B-cell precursor cells
- naive B-cells
- myeloid precursor cells
- monocytes
- dendritic cells (pDC & mDC)

Markers potentially shared with T-ALL blasts:

smCD3, cyCD3, CD2, CD4, CD5, CD7, CD8, CD45RA, (CD99)

cyCD3, CD2, CD4, CD5, CD7, CD8, (CD56), (CD99)

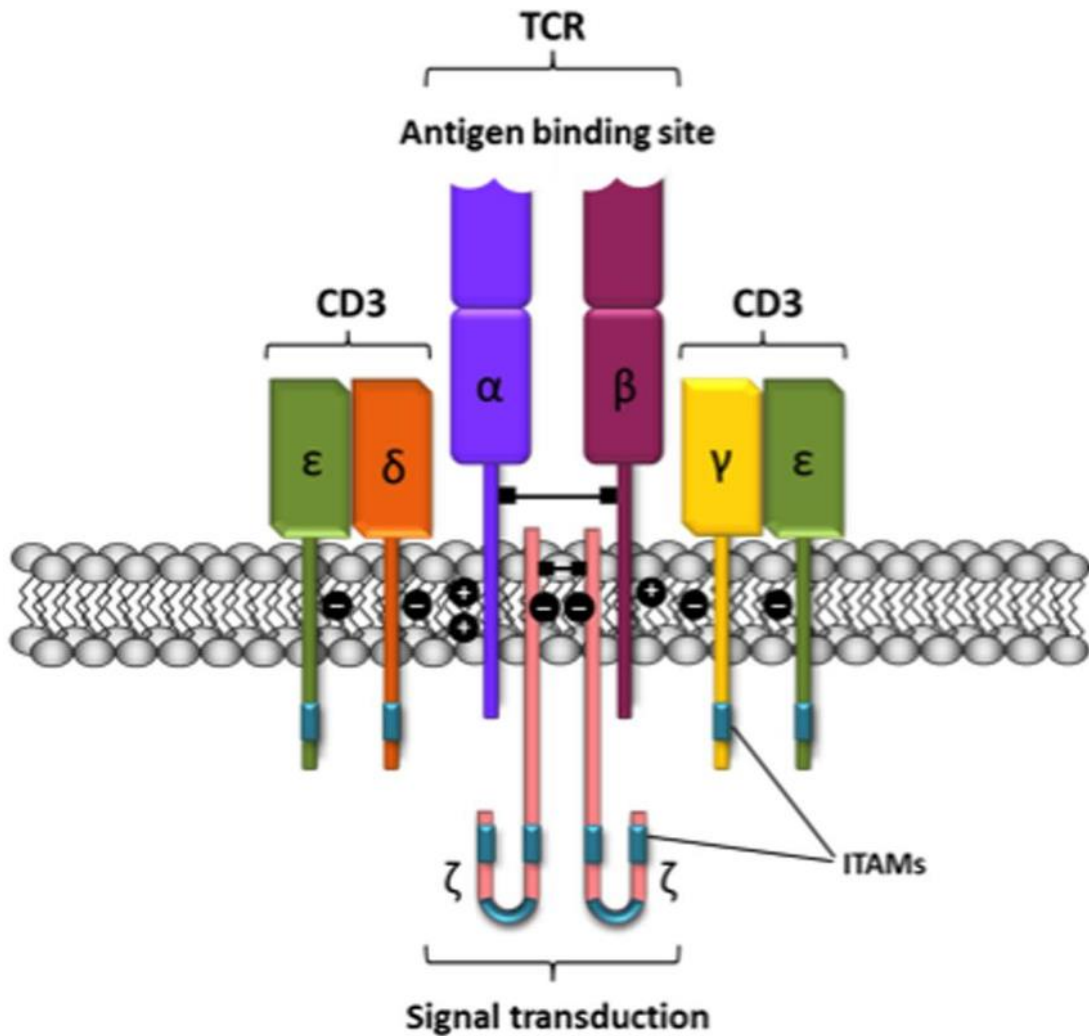
CD5, CD7, CD34, TdT, (CD99)

CD5

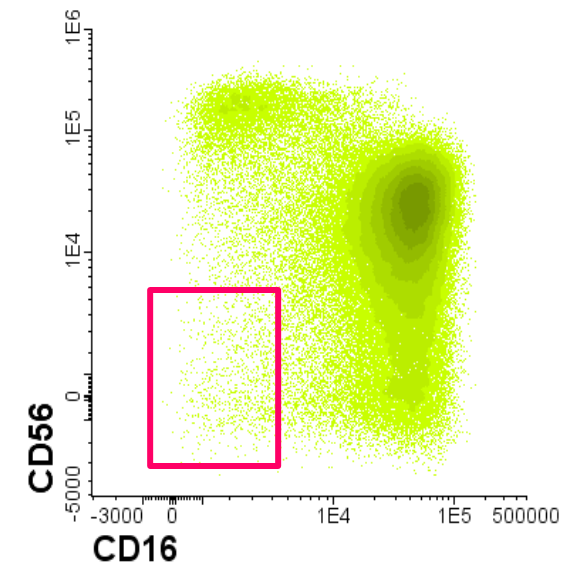
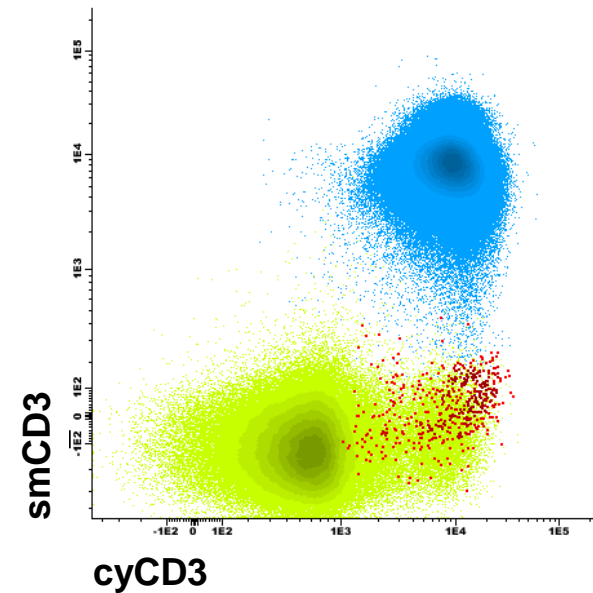
CD4, CD7, CD34, (CD117), (CD33), (CD13)

CD2, CD4, (CD33), (CD13)

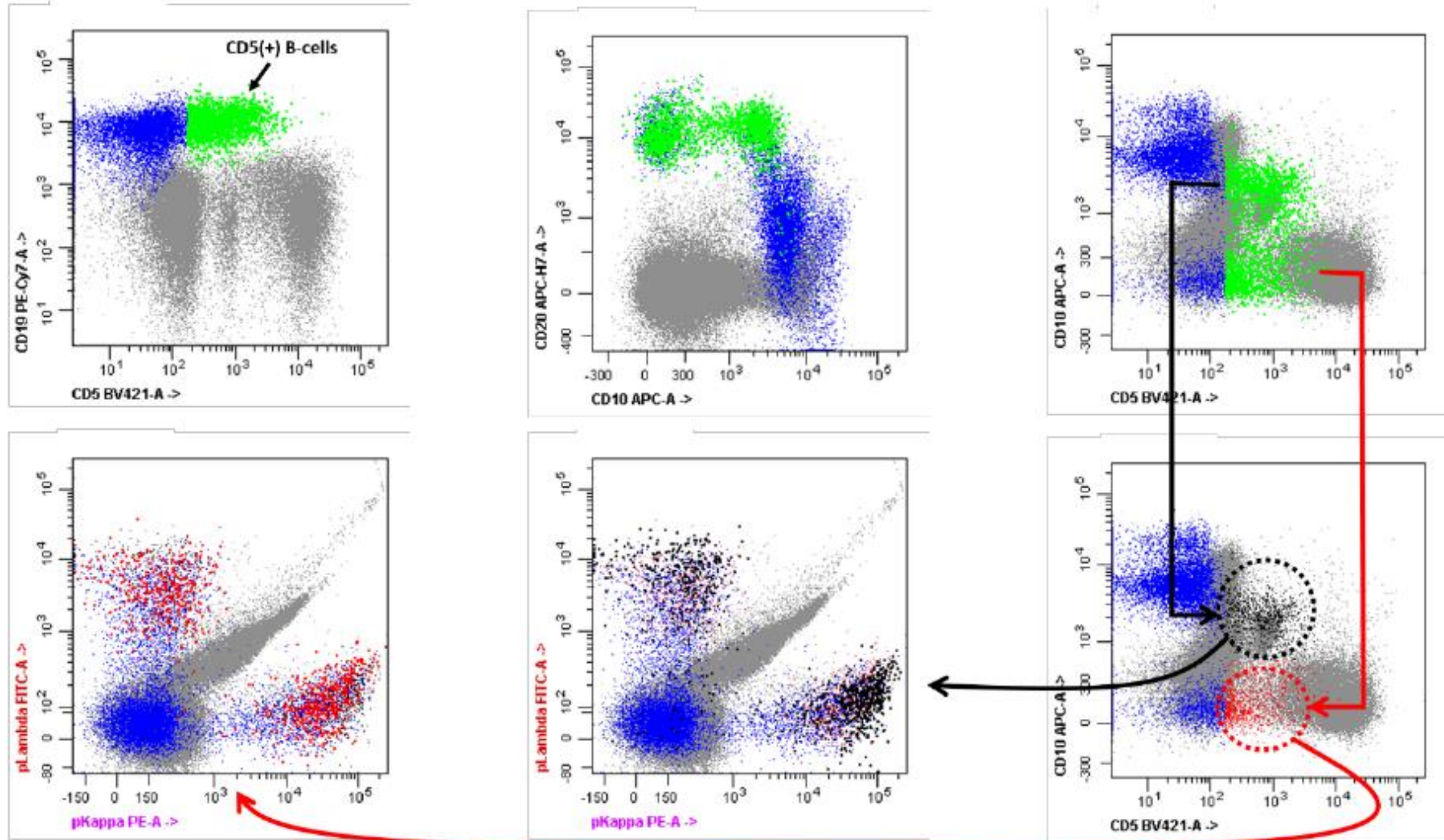
CD4, CD5, (CD33), (CD13)



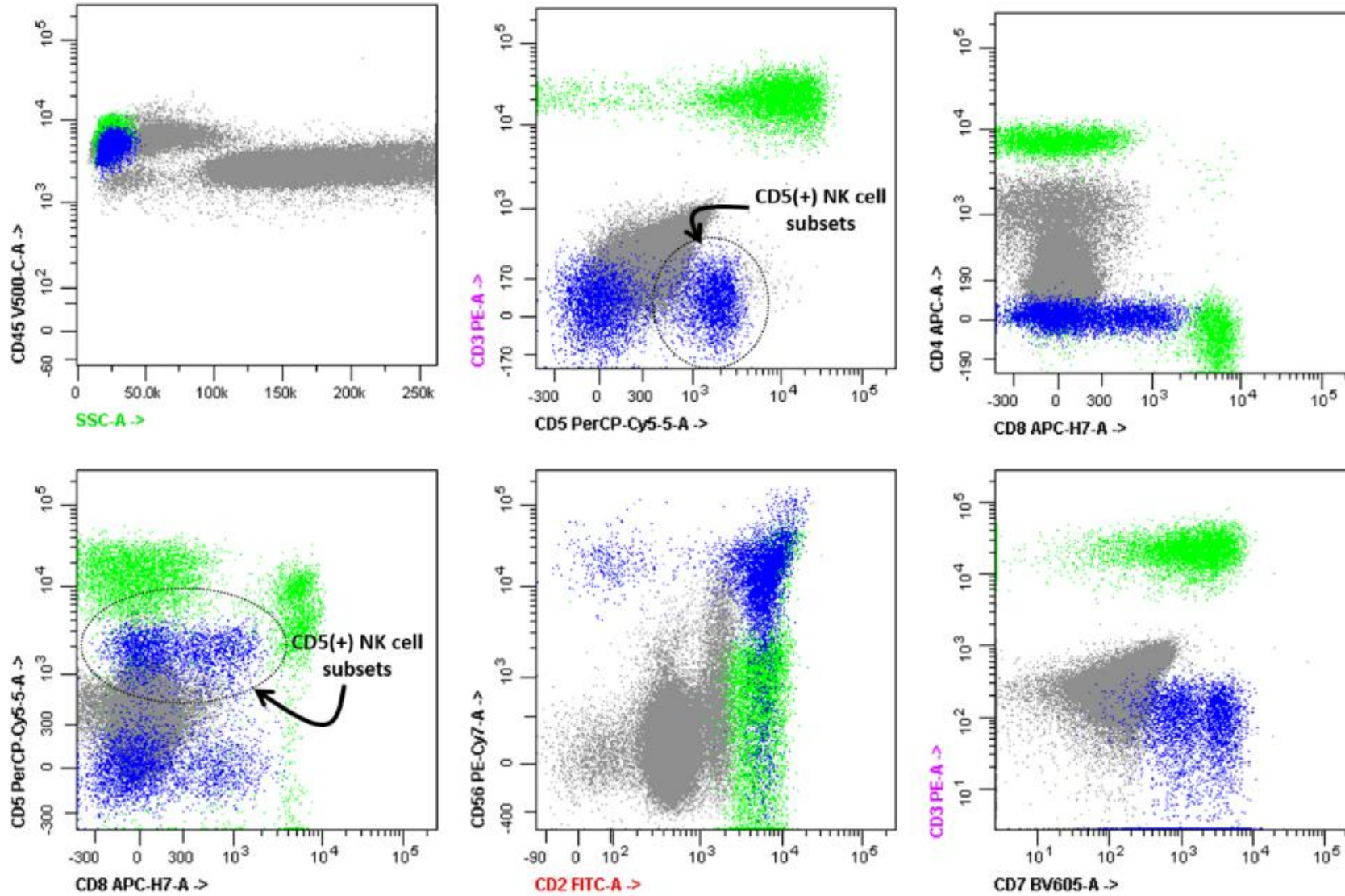
- NK-cells do not rearrange or productively transcribe T-cell receptor α -, β -, γ - or δ -chain genes and do not express the CD3 γ - or δ -subunits
- However, a subset of NK cells does express CD3 ϵ - and ζ -chains
- Not all NK-cells can be gated with CD16 and CD56 markers



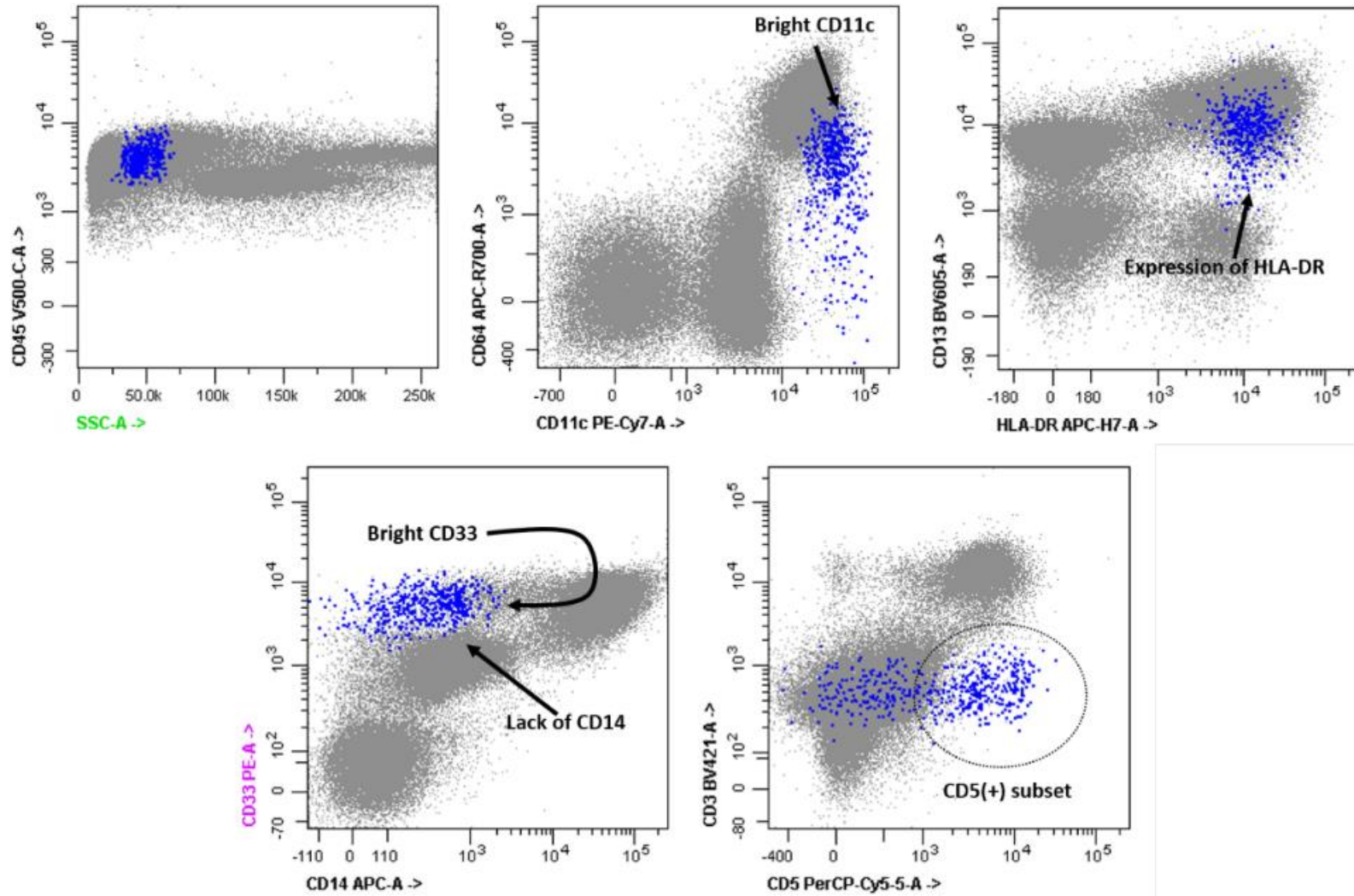
CD5+ BCP and mature cells...



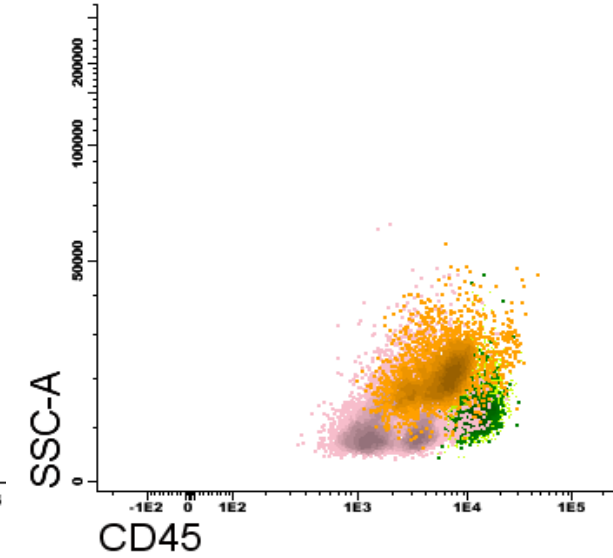
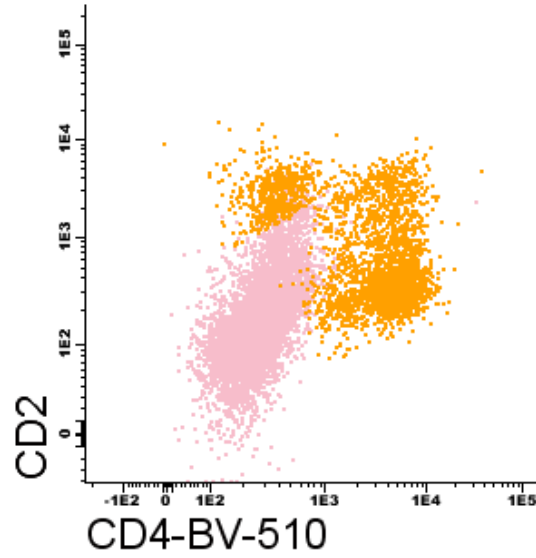
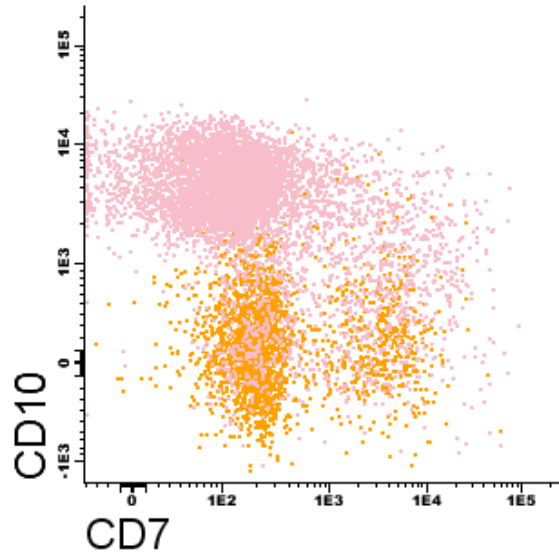
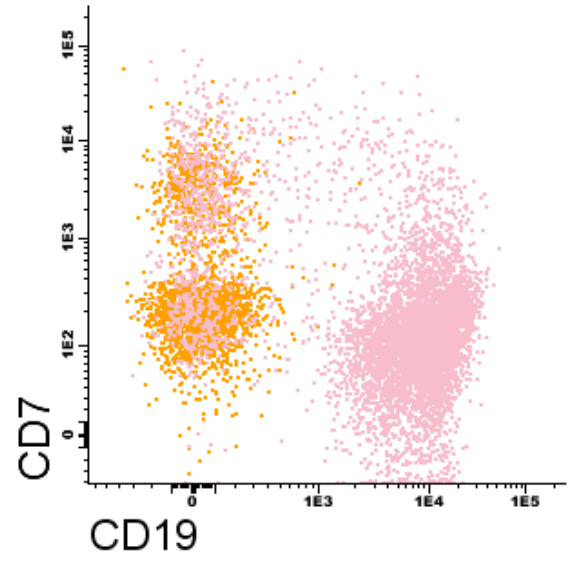
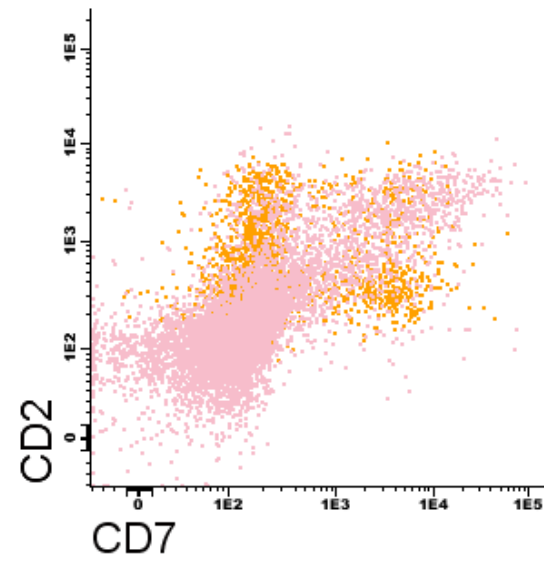
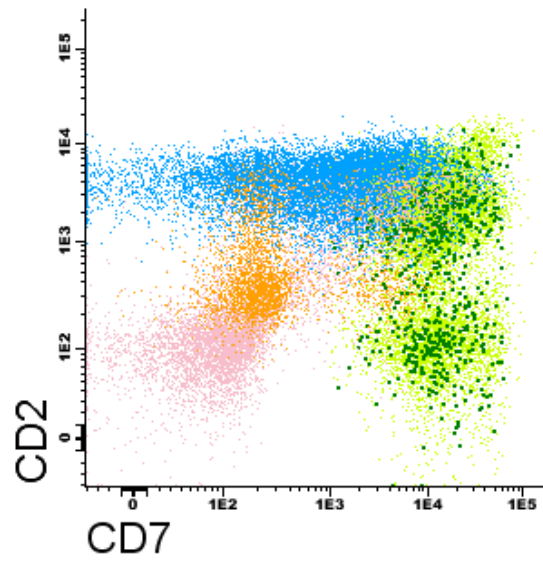
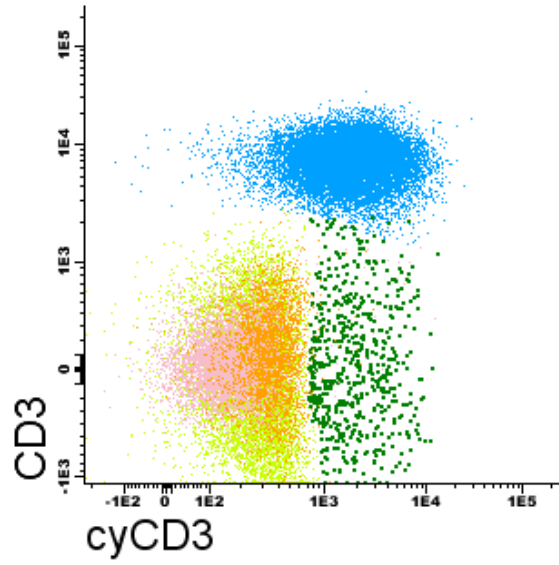
CD5+ NK cells...



CD5+ mDC's...



CD7+ CD2+ CD4+ myeloid cells and BCPs...



T-ALL MRD panel markers

„Classical” marker set

CD1a	CD2	cyCD3	smCD3	CD4	CD5	CD7	CD8	CD10	CD11a	CD11b	CD13	CD16	CD33	CD34	CD38	CD44	CD45RA
CD56	CD99	CD117															
TdT	HLA-DR	cyMPO	TCR $\alpha\beta$	TCR $\gamma\delta$	TCR β F1												

Example of an 8-color panel

	F1	F2	F3	F4	F5	F6	F7	F8
1.	cyCD3	CD3	CD7	CD45	CD5	TdT	CD99	CD34
2.	cyCD3	CD3	CD7	CD45	CD5	CD2	CD117	CD1a
3.	cyCD3	CD3	CD7	CD45	CD5	CD4	CD16+CD56	CD8
4.	cyCD3	CD3	CD7	CD45	CD5	CD45RA	CD13	CD33

T-ALL MRD panel markers

„Classical” marker set

CD1a	CD2	cyCD3	smCD3	CD4	CD5	CD7	CD8	CD10	CD11a	CD11b	CD13	CD16	CD33	CD34	CD38	CD44	CD45RA
CD56	CD99	CD117															
TdT	HLA-DR	cyMPO	TCR $\alpha\beta$	TCR $\gamma\delta$	TCR β F1												

Example of a 9-color panel *Wood BL, Methods Mol Biol 2013*

	PB	FITC	PE	PE-TR	PC5	PC7	A594	APC	APC7
1	CD16	cyCD3	CD7	–	CD56	CD5	CD38	smCD3	CD45
2	CD8	CD2	CD5	CD34	CD16+CD56	CD3	CD4	CD7	CD45

Example of a 10-color panel *Tembhare PR, Clin Cytom B, 2021*

	FITC	PE	ECD	PE-Cy5.5	PE-Cy7	APC	APC-A700	APC-A750	BV421	BV510
1	CD8	CD7	cyCD3	CD34	CD5	CD4	CD45	CD38	smCD3	CD16+CD56
2	TdT	CD7	cyCD3	CD13+CD33	CD117	CD1a	CD45	CD38	smCD3	CD16+CD56

T-ALL MRD panel

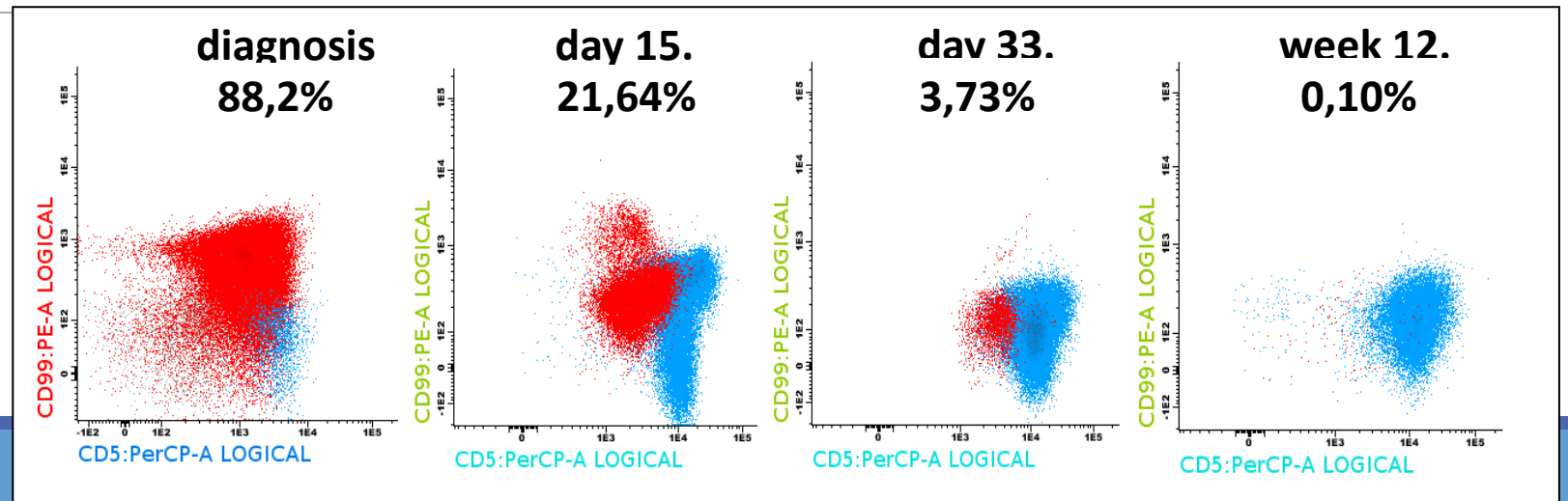
„Classical” marker set

CD1a	CD2	cyCD3	smCD3	CD4	CD5	CD7	CD8	CD10	CD11a	CD11b	CD13	CD16	CD33	CD34	CD38	CD44	CD45RA
CD56	CD99	CD117															
TdT	HLA-DR	cyMPO	TCRαβ	TCRγδ	TCRβF1												

Example of an 8-color panel

	F1	F2	F3	F4	F5	F6	F7	F8
1.	cyCD3	CD3	CD7	CD45	CD5	TdT	CD99	CD1a
2.	cyCD3	CD3	CD7	CD45	CD5	CD2	CD117	CD34
3.	cyCD3	CD3	CD7	CD45	CD5	CD4	CD16+CD56	CD8
4.	cyCD3	CD3	CD7	CD45	CD5	CD45RA	CD13	CD33

potential pitfall: downregulation of „immaturity” markers: TdT, CD99, CD34 and CD1a



New marker set 1

CD21	CD43	CD200	CD317	CD335
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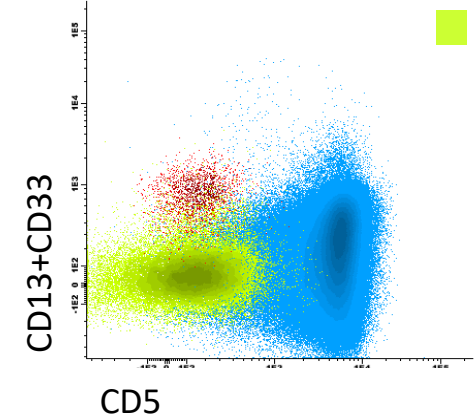
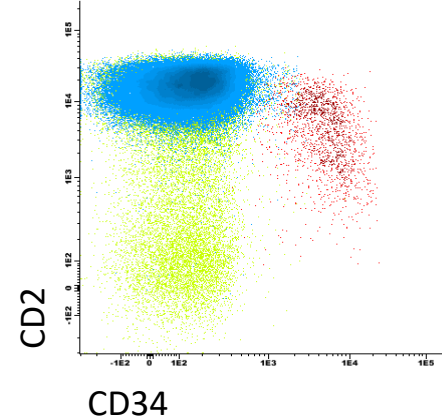
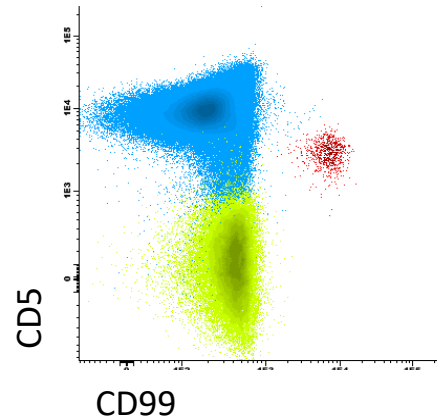
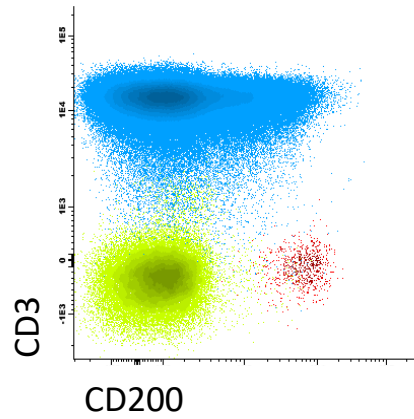
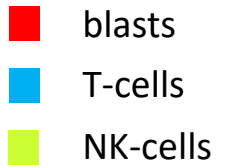
Example of a EuroFlow 8-color panel v.1

	FITC	PE	PerCPCy5.5	PE-Cy7	APC	APC-AF750	BV-421	BV-510
1.	CD16+CD56	CD45RA	CD5	cyCD3	CD7	CD3	CD34	CD45
2.	CD99	CD13+CD33	CD5	cyCD3	CD7	CD3	CD200	CD45
3.	CD43	CD1a	CD5	cyCD3	CD7	CD3	CD317	CD45
4.	TdT	CD21	CD5	cyCD3	CD7	CD3	CD335	CD45

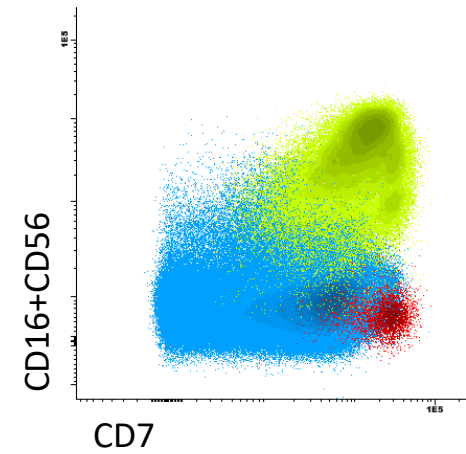
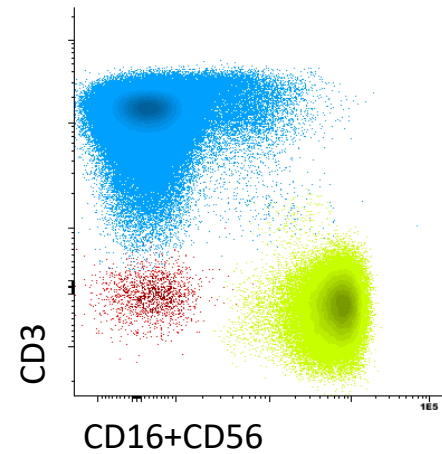
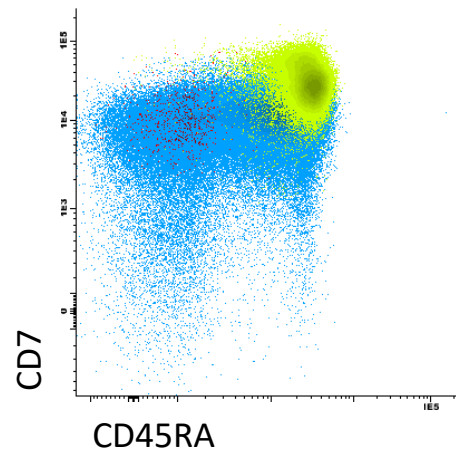
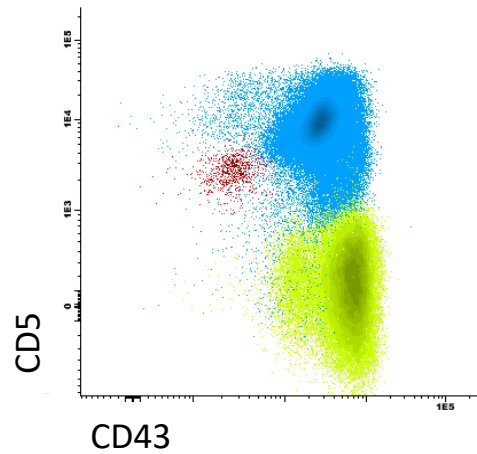


EuroFlow Evaluation of T-MRD markers

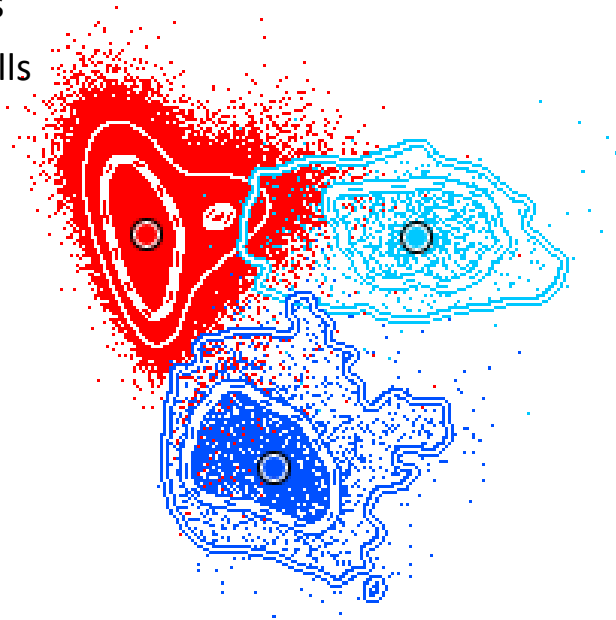
- some markers useful when positive: CD99, CD34, CD200, CD13+CD33, CD1a...



- ...others when negative: CD43, CD16+CD56, CD45RA



- blasts
- T-cells
- NK-cells



APS 1

Diagram Info

General PC1 PC2

Parameter	Significance
CD34:BV421-A LOGICAL	18.27
CD45:Alexa Fluor 700-A LOGICAL	11.79
CD2:PE-A LOGICAL	10.67
cyCD3-PE-Vio770:PE-Cy7-A LOGICAL	10.44
CD7:APC-A LOGICAL	9.82
FSC-A LINEAR	9.01
FSC-H LINEAR	7.65
CD3:APC-AF750-A LOGICAL	7.27
CD5:PerCP-Cy5-5-A LOGICAL	5.54
CD45RA:BV510-A LOGICAL	4.35

Rank	→	Points
1	→	3 points
2	→	2 points
3	→	1 point
4	→	0.5 points
≤ 5	→	0 points

	FITC	PE	PerCPCy5.5	PE-Cy7	APC	APC-AF750	BV-421	BV-510
1.	CD16+CD56	CD45RA	CD5	cyCD3	CD7	CD3	CD34	CD45
2.	CD99	CD13+CD33	CD5	cyCD3	CD7	CD3	CD200	CD45
3.	CD43	CD1a	CD5	cyCD3	CD7	CD3	CD317	CD45
4.	TdT	CD21	CD5	cyCD3	CD7	CD3	CD335	CD45

■ blasts
■ T-cells
■ NK-cells

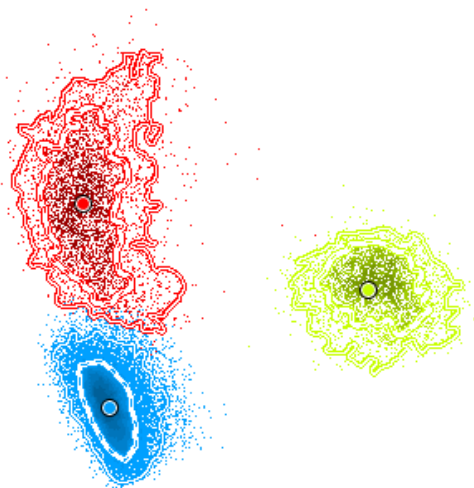


Diagram Info

General PC1 PC2

Parameter	Significance
CD5:PerCP-Cy5-5-A LOGICAL	24,89
CD 16+CD56:PE-A LOGICAL	21,55
CD3:APC-H7-A LOGICAL	20,75
cyCD3-PE-Vio770:PE-Cy7-A LOGICAL	18,72
CD7:APC-A LOGICAL	4,00
CD200:Pacific Blue-A LOGICAL	2,92
FSC-A LINEAR	1,82
FSC-H LINEAR	1,64
CD99:FITC-A LOGICAL	1,47
SSC-A Exp-SSC Low	1,30
CD45-HV-500:Pacific Orange-A LOGICAL	0,94

OK








Diagram Info

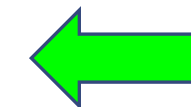
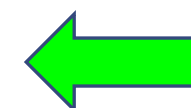
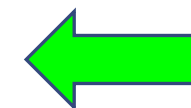
General PC1 PC2

Parameter	Significance
CD3:APC-H7-A LOGICAL	33,31
CD200:Pacific Blue-A LOGICAL	23,86
cyCD3-PE-Vio770:PE-Cy7-A LOGICAL	14,05
CD 16+CD56:PE-A LOGICAL	11,10
CD7:APC-A LOGICAL	7,00
CD5:PerCP-Cy5-5-A LOGICAL	3,77
CD99:FITC-A LOGICAL	2,52
CD45-HV-500:Pacific Orange-A LO...	2,40
SSC-A Exp-SSC Low	0,80
FSC-A LINEAR	0,78
FSC-H LINEAR	0,40

OK

- ✓ # / % of cases when a marker in top four
- ✓ top four scoring >10 in majority of cases
- ✓ independent evaluation of PC1 and PC2

	# evaluated	Negative	Positive	Useful %
CD43	17	7 =useful	10 =not useful	 41,2%
CD317	15	1 =useful	14 =not useful	 6,7%
CD200	16	6 =not useful	10 =useful	 62,5%
CD99 (Tu-12)	14	5 = not useful	9 =useful	 64,3%
CD16+CD56	15	15 =useful	0 =not useful	 100%
CD34	17	9 =not useful	8 =useful	 47,1%
CD45RA	17	8 =useful	9 =not useful	 47,1%





EuroFlow *Evaluation of T-MRD markers*

New marker set 2

CD81	CD55 67	CD55 J511KSC2.3
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Example of a EuroFlow 8-color panel v.2

	FITC	PE	PerCPCy5.5	PE-Cy7	APC	APC-AF750	BV-421	BV-510
1.	CD45RA	CD2	CD5	cyCD3	CD7	CD3	CD34	CD45
2.	CD99	CD13+CD33	CD5	cyCD3	CD7	CD3	CD200	CD45
3.	CD43	CD1a	CD5	cyCD3	CD7	CD3	CD16+CD56	CD45
4.	TdT	CD55 (67)	CD5	cyCD3	CD7	CD3	CD335	CD45
5.	CD81	CD55 (J511KSC2.3)	CD5	cyCD3	CD7	CD3	CD21	CD45



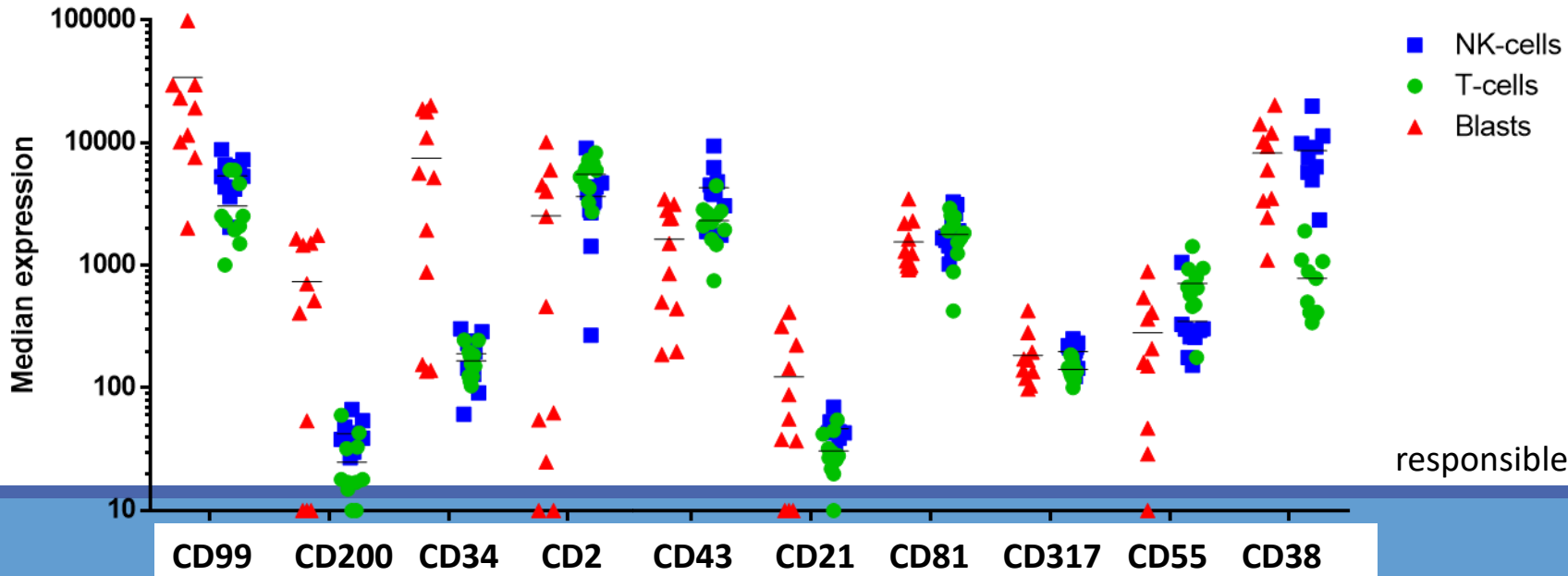
EuroFlow Evaluation of T-MRD markers

New marker set 3

CD4	CD8	CD38	CD71	CD117
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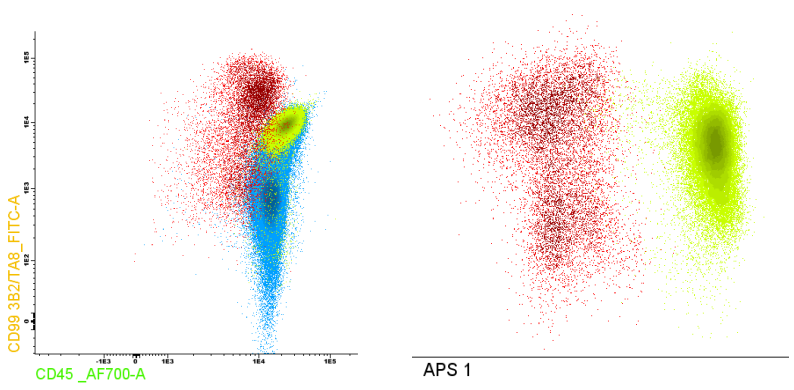
Example of a EuroFlow 12-color panel v.1

	FITC	PE	PerCPC y5.5	PE-Cy7	APC	AF700	APC-H7	V1	V2	V3	V4	V5
1	TdT+CD99	CD16+CD56	CD5	cyCD3	CD7	CD45	CD71	CD34	CD45RA	CD117	CD3	CD13+CD33
2.	CD8	CD200	CD5	cyCD3	CD7	CD45	CD38	CD1a	CD2	CD43	CD3	CD4



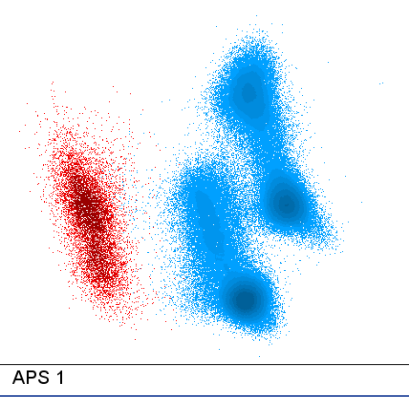
responsible scientist: Saskia Kohlscheen

CD99 3B2/TA8



PC1
Parameter rank 8 CD99 Significance 2.17

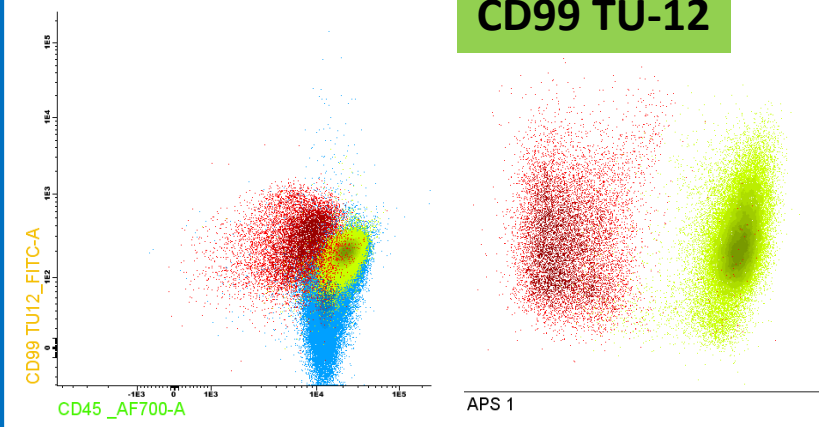
PC2
Parameter rank 1 CD99 Significance 19.10



PC1
Parameter rank 4 CD99 Significance 10.97

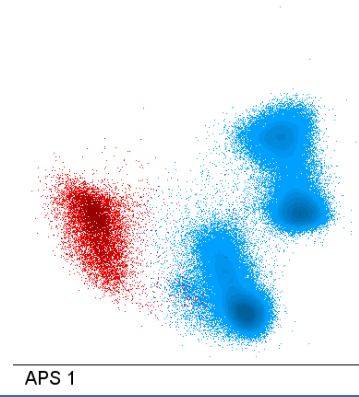
PC2
Parameter rank 4 CD99 Significance 11.03

CD99 TÜ-12



PC1
Parameter rank 9 CD99 Significance 2.73

PC2
Parameter rank 6 CD99 Significance 6.78

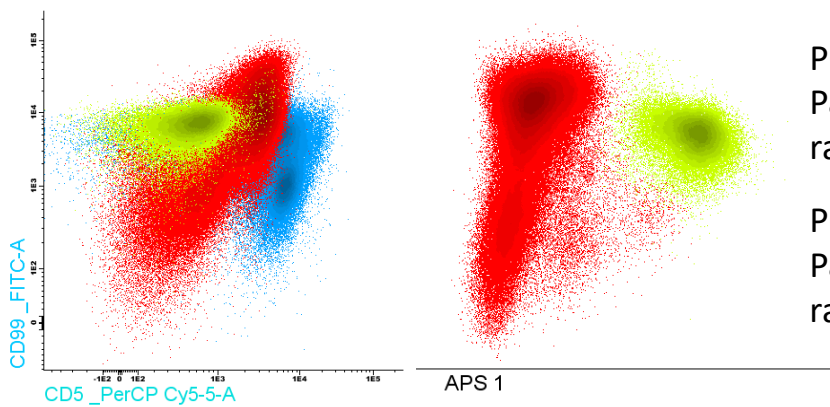


PC1
Parameter rank 6 CD99 Significance 6.96

PC2
Parameter rank 12 CD99 Significance 0.07



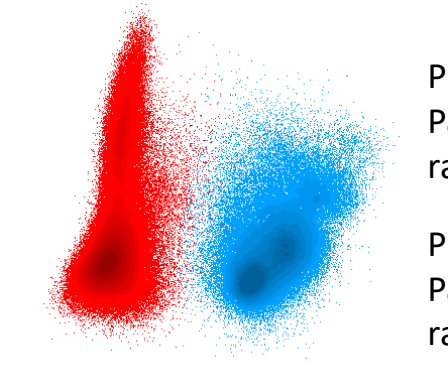
CD99 3B2/TA8



PC1
 Parameter Significance
 rank 7 CD99 2.26

PC2
 Parameter Significance
 rank 2 CD99 18.88

APS 1

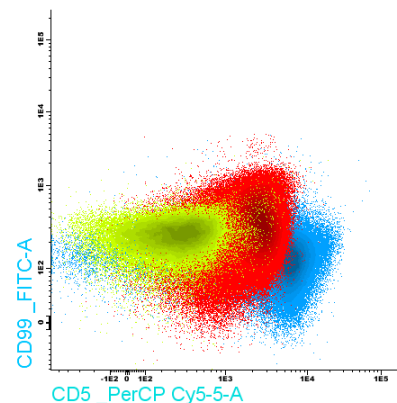


PC1
 Parameter Significance
 rank 8 CD99 4.61

PC2
 Parameter Significance
 rank 4 CD99 13.14

APS 1

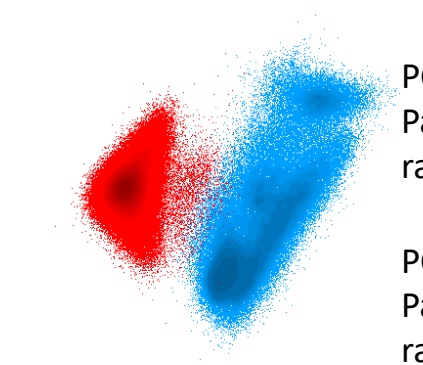
CD99 TÜ-12



PC1
 Parameter Significance
 rank 9 CD99 1.70

PC2
 Parameter Significance
 rank 4 CD99 4.68

APS 1



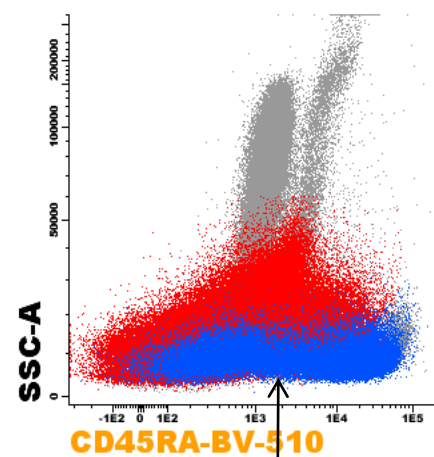
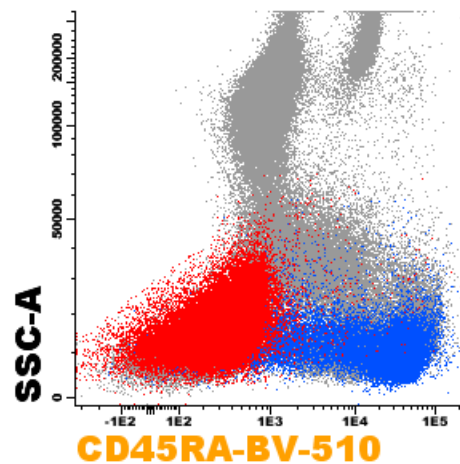
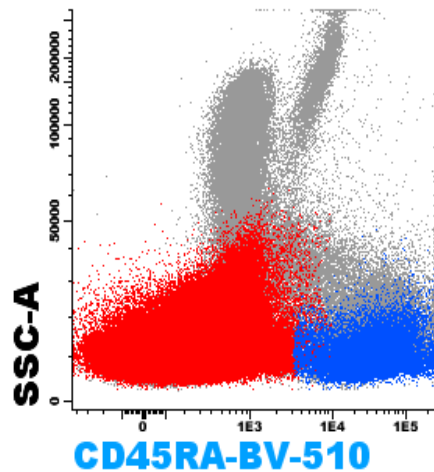
PC1
 Parameter Significance
 rank 7 CD99 5.27

PC2
 Parameter Significance
 rank 12 CD99 0.58

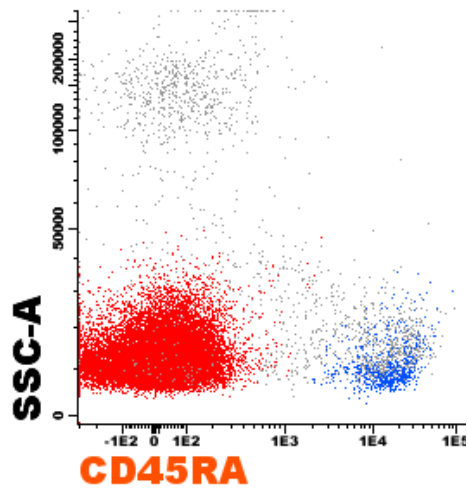
APS 1



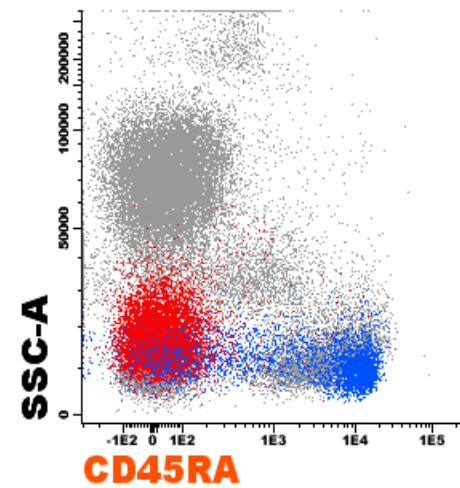
CD45RA: HI100



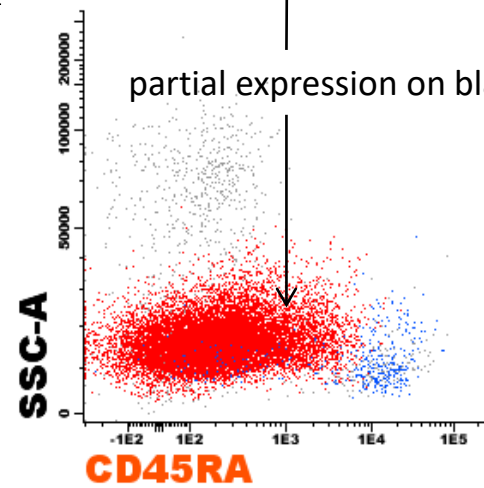
CD45RA: L48



3253



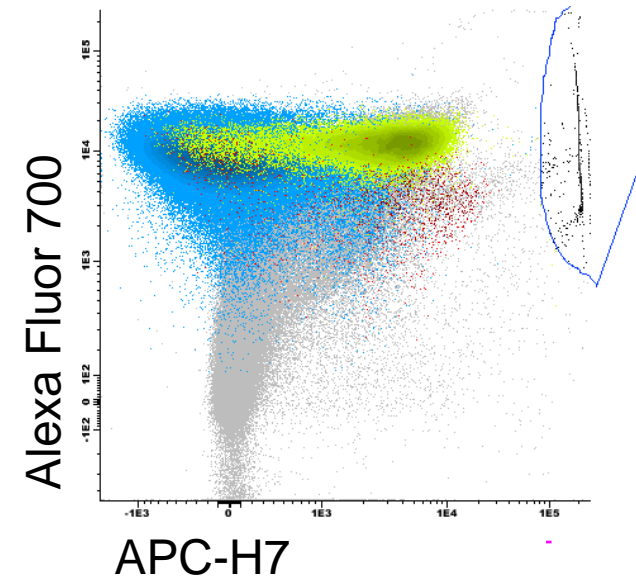
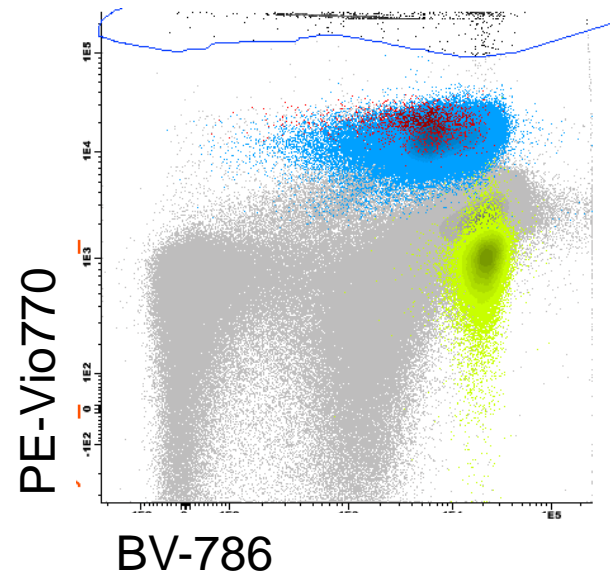
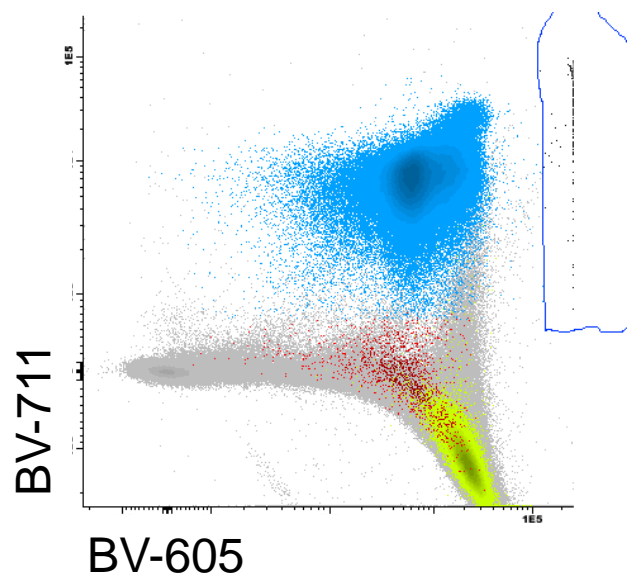
3091



3244

Technical issues

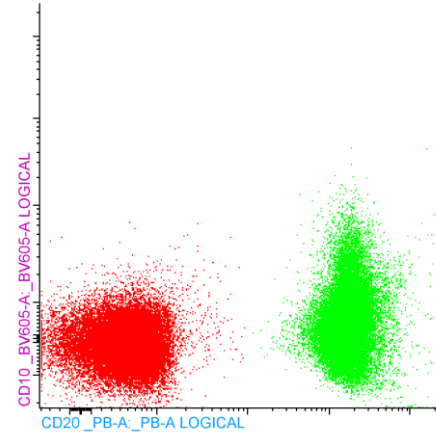
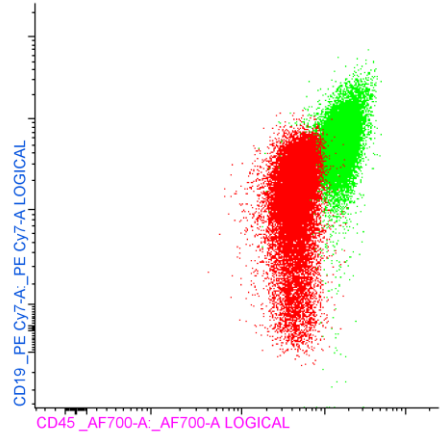
- compensation.... (BV dyes, far red dyes)
- off-scale events (BV dyes, PE-Vio770, APC-H7)



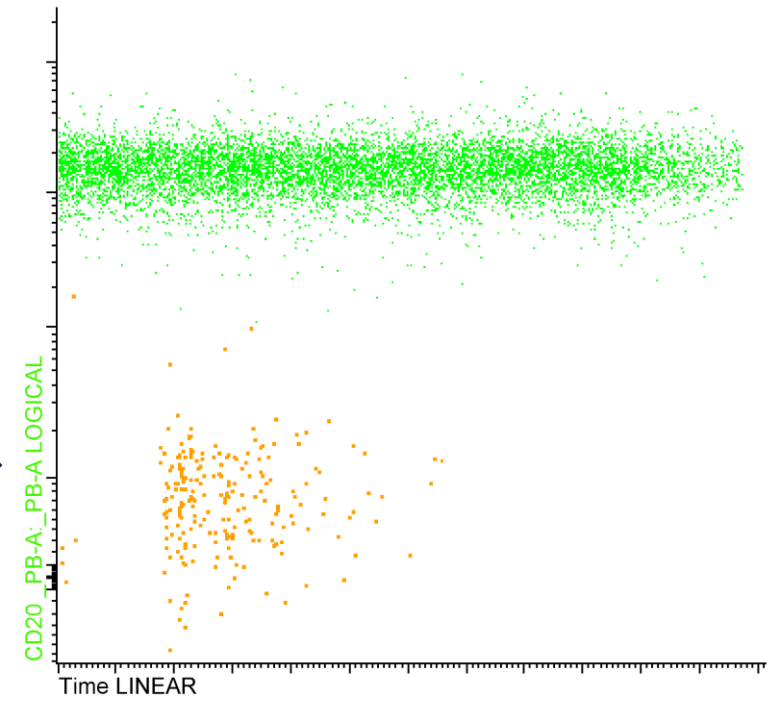
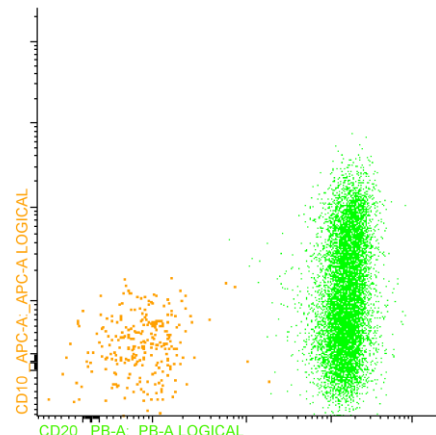
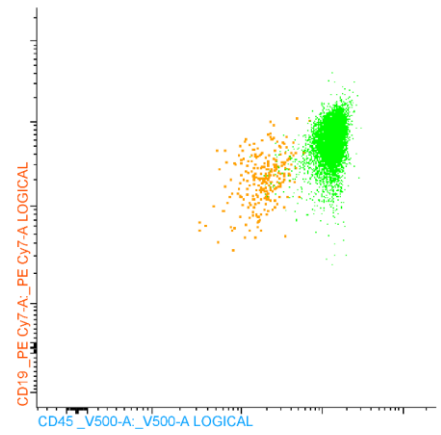
Technical issues

- *sample carry-over*

High density sample



MRD sample



Conclusions and recommendations

- increase your sensitivity level of MRD detection with the use „bulk lysis” protocol
- supplement your T-MRD panel with new MRD markers facilitating the discrimination of the residual leukemic cells from their normal counterparts
- learn how to discriminate MRD from normal „competing” populations
- prepare for occurrence of phenotypic shifts of leukemic blasts
- mind your protocol:
 - trust your intracellular staining procedure and reagents
 - use appropriate antibody clones that recognize cytoplasmic domains
 - be aware of staining differences between the antibody clones used in your diagnostic and MRD settings
 - keep in mind that only UCHT1 , VIT-3b, Gi 9-41 and SK7/Leu-4 are able to detect CyCD3!
 - carefully titrate your antibodies
- check for possible sample carry-over
- await the EuroFlow manuscript on MRD analysis in T-ALL



Thank you for your attention!



UFRJ

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