Immunophenotyping in Acute Leukemias

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What is Flow Cytometry?

Single
Cyto = cells

Metry = measurement

Flow = In a flow or stream

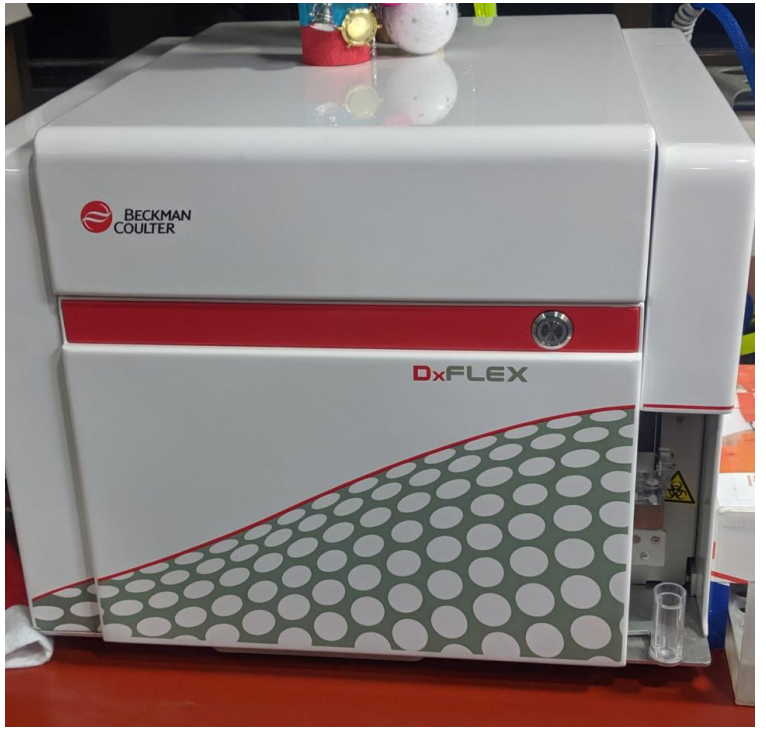

Waste


BD FACSCanto II
BD
Helping all people live healthy lives
BD FACSCanto™ II Flow Cytometry System
Innovation with Impact


BECKMAN COULTER
CytoFLEX

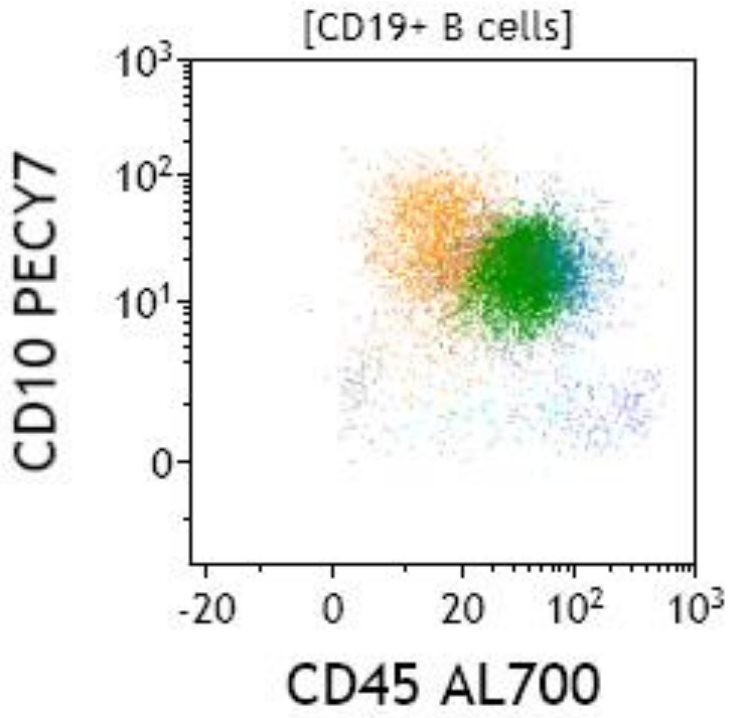

BD LSRFortessa™

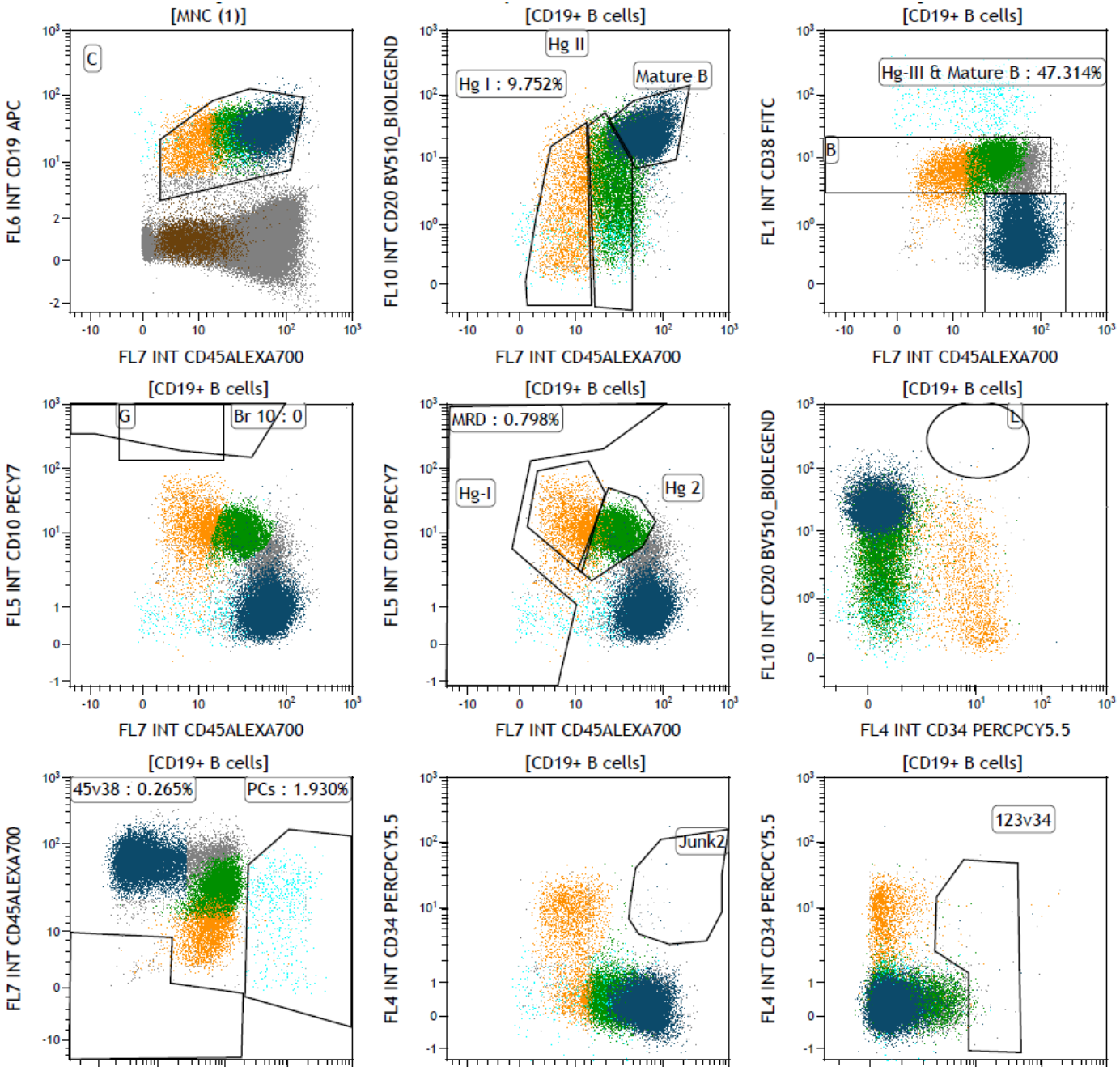

BECKMAN
COULTER
CytoFLEX LX


BECKMAN
COULTER
DxFLEX

Flow cytometry

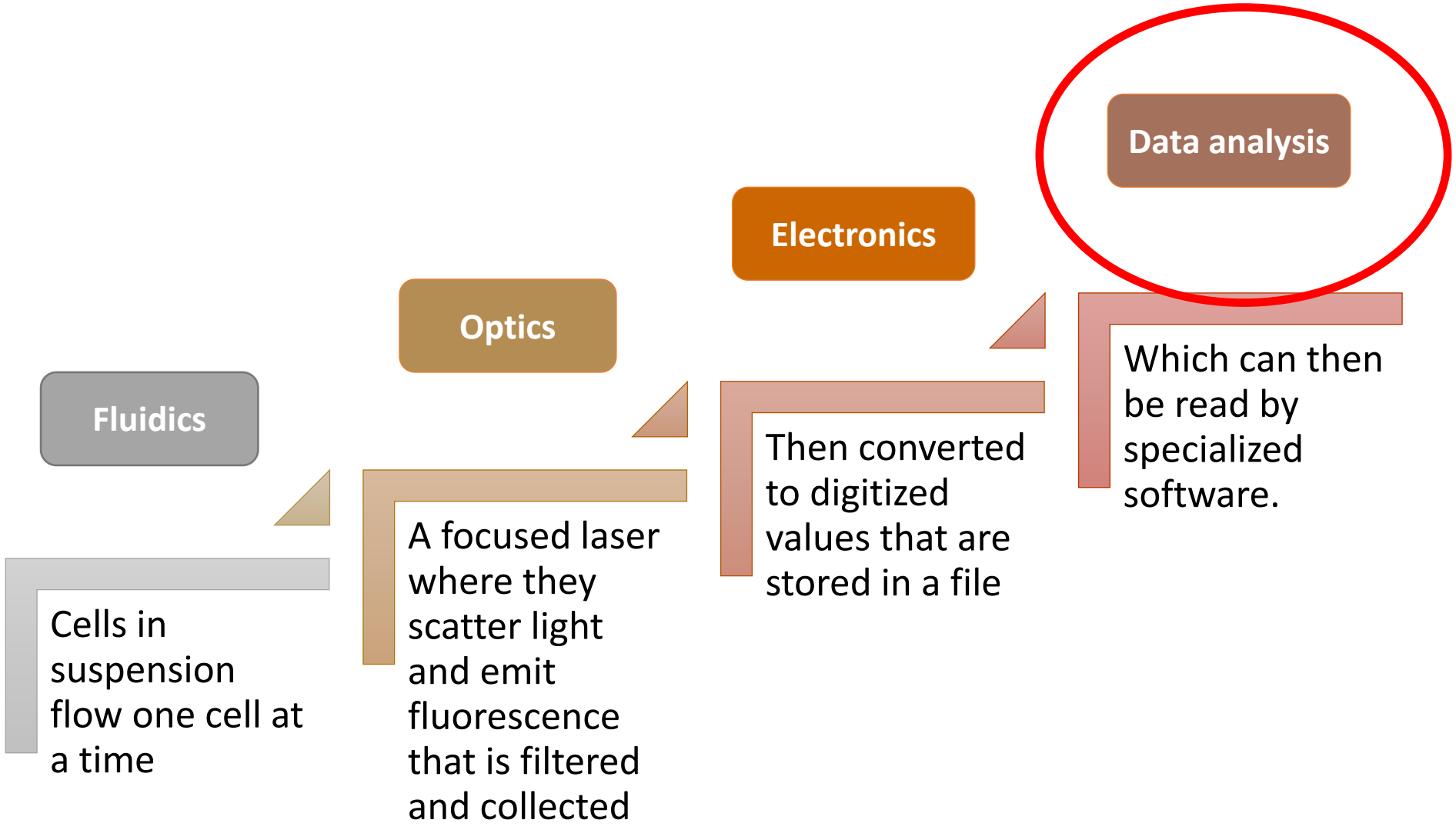
- No assessment of architecture, however pattern based analysis
- Pin-point location of a cell in a n-dimensional place
- Rapid, independent analysis of a large number of cells (up to 5-10 millions)



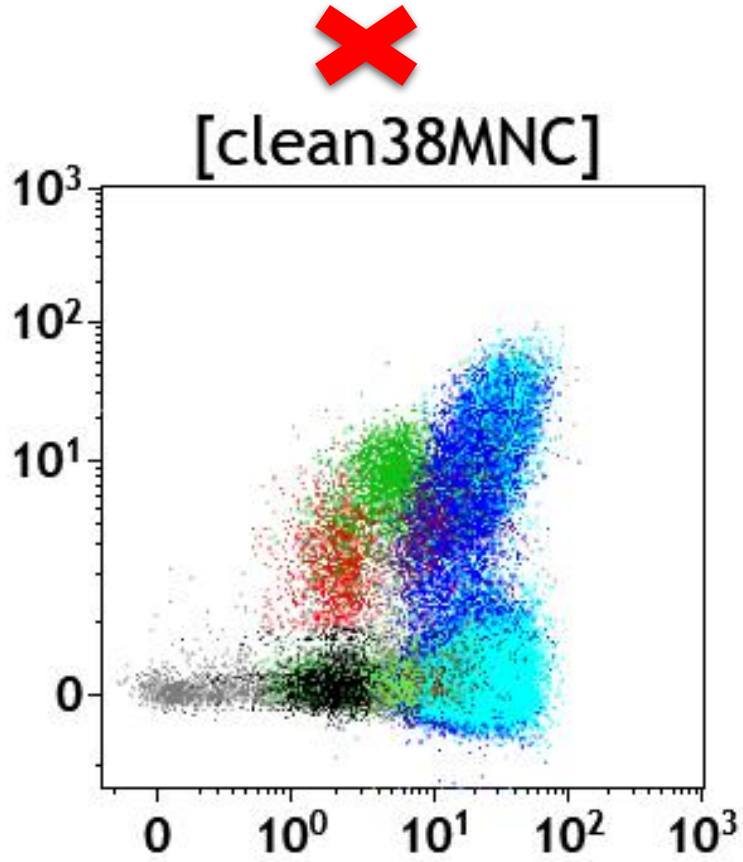


Tembhare et al. Cytometry Part B 2019;00B: 1-11

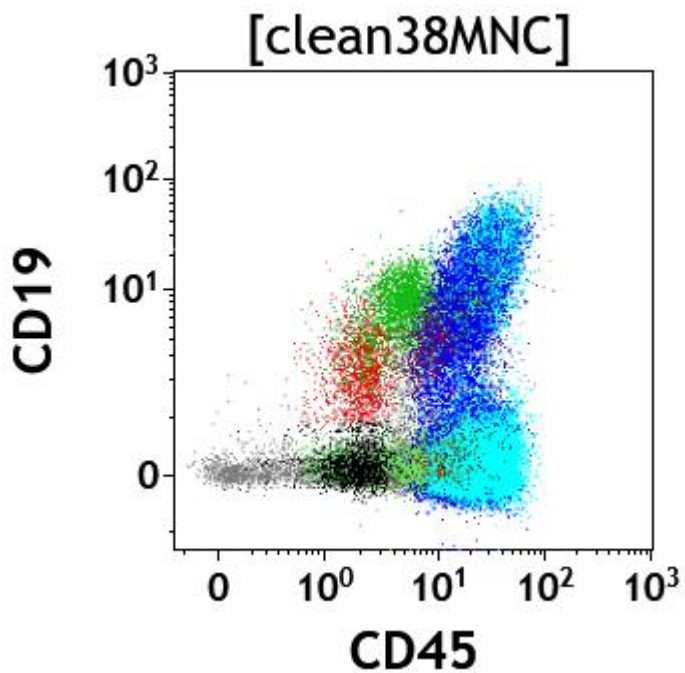
What Happens in a Flow Cytometer?

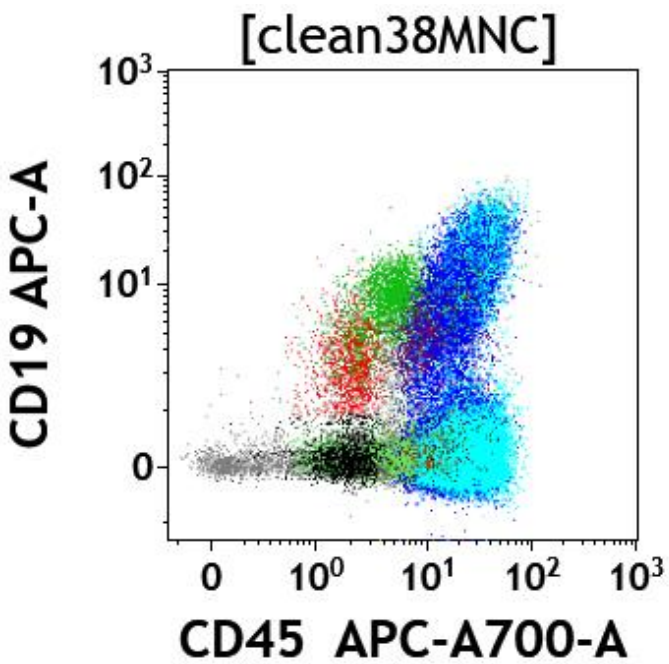


Is it a correct FCM plot?

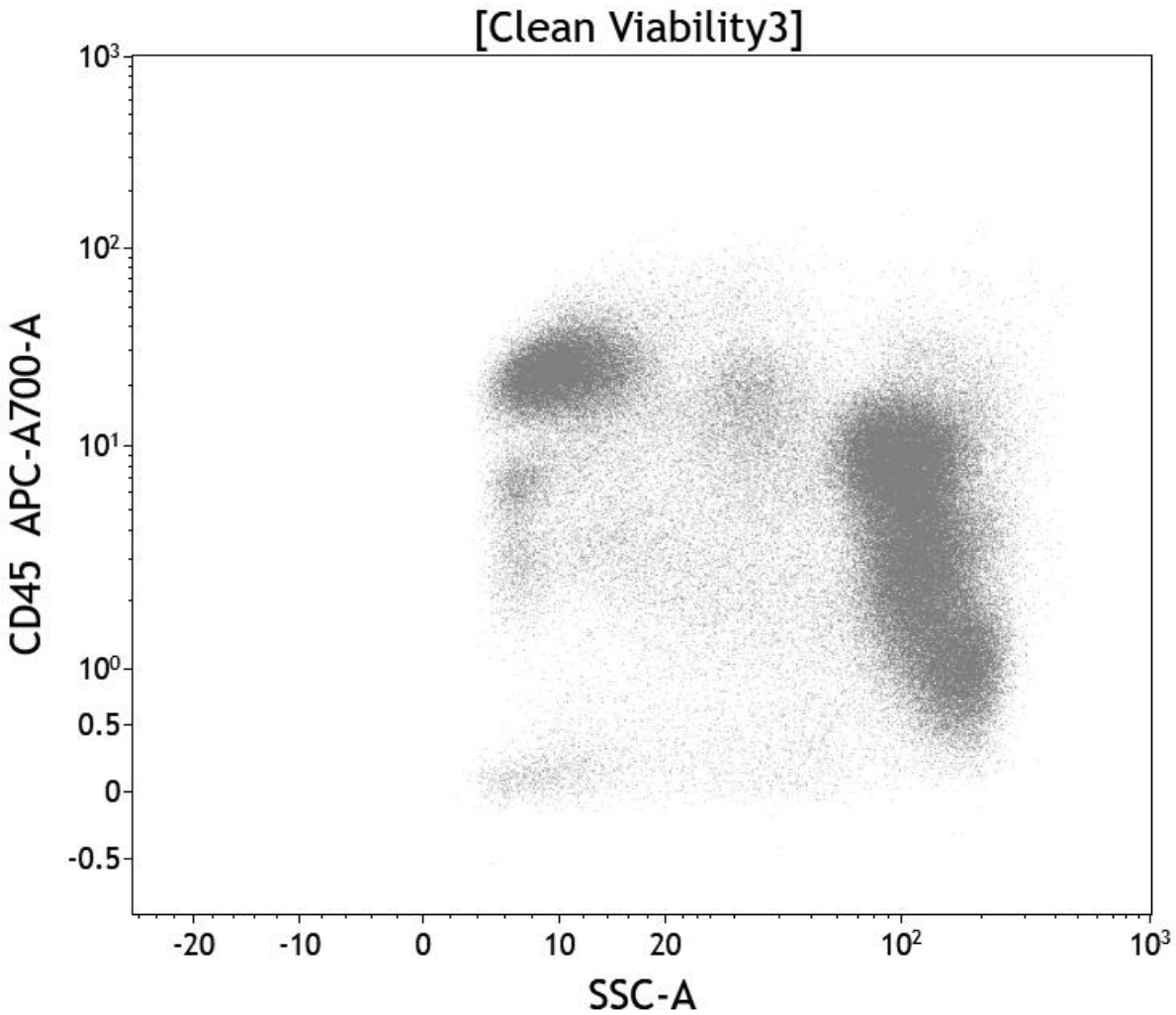


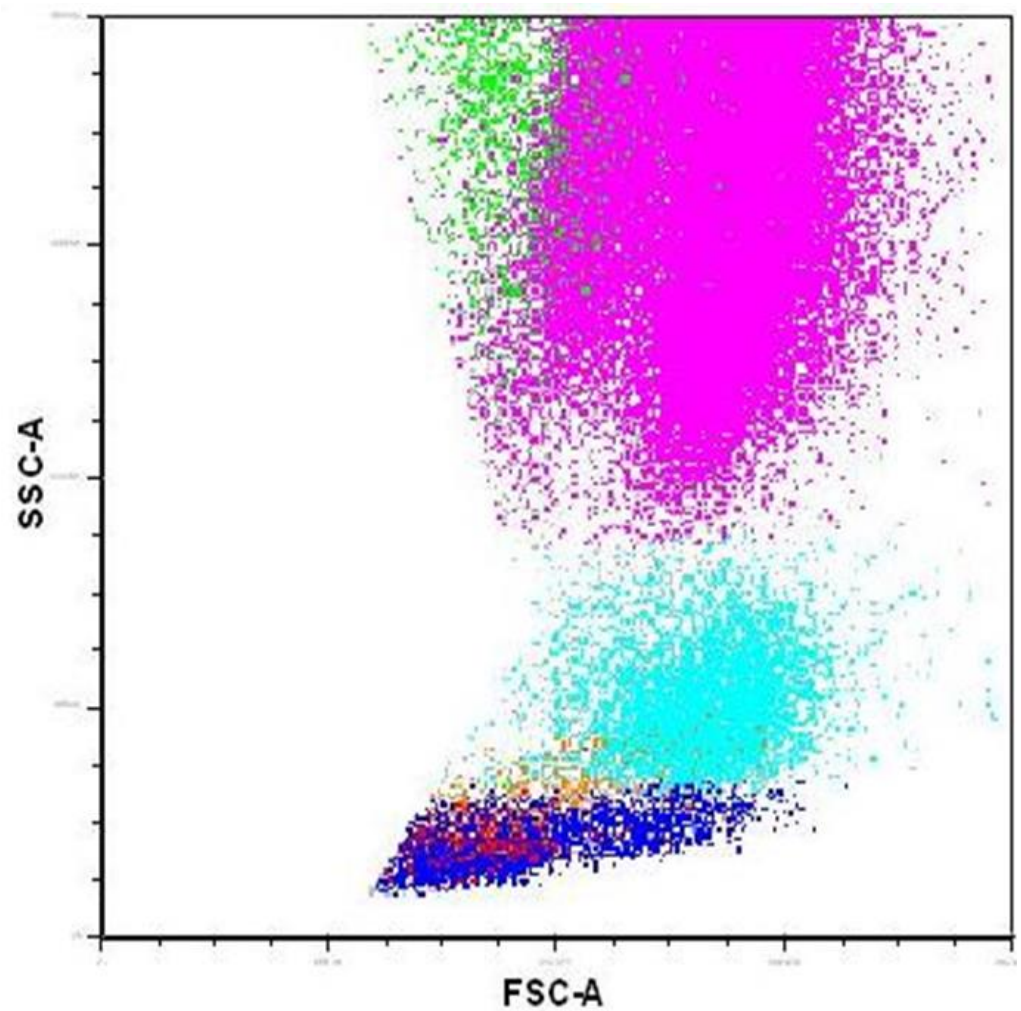
Is it a correct FCM plot?



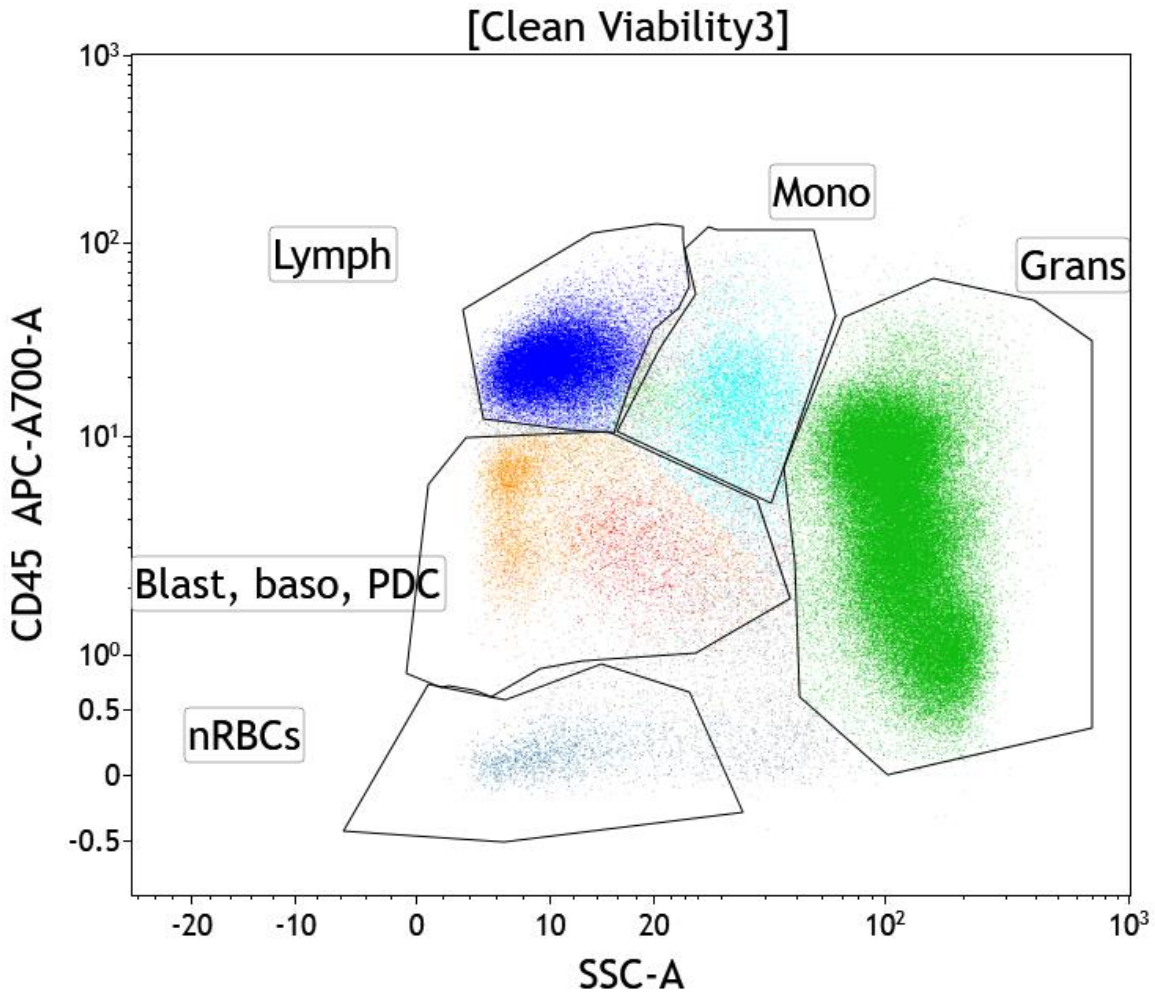


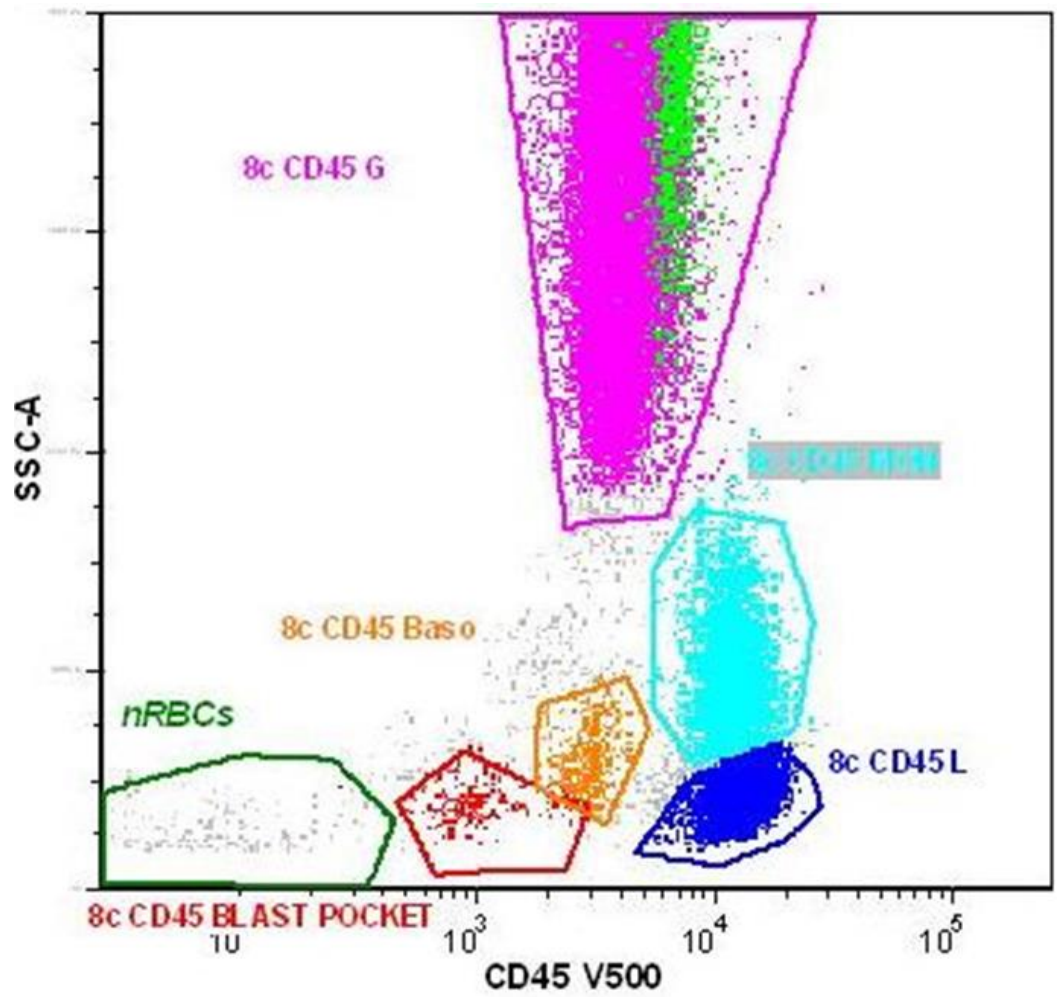
Basic Analysis Approach



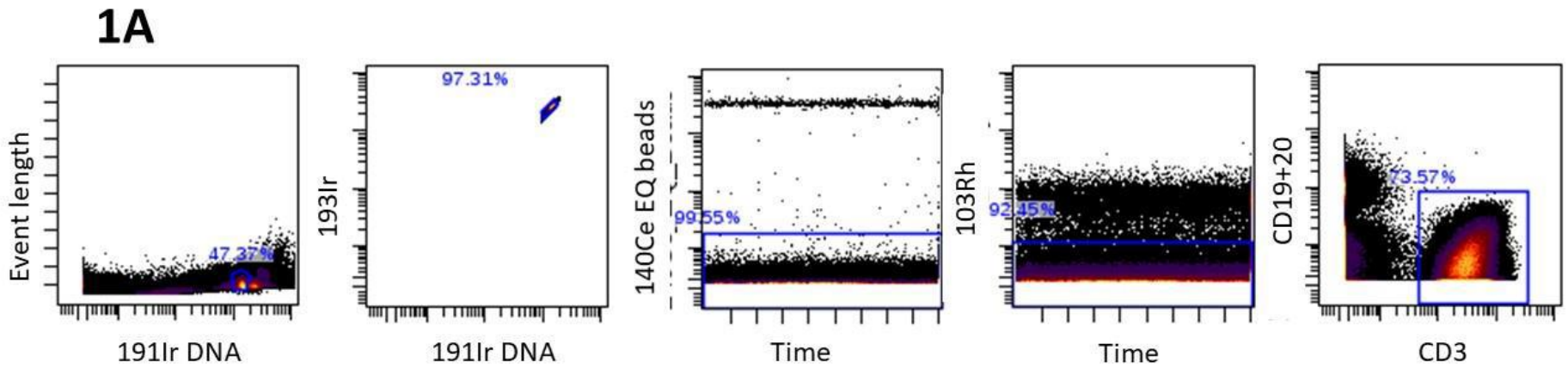


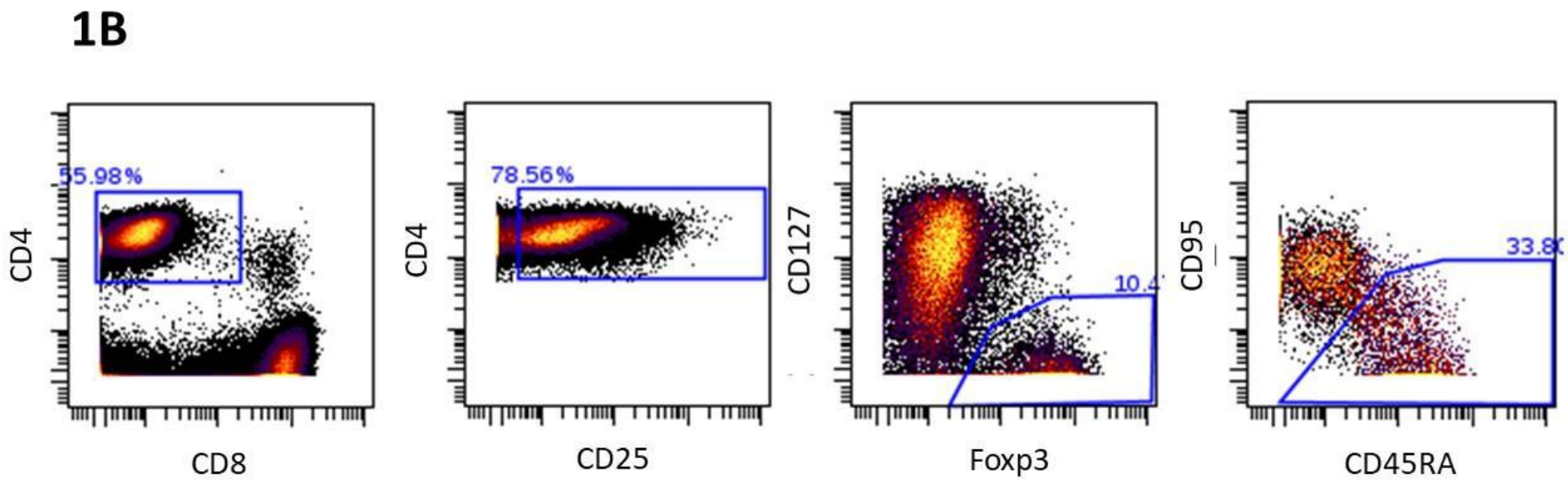
Basic Analysis Approach





Gating


1A
Event length
47.37%
191Ir DNA
193Ir
97.31%
191Ir DNA
140Ce EQ beads
99.55%
Time
103Rh
92.45%
Time
CD19+20
73.57%
CD3


1B
CD4
55.98%
CD8
CD4
78.56%
CD25
CD127
10.4
Foxp3
CD95
33.8
CD45RA

Sample processing

- *Single cell suspension*: all specimens with cells in suspension

  PB, BMA, CSF, PF, BAL

  Solid tissue
  - Fine needle aspirations
  - Tissue suspensions - slicing, mincing and teasing = Filtering

- *Sample stabilization:* Anticoagulant - EDTA or Heparin

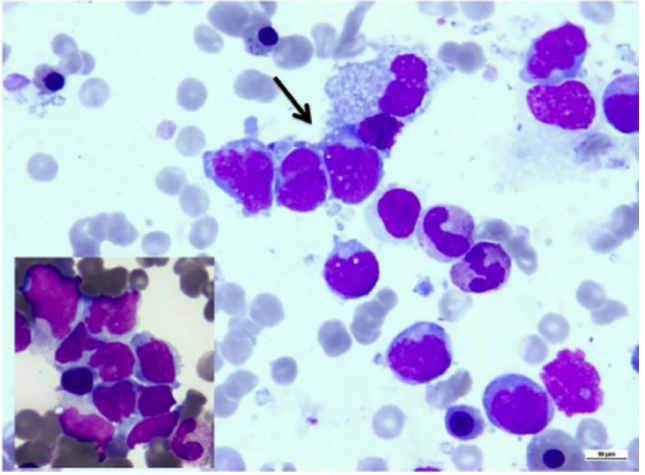
- *Enrichment of cells:* For leucocytes - RBC Lysis - NH_4CL or

  - Density gradient centrifugation – Ficoll medium

  - *Antibody staining:* Separate cells-wash-incubate with Ab-F in dark

  - *Acquisition:* Acquire the stained cells at earliest or
    Fixed and store in refrigerator

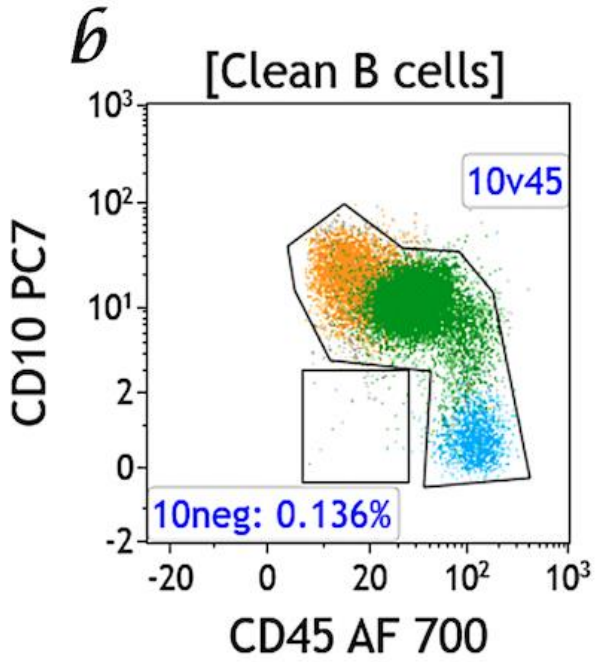
  - *Data Analysis*: Needs experience and knowledge



Clinical history+ Morphology

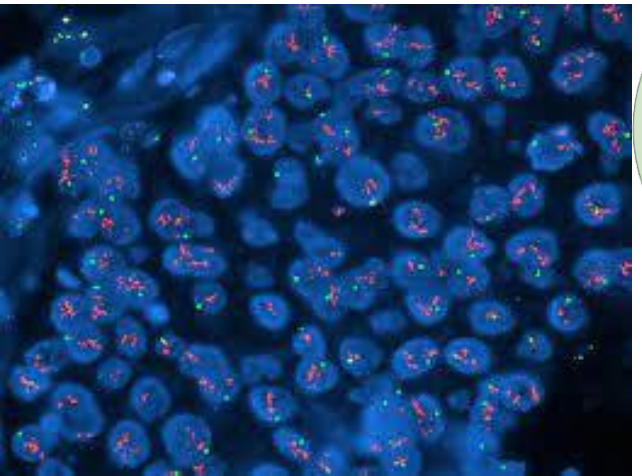
FCM Immuno-phenotyping

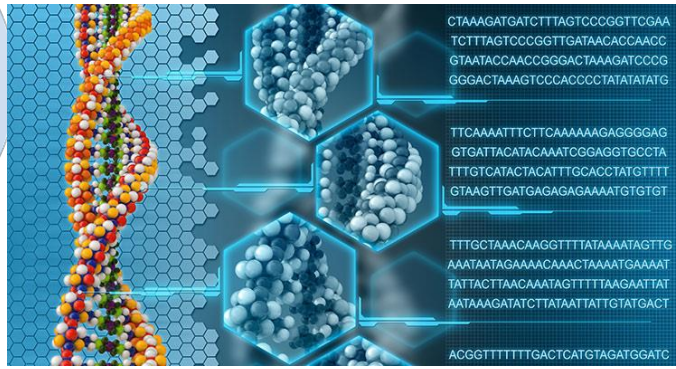
Diagnosis of Acute Leukemia



FISH + Conventional Cytogeentics

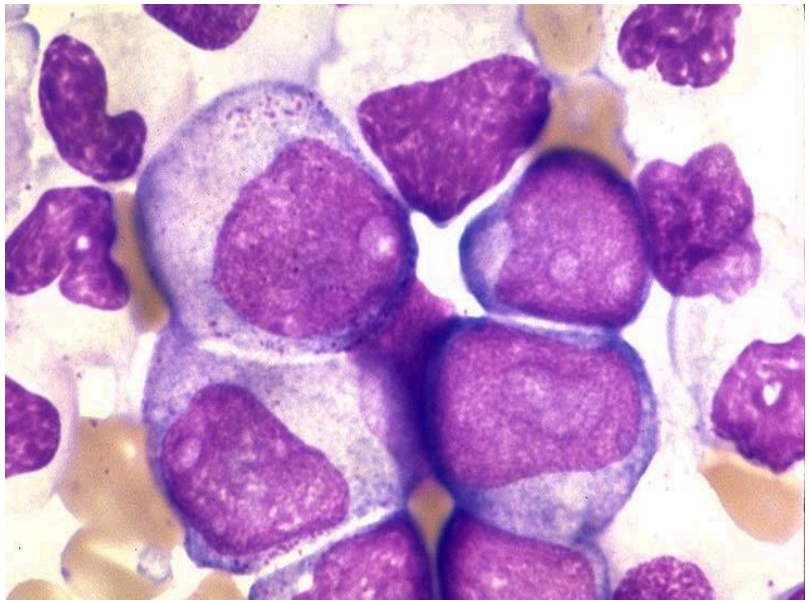
Molecular techniques





Role of FCM in AL

- **Confirmation** of presence of **abnormal** blasts
- Accurate **quantification** of blasts
- **Lineage determination** and **characterization** of abnormal blasts.

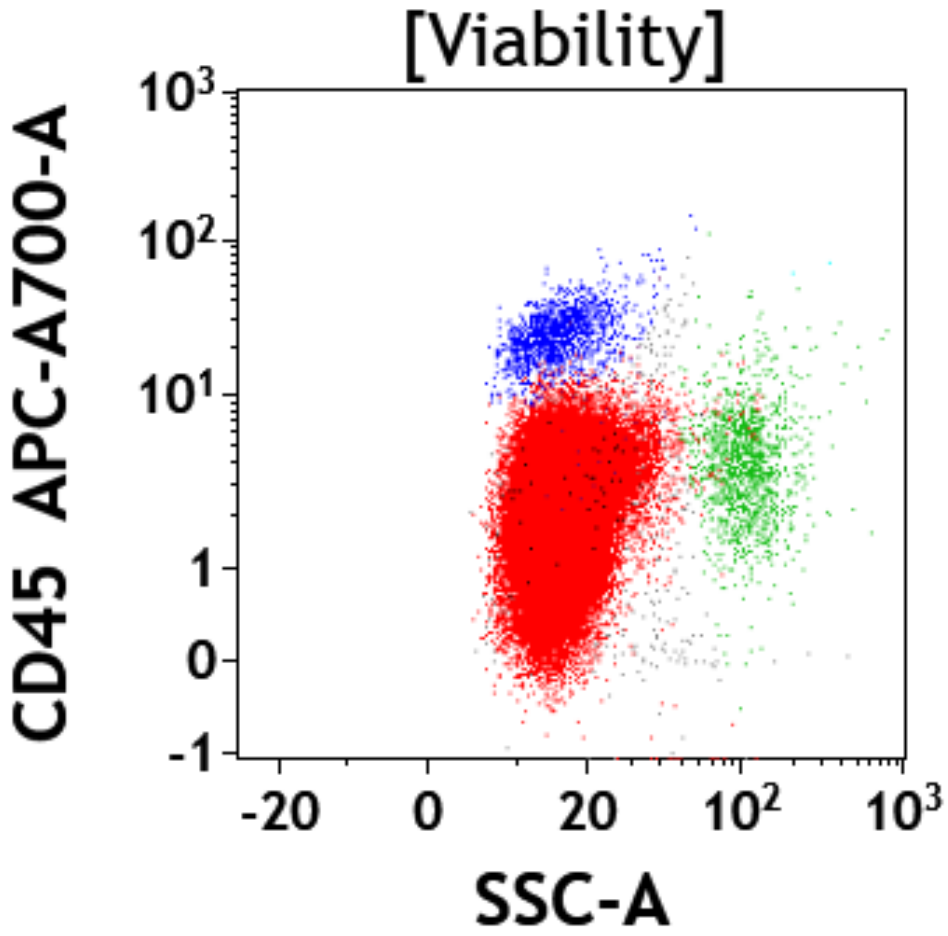


- FCM can provide reliable **prognostic information**
- Typical FCM marker expression can reliably **predict disease genotype**.
- FCM evaluation of biomarkers can predict **response to targeted therapy**
- FCM based **pattern-analysis** identifies aberrant maturation

- FCM is a practically useful, **highly sensitive** technique to **monitor treatment response** and quantitate measurable residual disease (MRD).

What are the FCM strategies to identify blasts?

- Low side light scatter
- Weak CD45 expression

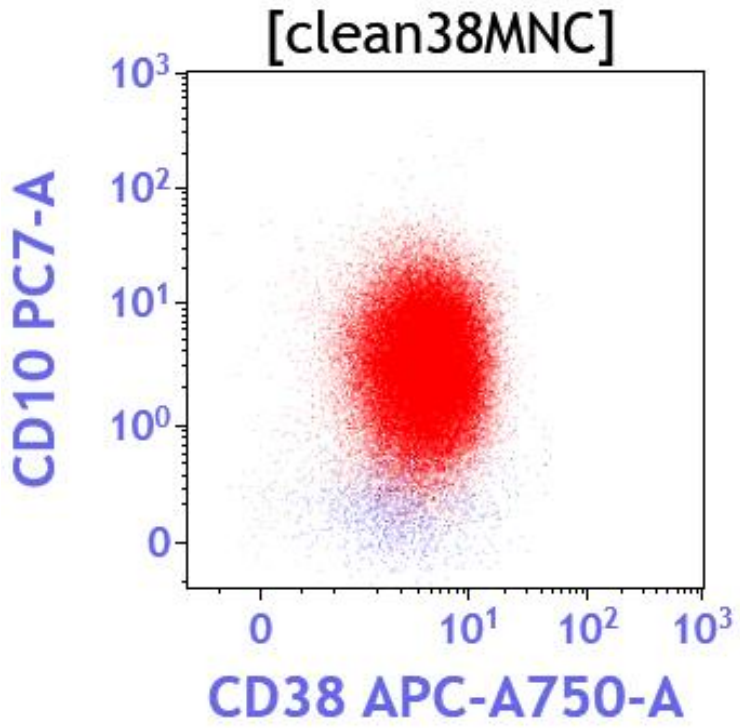


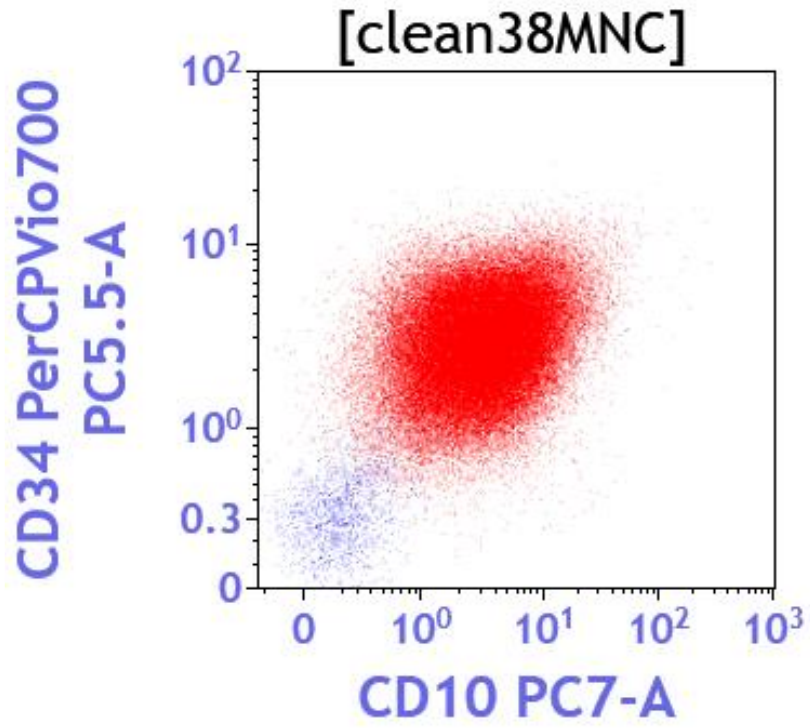
What are the FCM strategies to identify blasts?

Markers of immaturity

such as **CD34** and **TdT**

CD117, CD1a





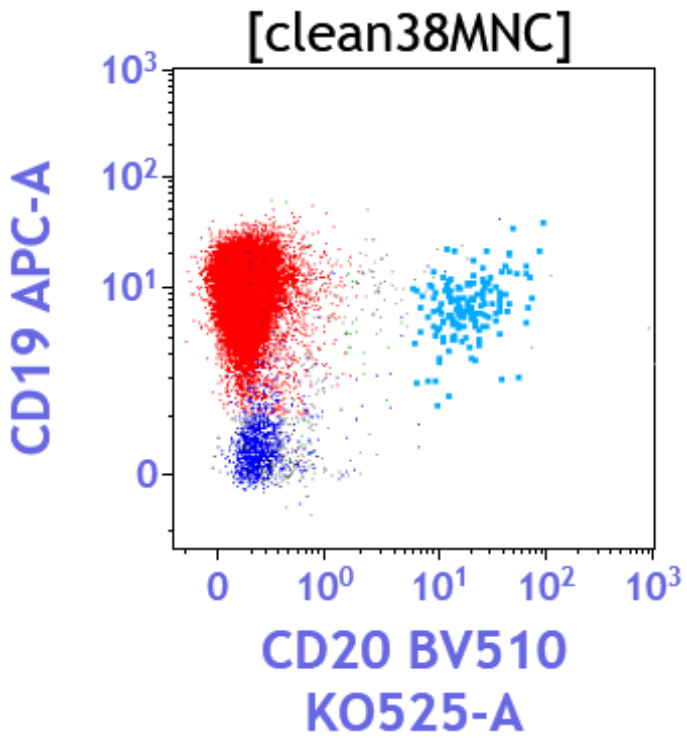
What are the FCM strategies to identify blasts?

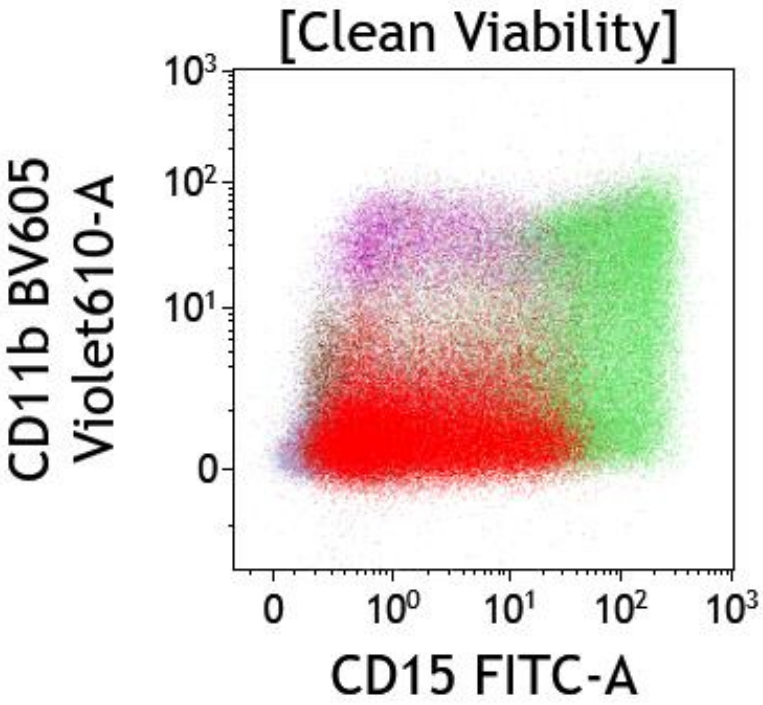
- Lack **maturity-associated** markers

Myeloblasts - CD11b, CD15, CD16.

B lymphoblasts – CD20, CD45, surface immunoglobulin heavy and light chains

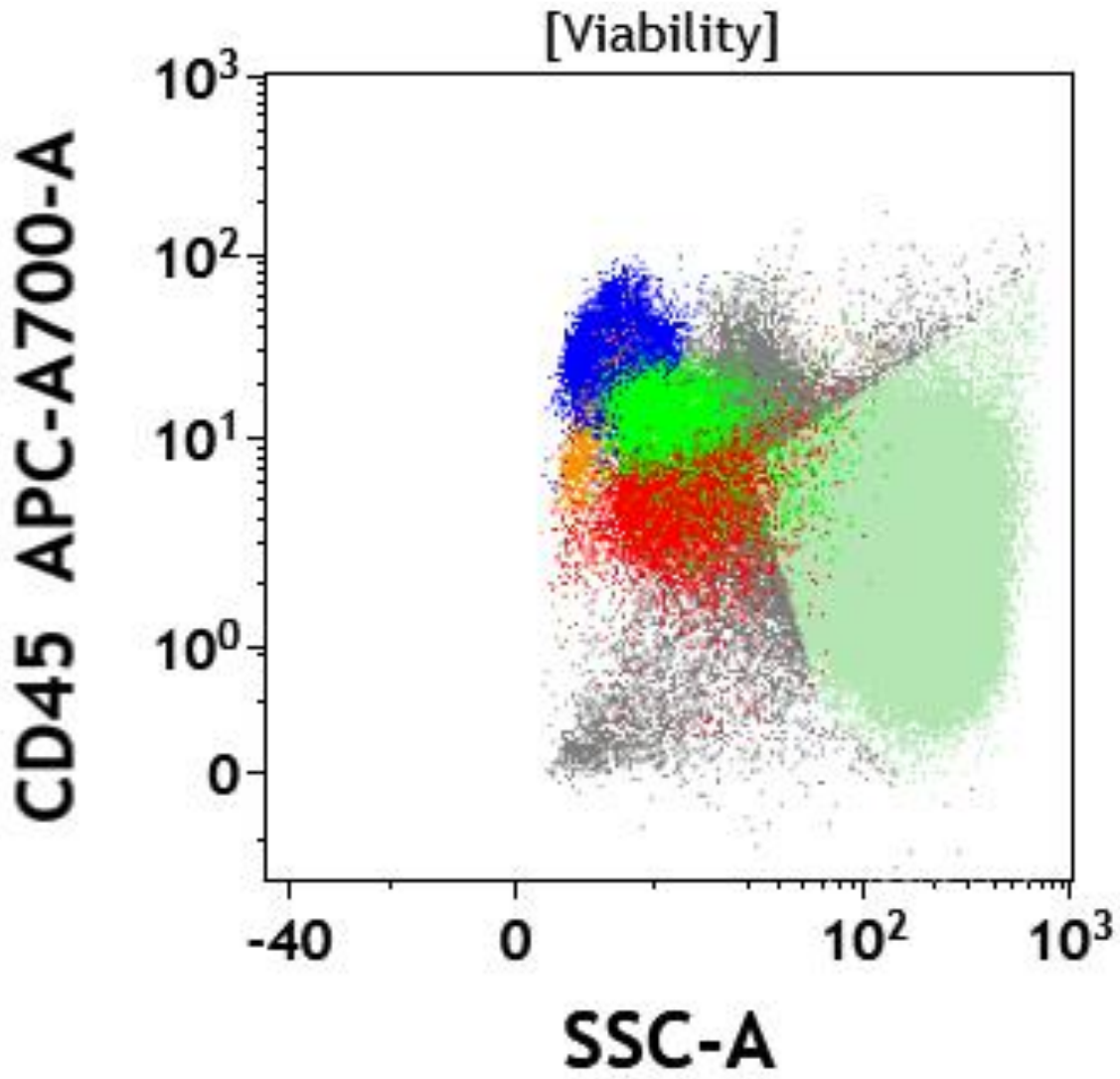
T lymphoblasts – Surface CD3





Is only CD45/SSC enough?

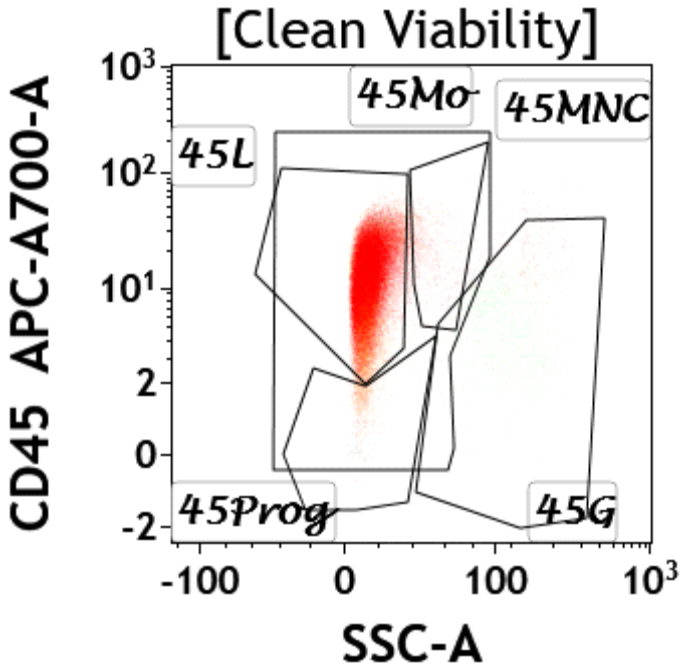
- Blasts
- PDC
- Basophils
- NK cells
- Erythroid
- **All normal precursors**

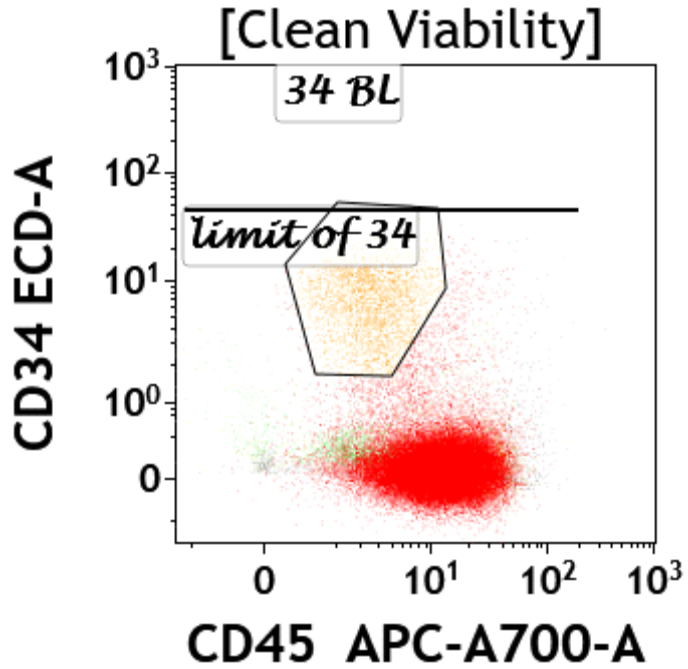


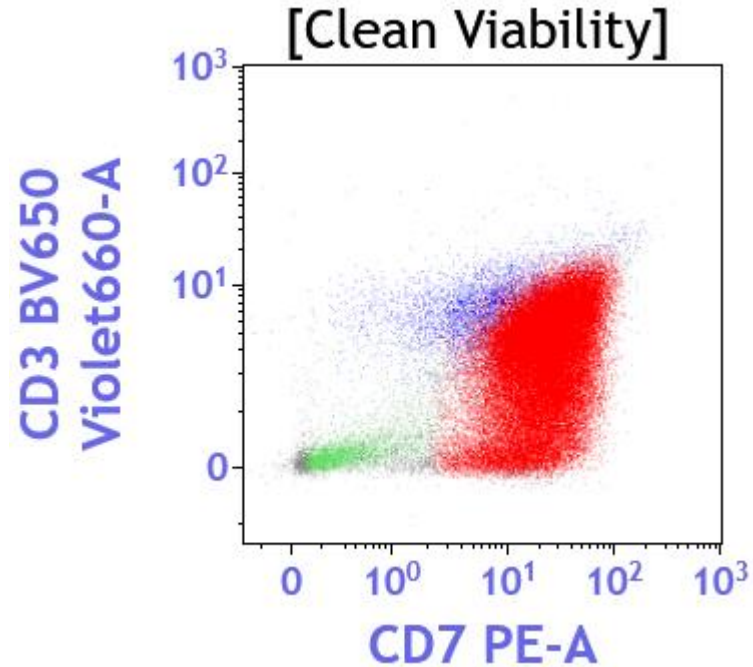
Case-1

- 16/M
- Mediastinal lymphadenopathy and supraclavicular LN
- CBC: Hb 10.1 , TC 26.9 , Plt 174

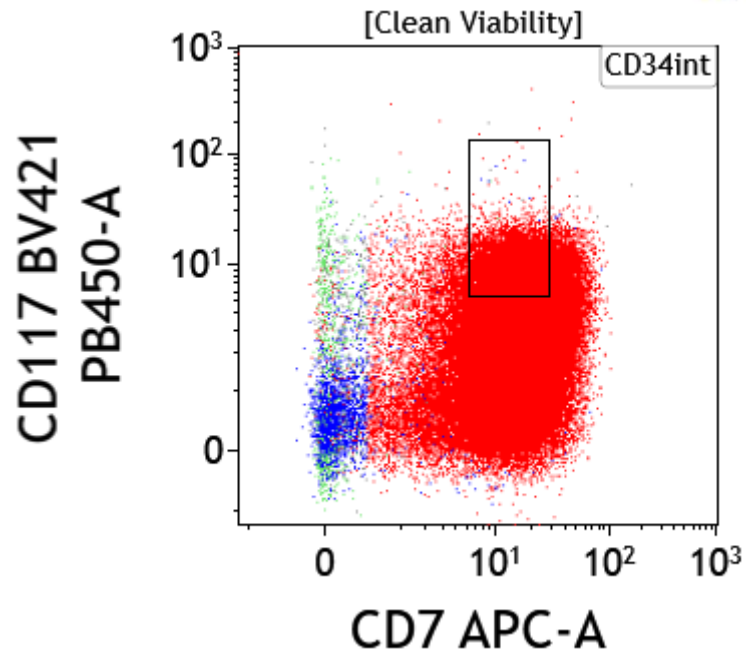
- PB for flow


[Clean Viability]
45Mo
45MNC
45L
45Prog
45G
CD45 APC-A700-A
SSC-A


[Clean Viability]
34 BL
limit of 34
CD34 ECD-A
CD45 APC-A700-A


[Clean Viability]
CD3 BV650 Violet660-A
CD7 PE-A

Blasts?


[Clean Viability]
CD34int
CD117 BV421 PB450-A
CD7 APC-A

Enumeration of Blasts

Flow cytometric count lower than manual count

- **Dilution with peripheral blood**
- Some blasts lack expression of CD34 and CD117, CD45 expression may vary
- **Morphologic blast count = Sum of all precursors**

Flow cytometric count higher than manual count

- Loss of NRBCS during red cell lysis.
- Ficoll Hypaque separation
- Blast identifications may be difficult due to poor preservation or may be disrupted during smear preparation

Immunophenotypic markers relevant to Acute Leukemia

Markers of Immaturity – TdT, CD34

Lineage Associated markers

Myeloid	**- CD13, CD33, CD117, CD64, CD11b, CD15, CD16**
Monocytic	**- CD13, CD33, CD36, CD64, CD117, CD11b, CD11c, CD14, CD4, cLysozyme**
Erythroid	**- CD36, CD71, CD105, CD235a (Glycophorin A), Hb**
Megakaryocytic	**- CD36, CD41, CD42b, CD61 andCD62**
Basophil	**- CD203, bright CD123, HLADR negative**
PDC	**- CD123, CD303, CD304, CD4, CD56, CD33**
	- Other on subset CD2, CD5, CD7
B cell	**- CD19, CD22, CD20, cCD79a, CD10, sIgM, sIgD, light chains –K/L**
T cell	**- CD1a, CD2, CD5, CD7, CD10, CD4, CD8, CD3, TCRγδ, TdT**
NK cell	**- CD16, CD56, CD57, CD94, CD161, NKp46, KIR**

Asynchronous expression of markers

Loss of normally expressed markers

Lineage Infidelity markers

(Leukemia associated immunophenotype; LAIP)

Lymphoid markers in AML - CD7, CD56, CD2, CD5 and CD19.

Myeloid markers in ALL – CD66c, CD13, CD33, CD117, CD15

B

Lineage assigning markers

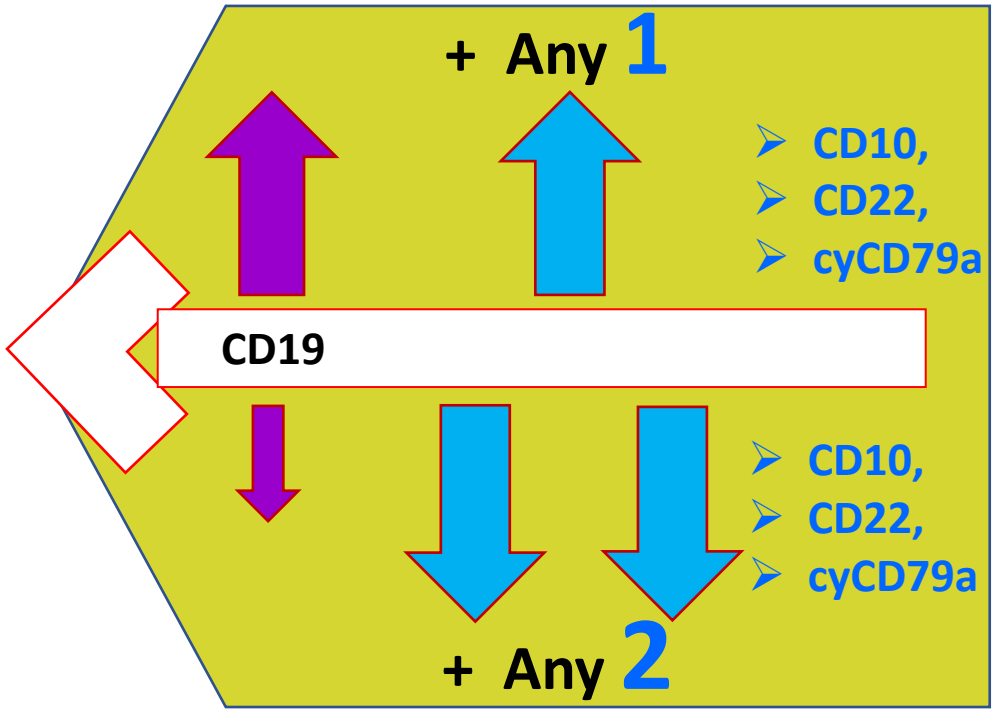
Myeloid lineage – cyMPO

Monocytic lineages -NSE, CD11c, CD14, CD64, lysozyme

T-cell lineage - sCD3/cyCD3

B-cell lineage - CD19, CD10, C[illegible]79a

B



Arber et al. Blood. DOI 10.1182/blood-2016-03-643544

What markers do I need in my panel?

Table 1. The AIEOP-BFM consensus antibody panel for pediatric ALL

Mandatory and optional markers (each combined with CD45)	
Intracellular[a, b]	iCD3, iCD22, iCD79a, iIgM (μ-chain), iLysozyme, iMPO
Surface[a]	CD2[c], CD3, CD5, CD7; CD10, CD19, CD20; CD11c, CD11b, CD13, CD14, CD15, CD33, CD64, CD65[d], CD117; CD34, (CD45), CD56, HLA-DR
	if T-ALL: CD1a, CD4, CD8, TCRαβ, TCRγδ
	if B-IV suspected: κ-chain, λ-chain (surface staining after pre-washing or intracellular)
Optional / Recommended	all cases: NG2[e], CD371[c, f]
	if BCP-ALL: CD11a[c], CD22, CD24, CD38, CD44, CD58, CD66c, CD123[c], CRLF2[c, g]
	if T-ALL: CD99, iTdT
	if BAL according to general panel: CD24, iTdT

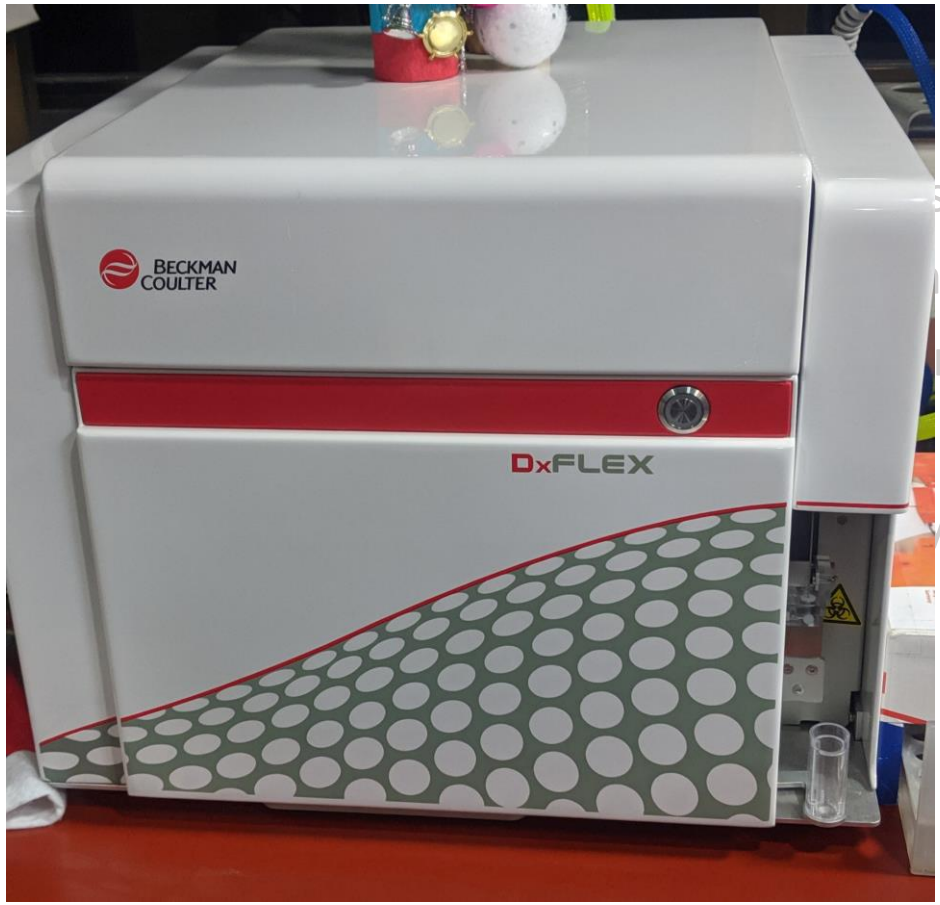
Dworzak et al. Clinical Cytometry doi.org/10.1002/cyto.b.21518

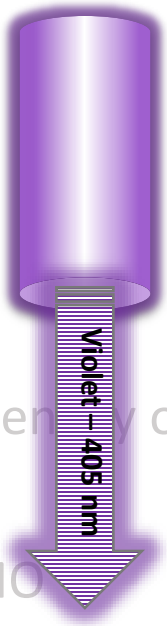
Thought process behind panel designing

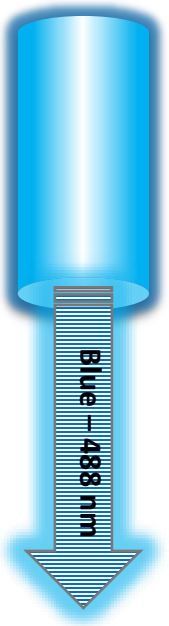
- Accurate lineage assignment
- Should be able to identify relevant subgroups, such as ETPALL, CRLF2-rearranged BCR-ABL1 like B-ALL, RAM-AML
- Should document enough immunophenotypic information for MRD assessment
- Predict relevant genomic subgroups

How to design my panel?

- **Know your instrument**



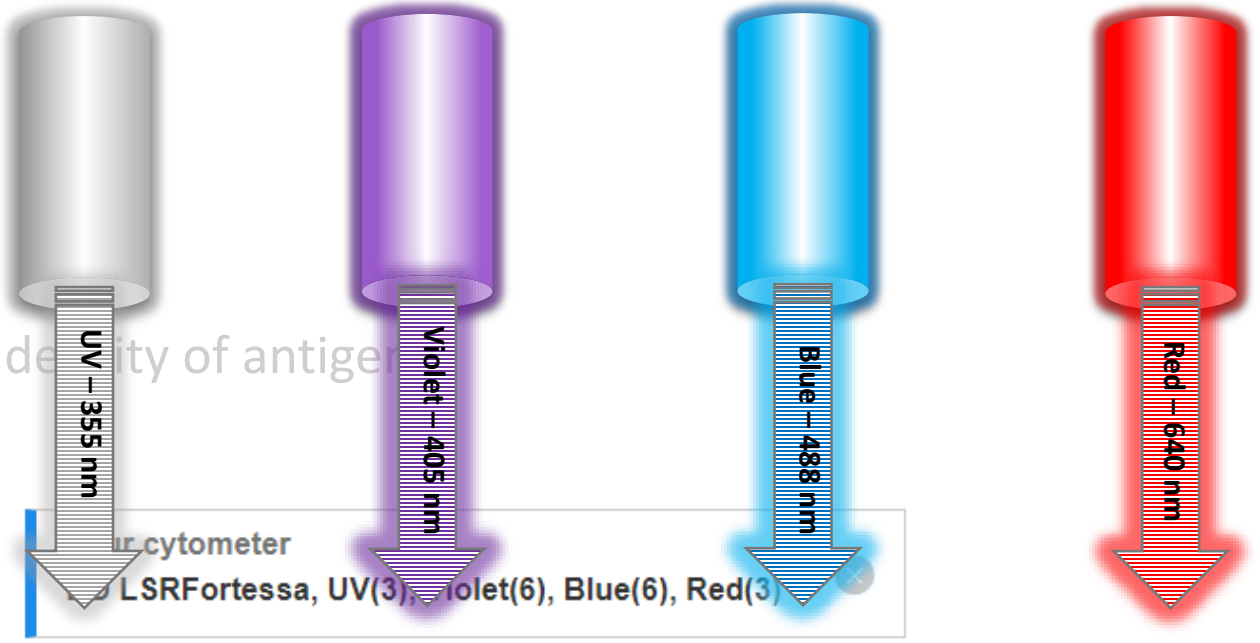




Violet 405nm	Blue 488nm	Red 638nm
450/45	525/40	660/10
525/40	585/42	712/25
610/20	610/20	780/60
660/10	690/50	
780/60	780/60	

How to design my panel?

- **Know your instrument**



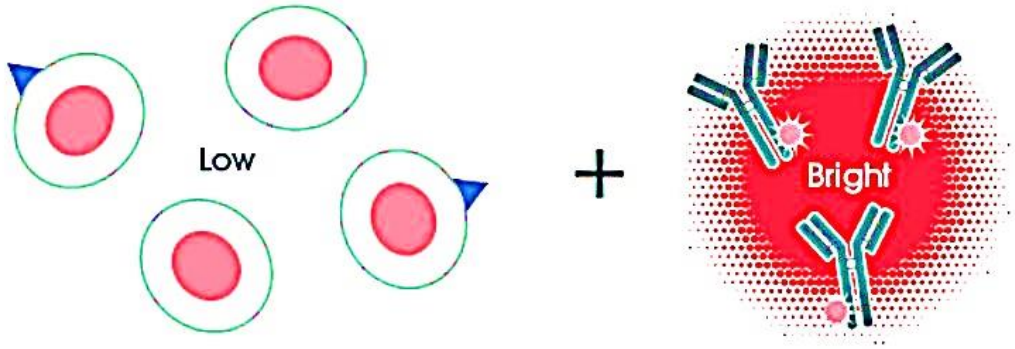
UV 355nm	Violet 405nm	Blue 488nm	Red 640nm
379/28	450/50	530/30	670/14
450/50	525/50	575/26	730/45
740/35	610/20	610/20	780/60
	660/20	670/30	
	710/50	695/40	
	780/60	780/60	

How to design my panel?

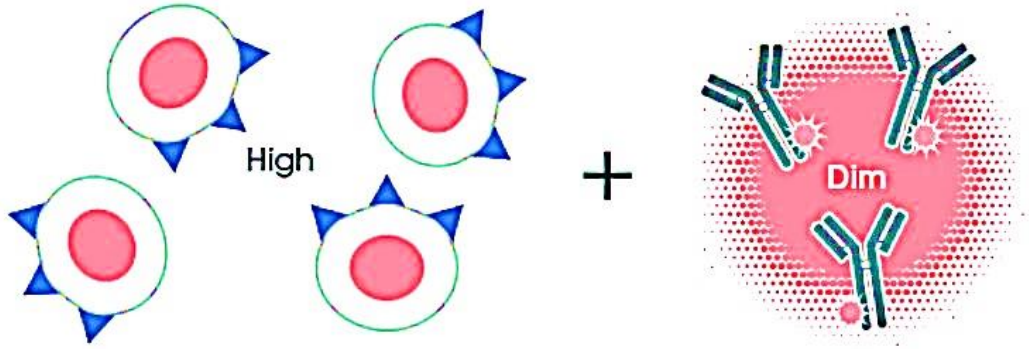
- Know your instrument

- **Match fluorochromes by brightness to the density of antigens**

Low/unknown antigen expression and/or low cell populations
= use brighter fluorochromes, eg PE



High antigen expression and/or high cell populations
= use dimmer fluorochromes, eg PerCP



How to design my panel?

- Know your instrument

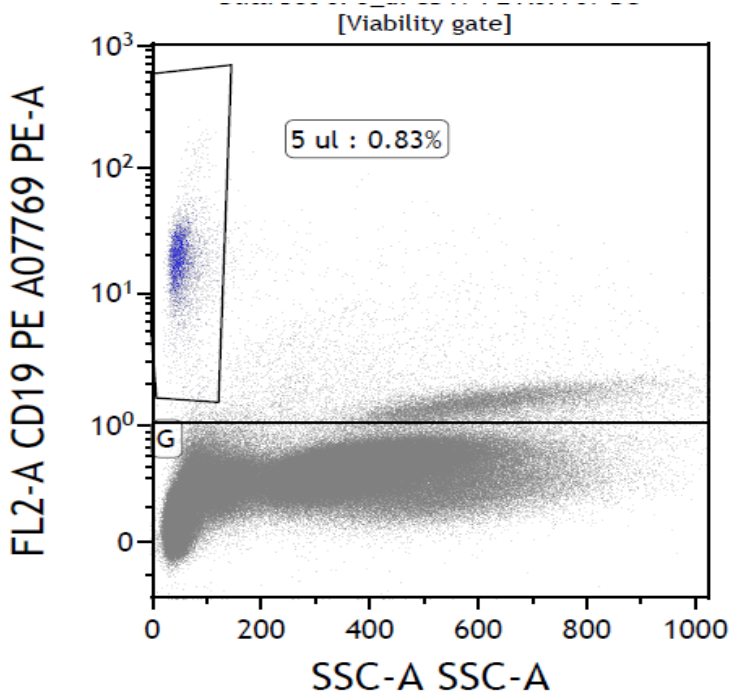
- Match fluorochromes by brightness to the density of antigens

- **Evaluate the spillover of fluorochromes - FMO**
- **Minimize spillover of adjacent fluorochromes**
- **Be informed about spillover and background in each channel of your panel**

- **Use appropriate controls and validate your panel**

- **Calculate the cost of the assay**

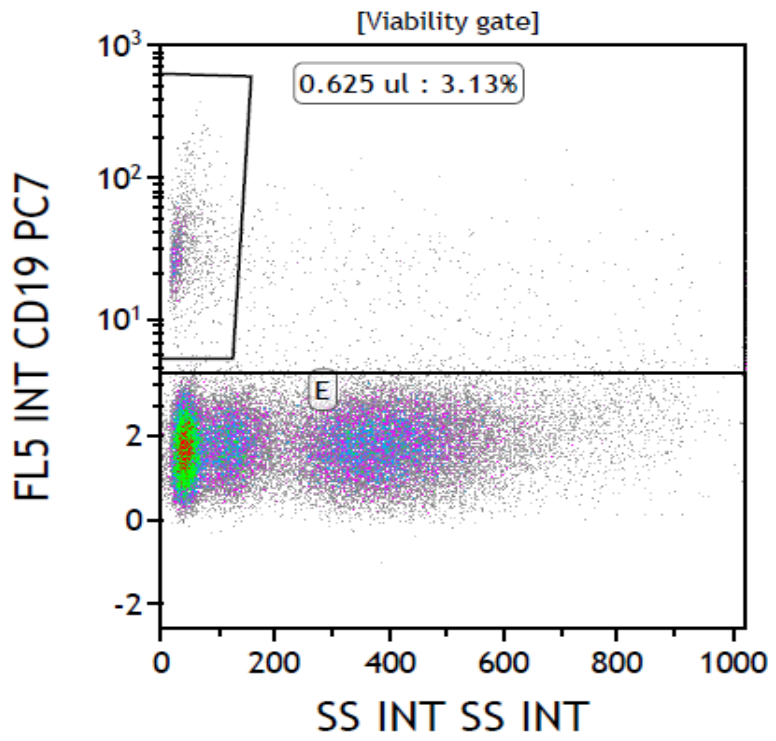
Must confirm yourself



Gate	Y-GMean
All	0.36
5 ul	15.94
G	0.33

CD19 PE A07769 BC

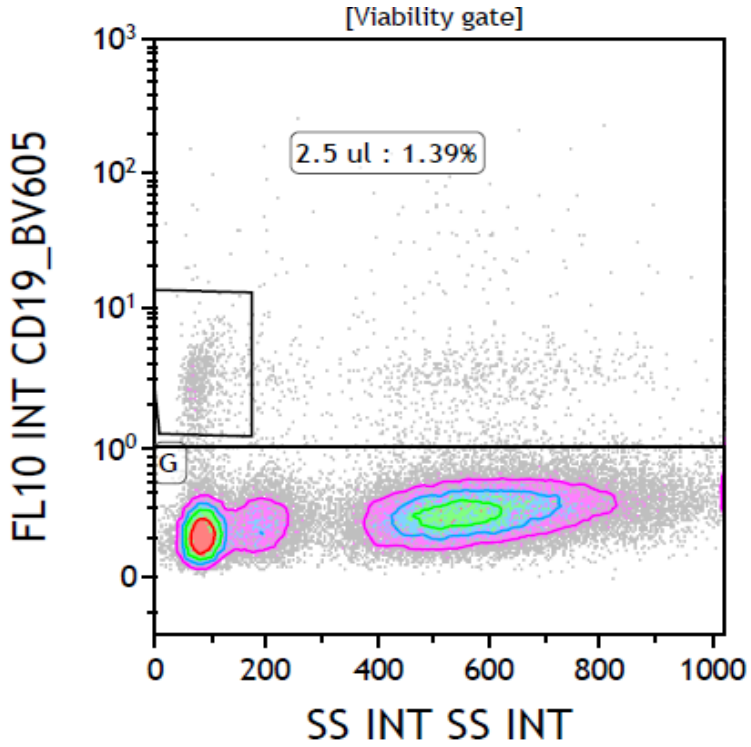
Conc	20_ul	10_ul	5_ul	2.5_ul	1.25_ul	0.625_ul	0.312_ul	0.156_ul
Signal	25.47	20.55	15.94	9.45	5.65	3.56	1.91	1.06
Noise	0.37	0.34	0.33	0.32	0.32	0.3	0.32	0.31
s/n Ratio	68.84	60.44	48.30	29.53	17.66	11.87	5.97	3.42



Gate	Y-GMean
All	1.92
0.625 ul	28.93
E	1.69

CD19_PC7 BC IM3628

Conc	10_ul	5_ul	2.5_ul	1.25_ul	0.625_ul	0.312_ul
Signal	79.78	93.16	51.03	39.08	28.93	21.9
Noise	17.21	28.82	4.39	2.62	1.69	1.1
s/n Ratio	4.64	3.23	11.62	14.92	17.12	19.91



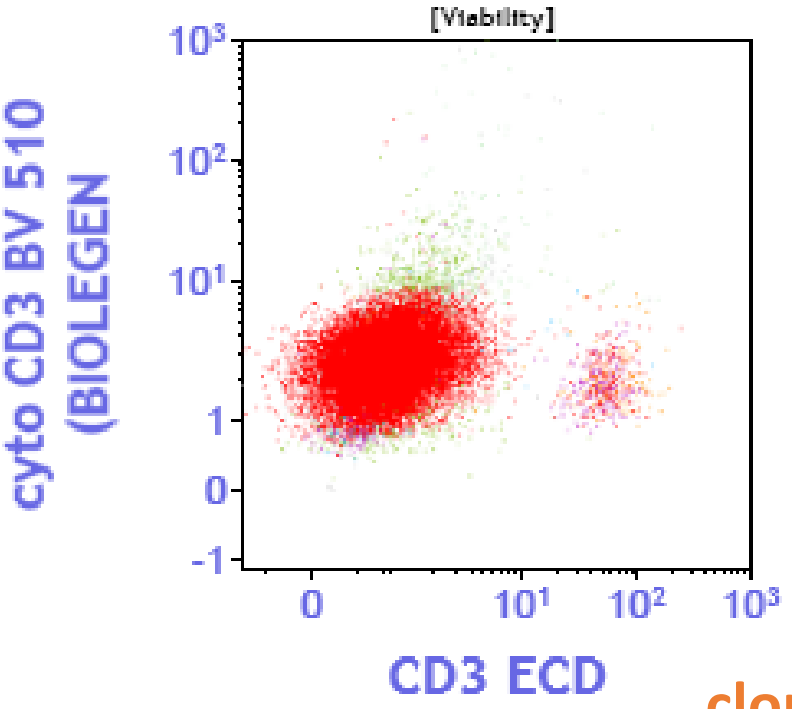
Gate	Y-GMean
All	0.36
2.5 ul	2.95
G	0.33

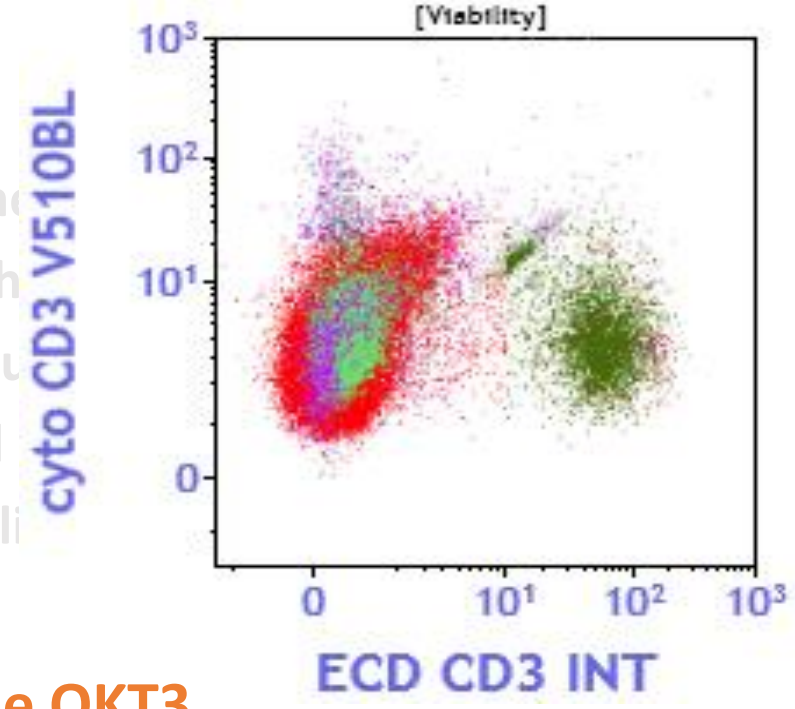
CD19 BV605_300556_BIOLEGEND

Conc	5_ul	2.5_ul	1.25_ul	0.625_ul	0.312_ul	0.156_ul
Signal	3.4	2.95	2.86	2.48	2.45	1.97
Noise	0.35	0.33	0.32	0.31	0.32	0.31
s/n Ratio	9.71	8.94	8.94	8.00	7.66	6.35

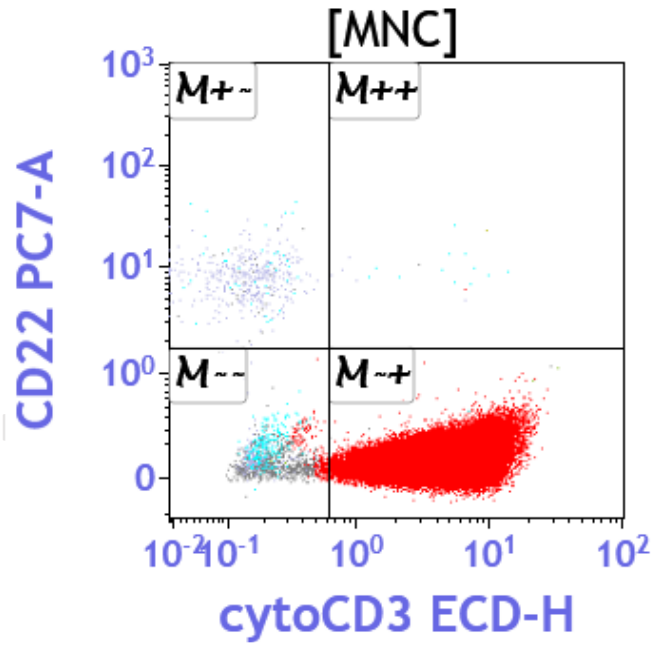
How to design my panel?

clone UCHT1





clone OKT3



- **Multicolor staining approach is compulsory (i.e., ≥4 colors; 6-color approach recommended) CD45-backbone+**
- **Precaution about intracellular markers: MPO, cCD3**

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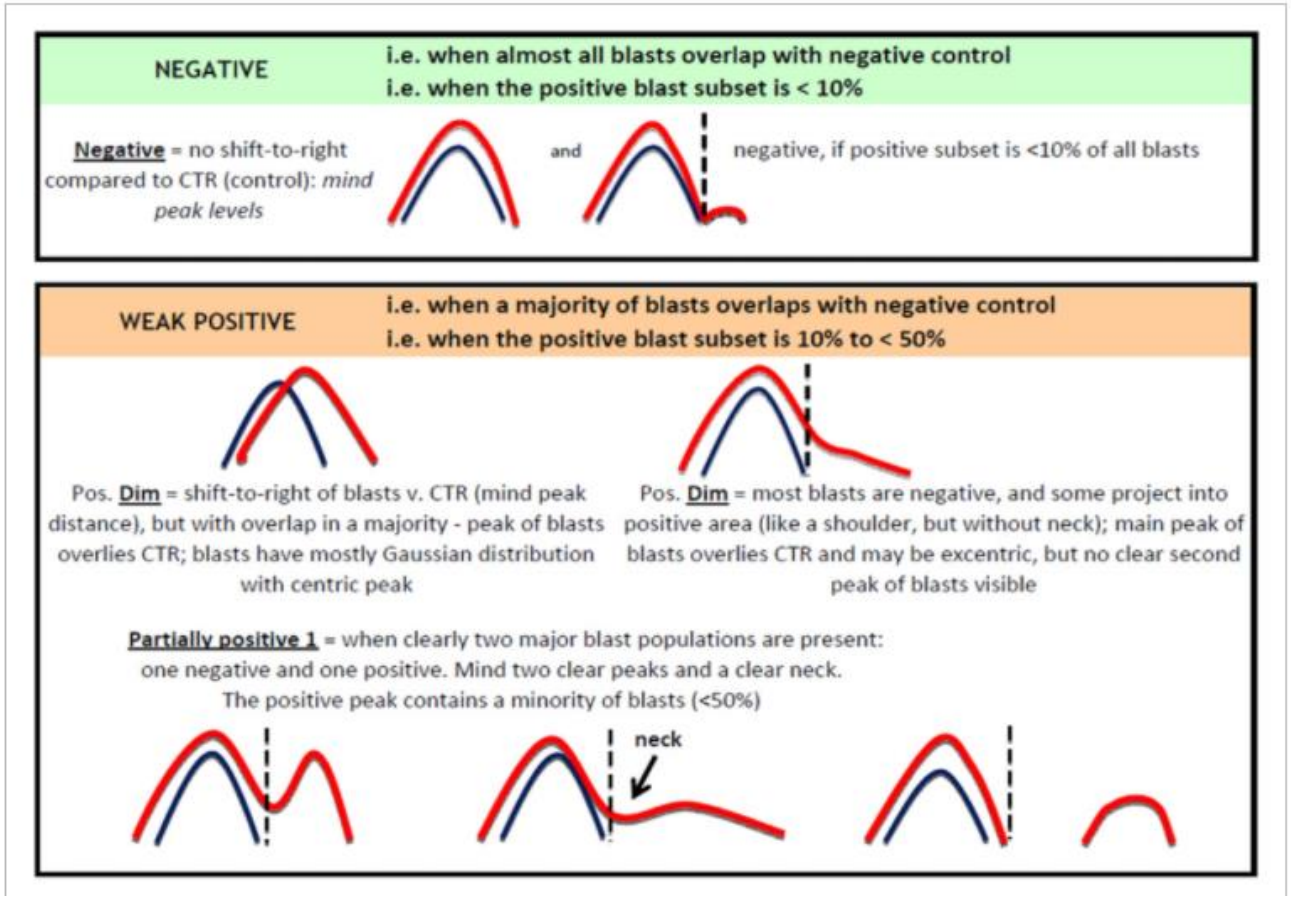
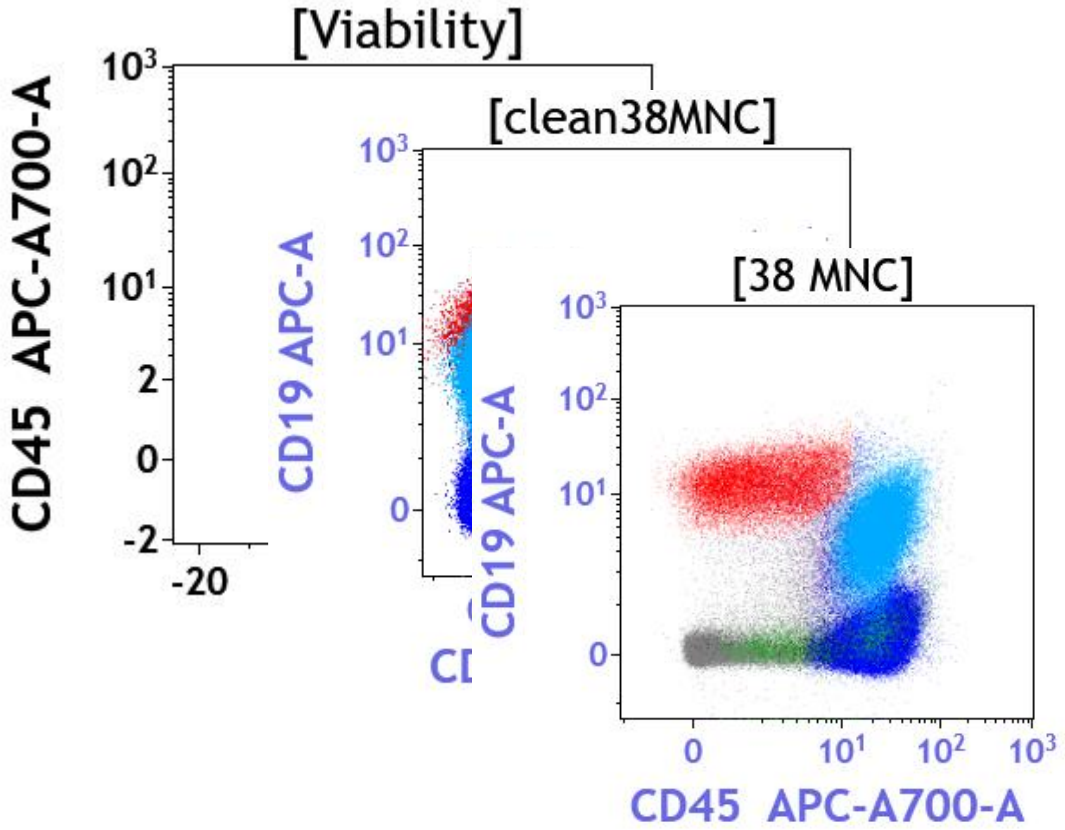
FCM AL Panel in TMH

Tube No.	BV786	BV650	BV605	BV510	BV421	FITC	PE	ECD	PC5.5	PC7	APC	APC AF_700	APC AF_750
1	CD25	X	X	CD20	CD123	CD304	CD86	CD73	CD34	CD10	CD19	CD45	CD38
2	CD16	CD14	CD11b	HLA-DR	CD117	CD15	CD13	CD19	CD34	CD56	CD7	CD45	CD38
3	CD14	CD11c	X	HLA-DR	CD36	CD163	CD123	CD64	CD33	CD117	CD34	CD45	CD38
4	CD56	X	X	sCD3	CD5	CD4	CD7	CD34	TCR γδ	CD2	CD1a	CD45	CD8
5	X	X	X	x	CD117	AMPO	cyto CD79a	cytoCD3	x	CD22	CD34	CD45	CD11b
Ploidy	X	X	X	X	**FxCycle**	X	X	CD19	X	CD10	CD34	CD45	CD38
CRLF2	X	X	X	X		**NG2**	X	CD19	**TSLP**	CD10	CD34	CD45	CD38
Add Cyto	CD117	X	x	CD20 + CD3	CD5	CD38	Pax-5	CD34	CD19	CD10	CD7	CD45	CD11b
Add B-ALL	X	X	X	CD20	CD44	CD58	CD66c	CD34	CD19	CD10	ROR1	CD45	CD81
Add MGK	X	X	X	HLADR	CD36	CD41	CD61	CD34	CD33	CD117	CD42b	CD45	CD38

How to describe immunophenotype?

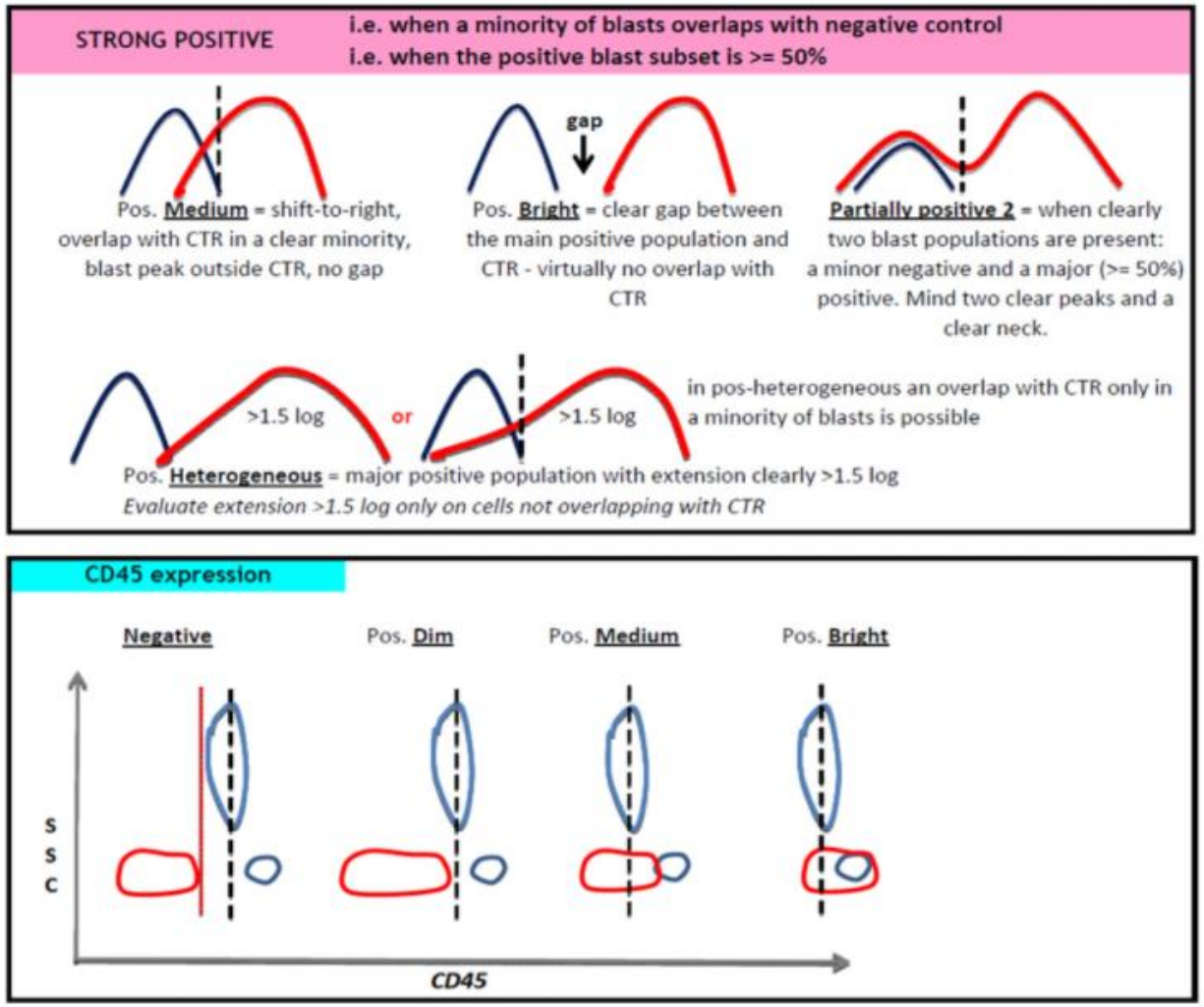
A threshold **≥10%** of gated blasts is used for all antigens (independently from surface or intracellular location) to consider an antigen as “positive.”

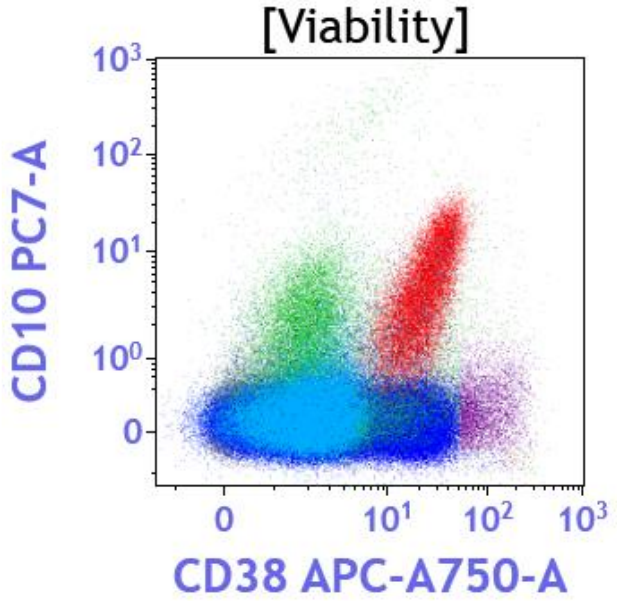


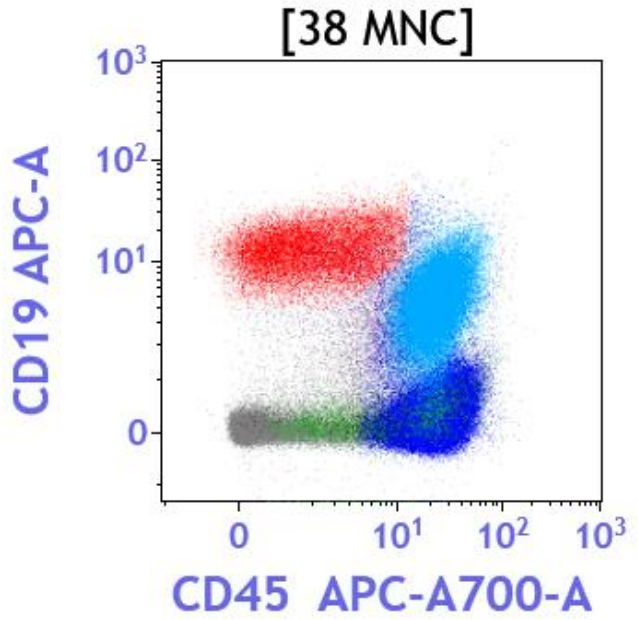


Dworzak et al. Clinical Cytometry doi.org/10.1002/cyto.b.21518

How to describe immunophenotype?







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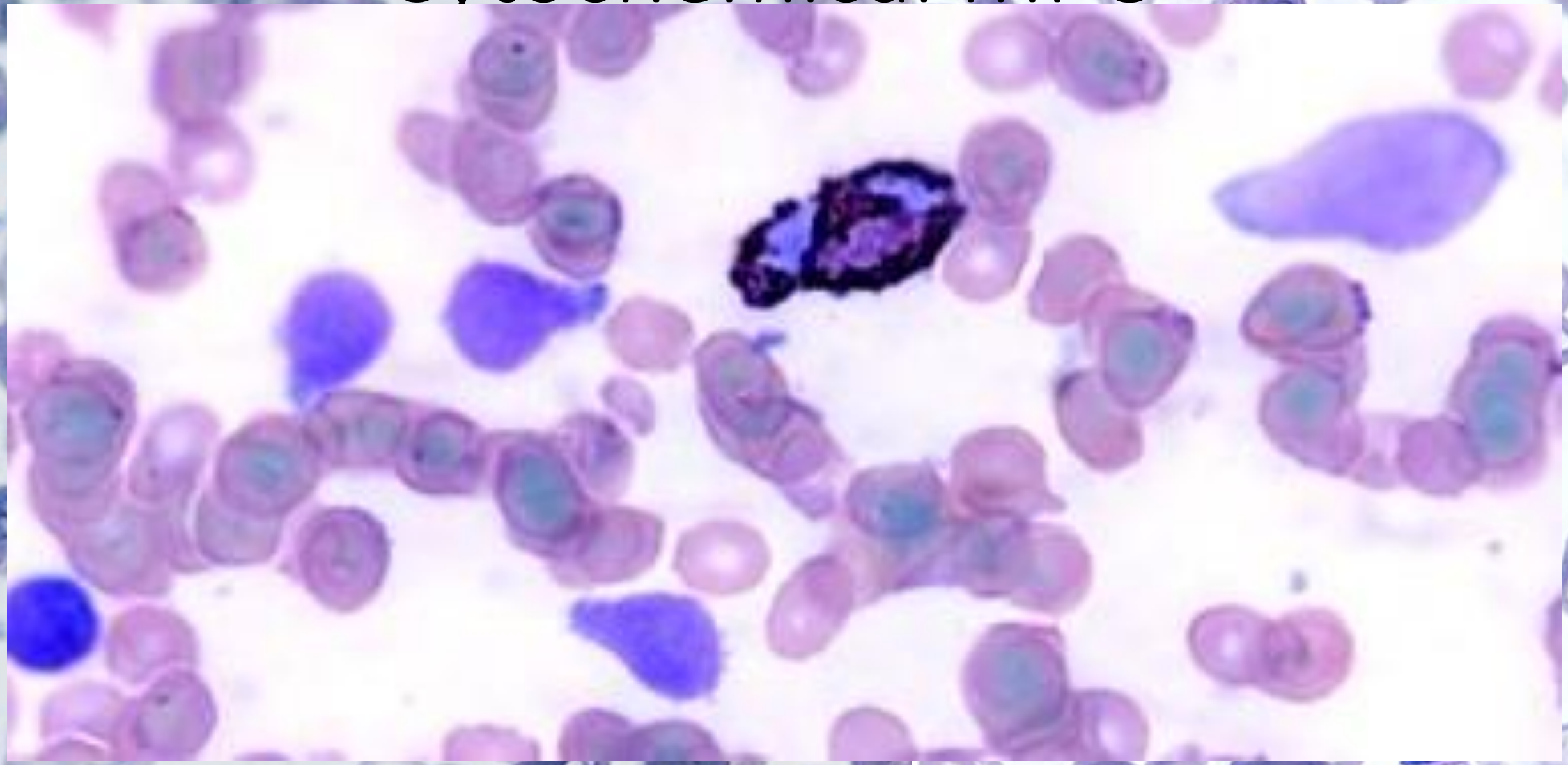
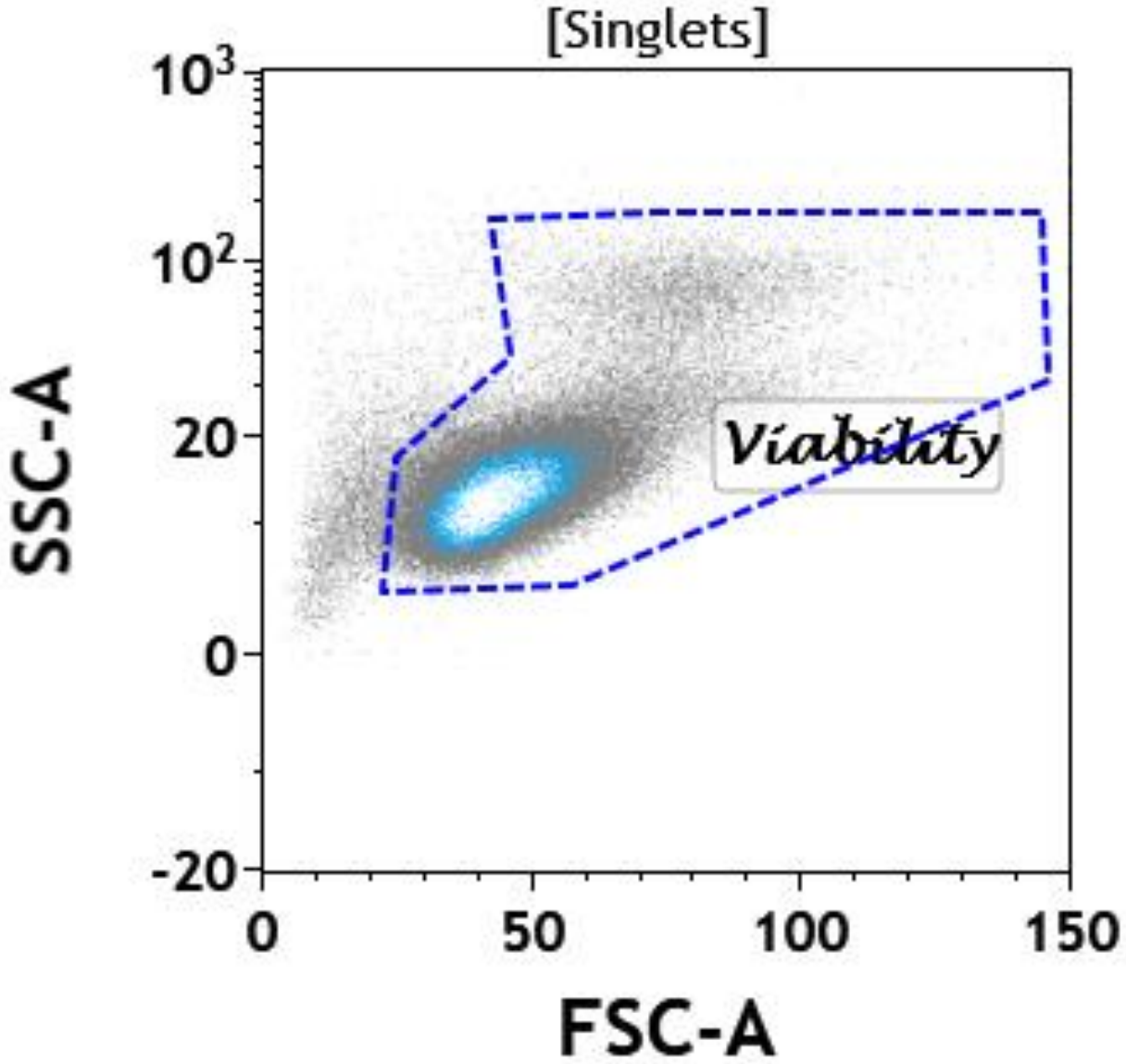
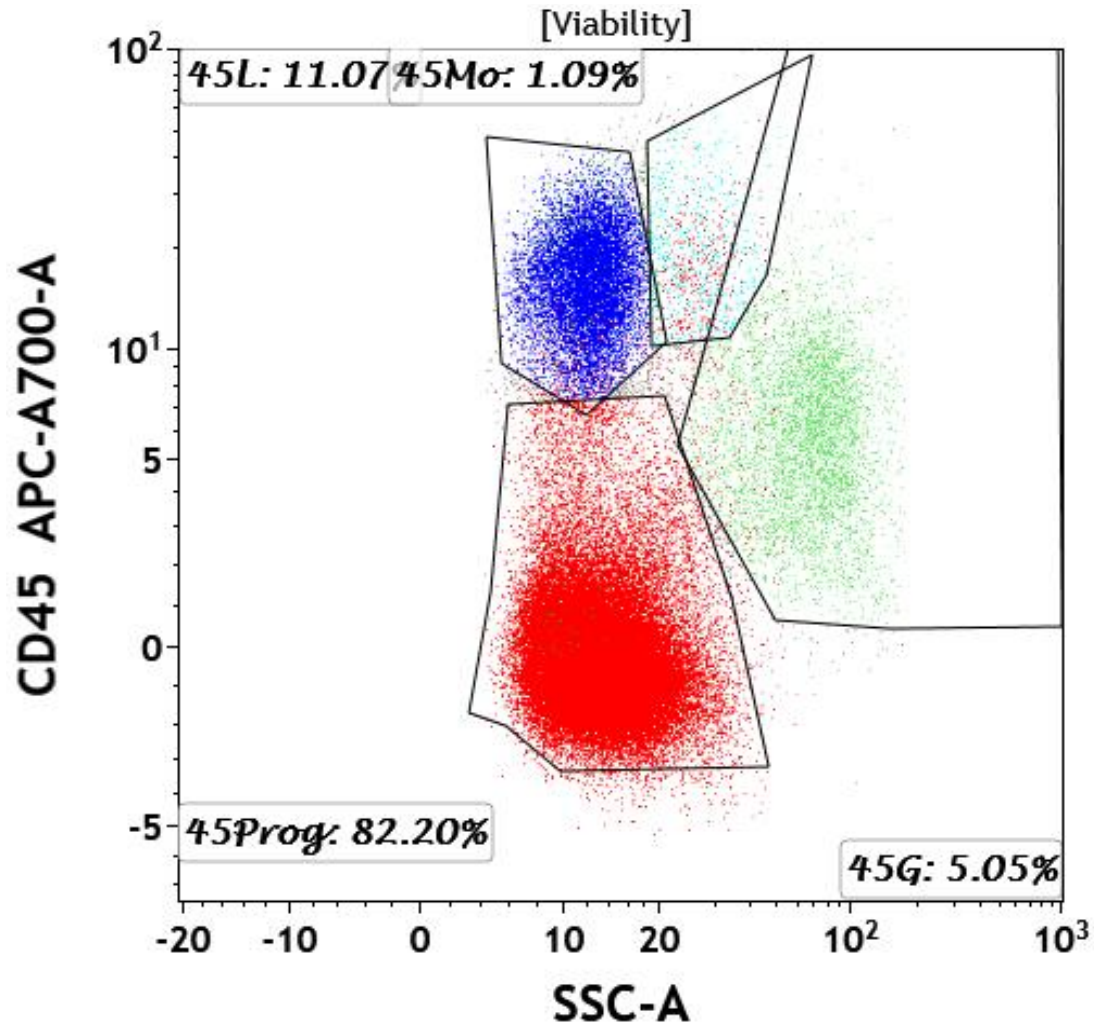
Case-2

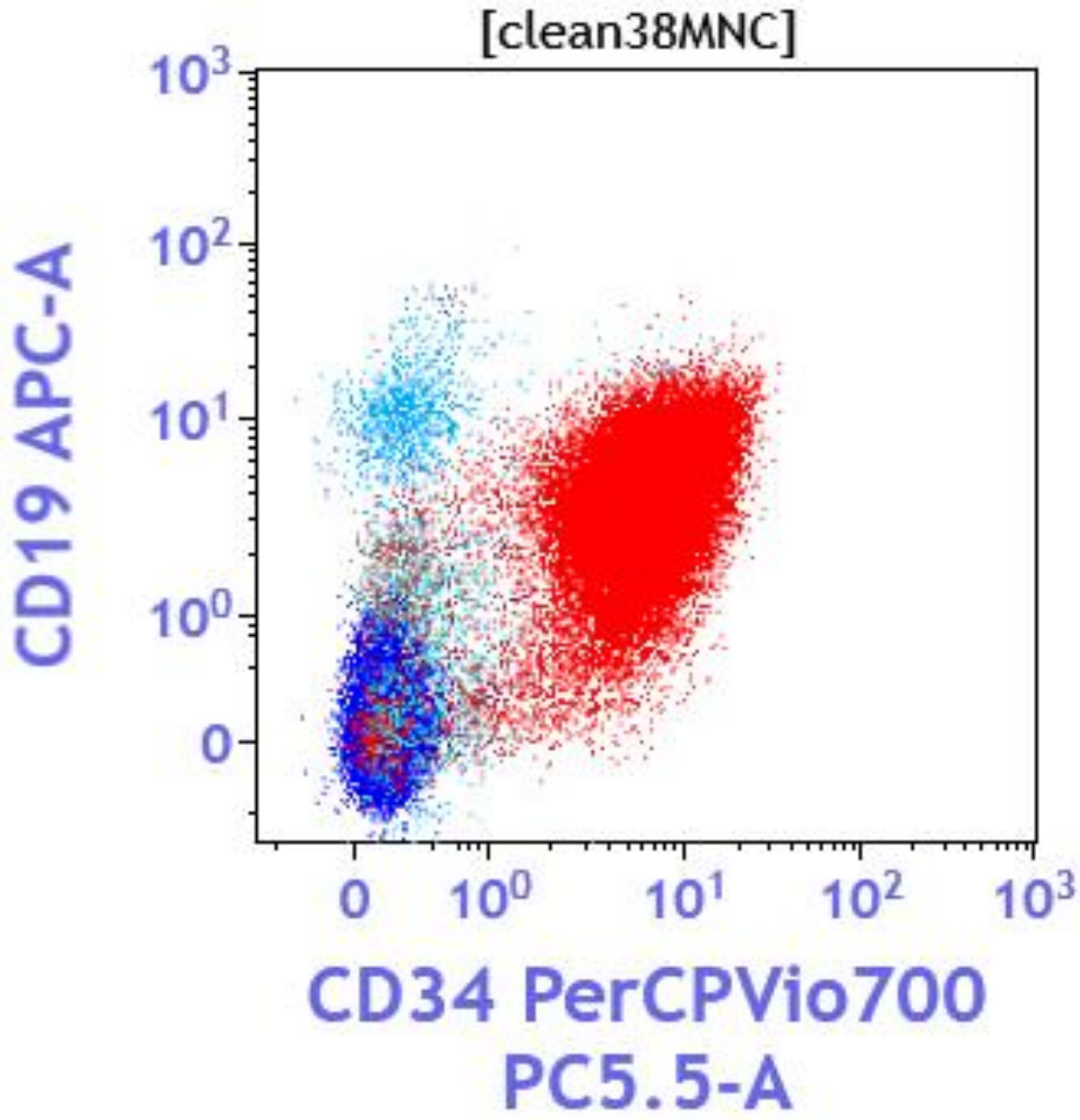
- 50 yrs male
- Generalized weakness and dyspnea on exertion for 1.5 months
  Fever -off and on
- CBC(23.8.19): Hb 7.1 , TC 69k , Plt 74k
- Peripheral blood Smear: 90% Blasts
- Bone marrow examination (19.8.19): acute leukemia

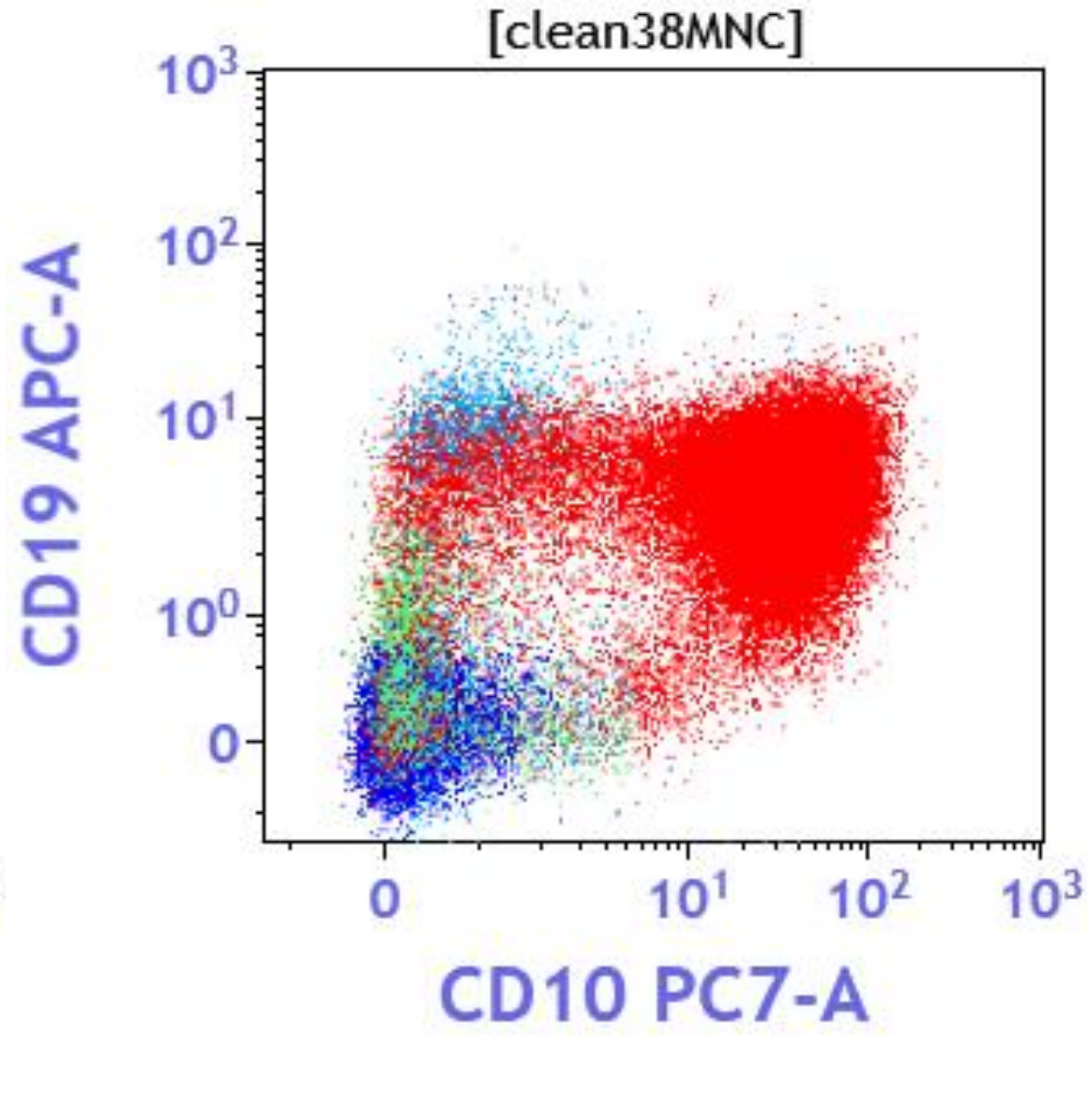
Cytochemical MPO




[Singlets]
SSC-A
FSC-A
Viability
10^3
10^2
20
0
-20
0
50
100
150


[Viability]
CD45 APC-A700-A
SSC-A
45L: 11.07
45Mo: 1.09%
45Prog: 82.20%
45G: 5.05%
10^2
10^1
5
0
-5
-20
-10
0
10
20
10^2
10^3


[clean38MNC]
CD19 APC-A
10^3
10^2
10^1
10^0
0
0
10^0
10^1
10^2
10^3
CD34 PerCPVio700
PC5.5-A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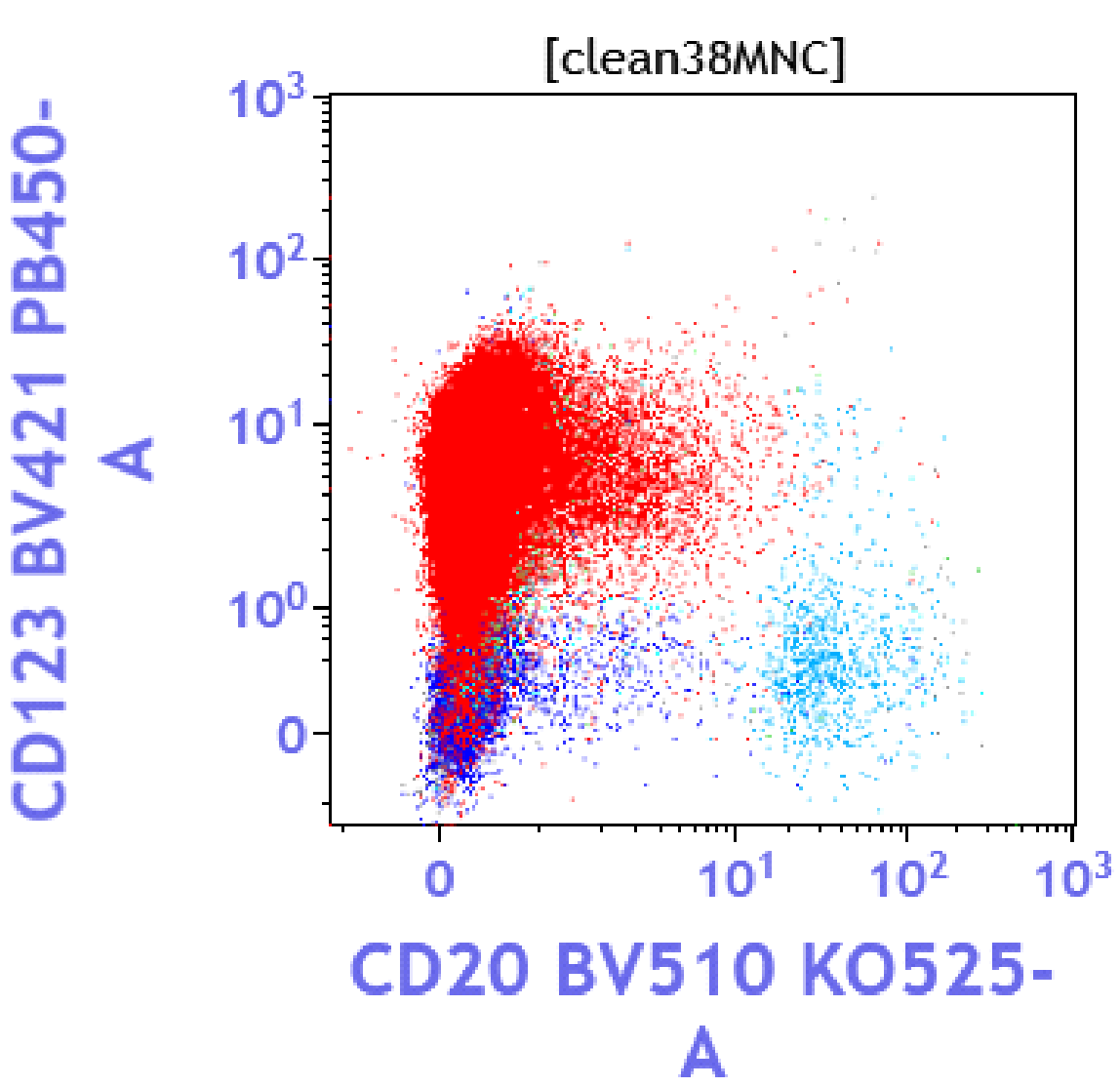
CD19 APC-A
10^3
10^2
10^1
10^0
0
0
10^1
10^2
10^3
CD10 PC7-A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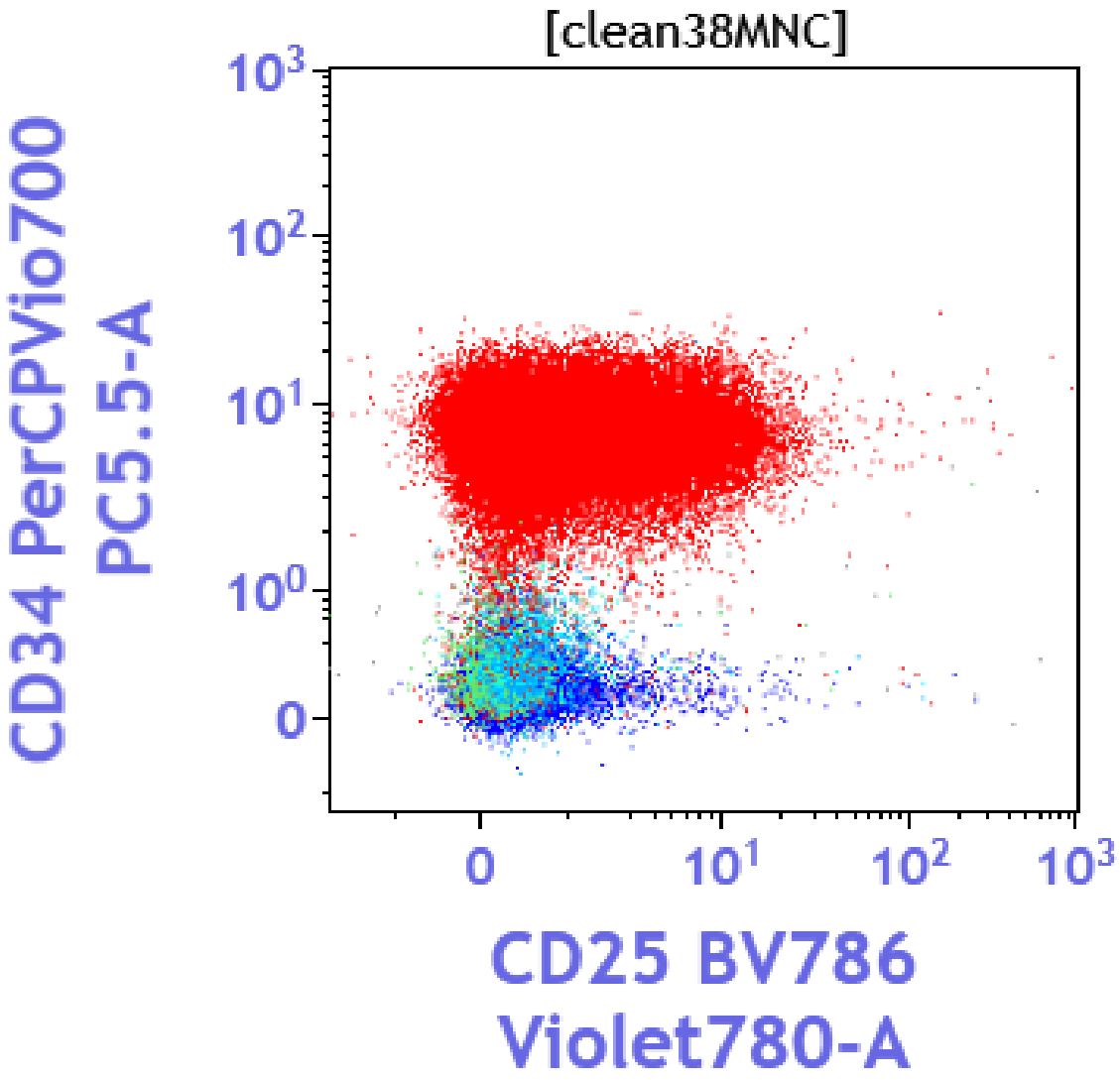
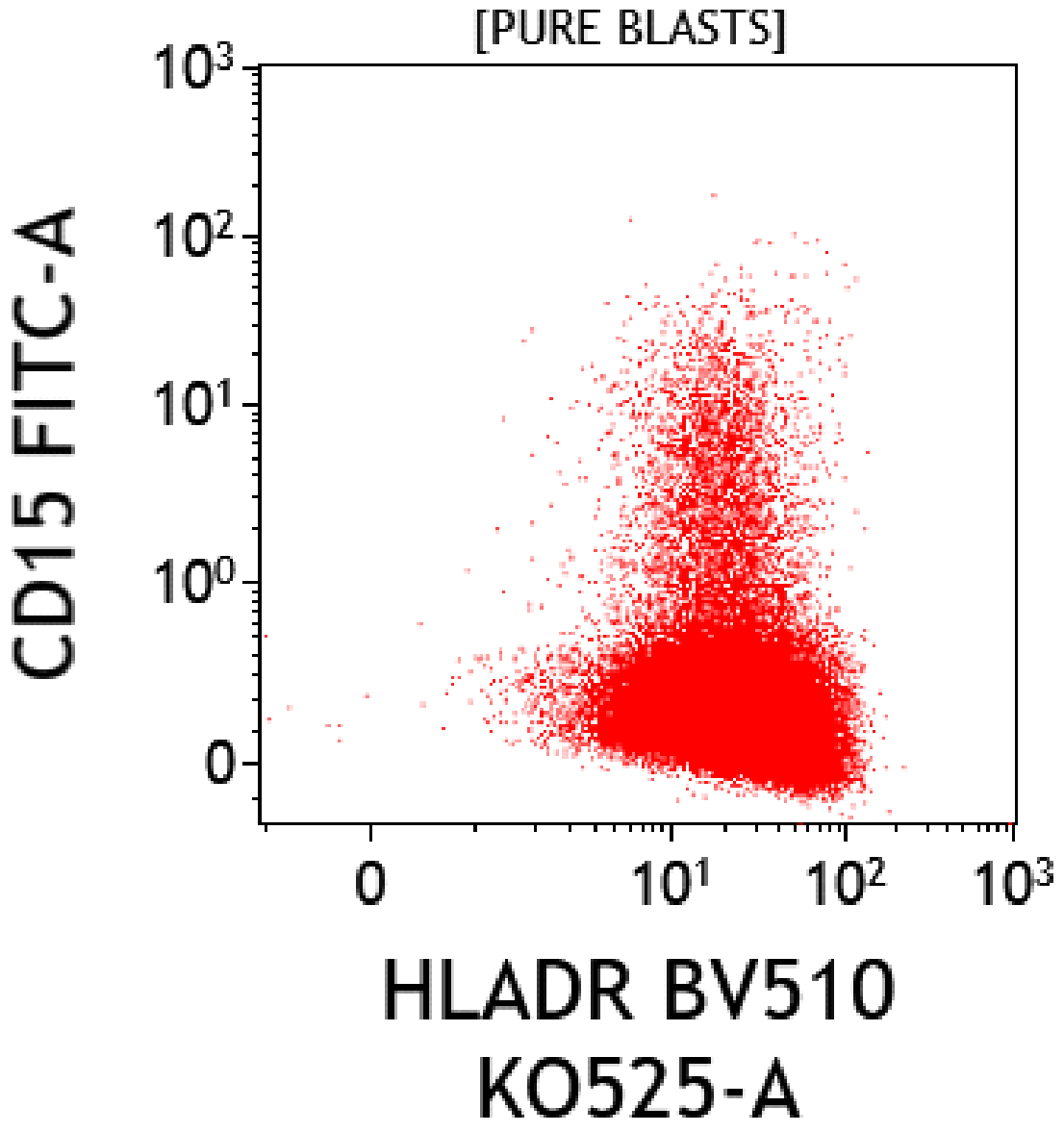
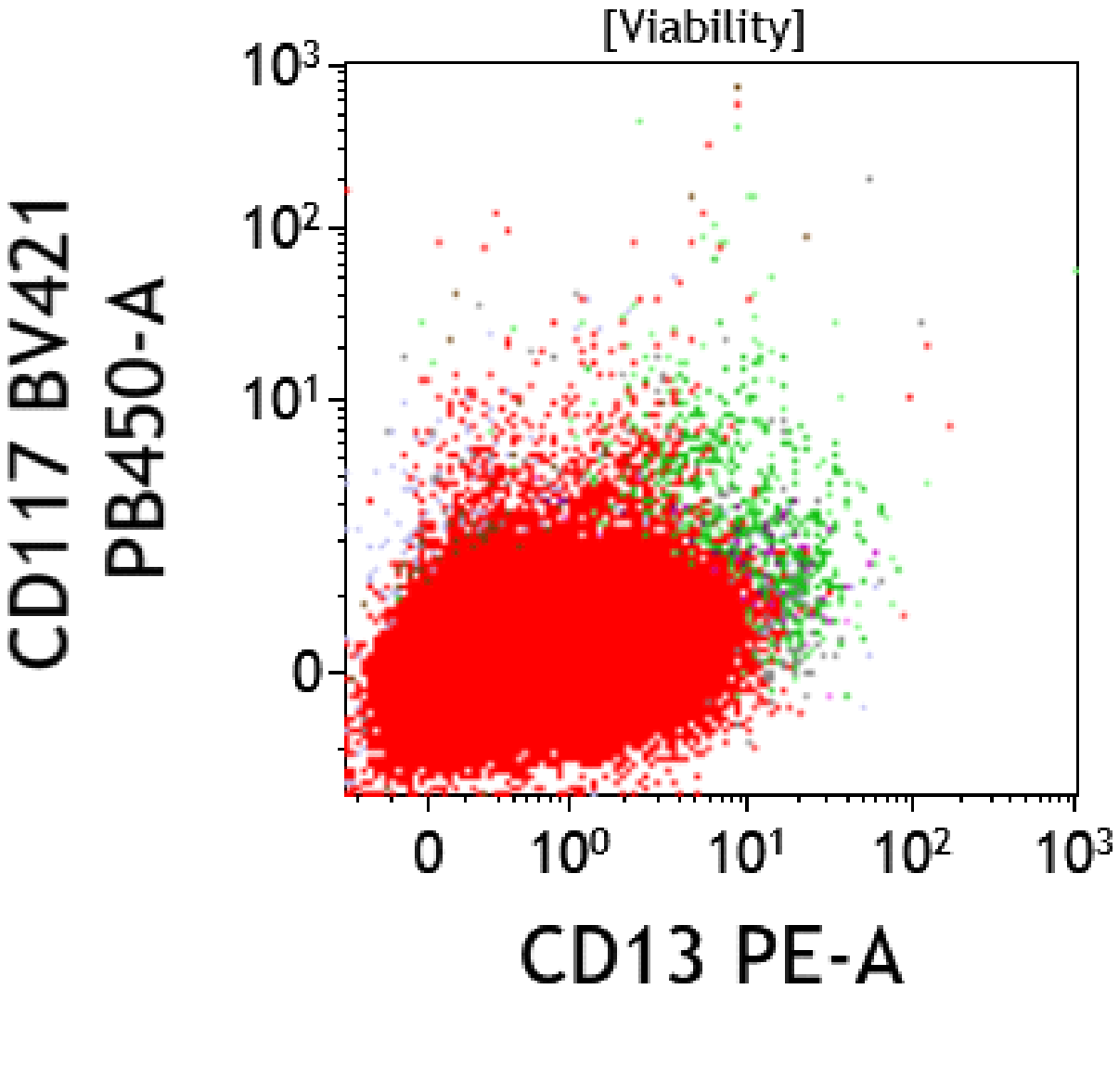
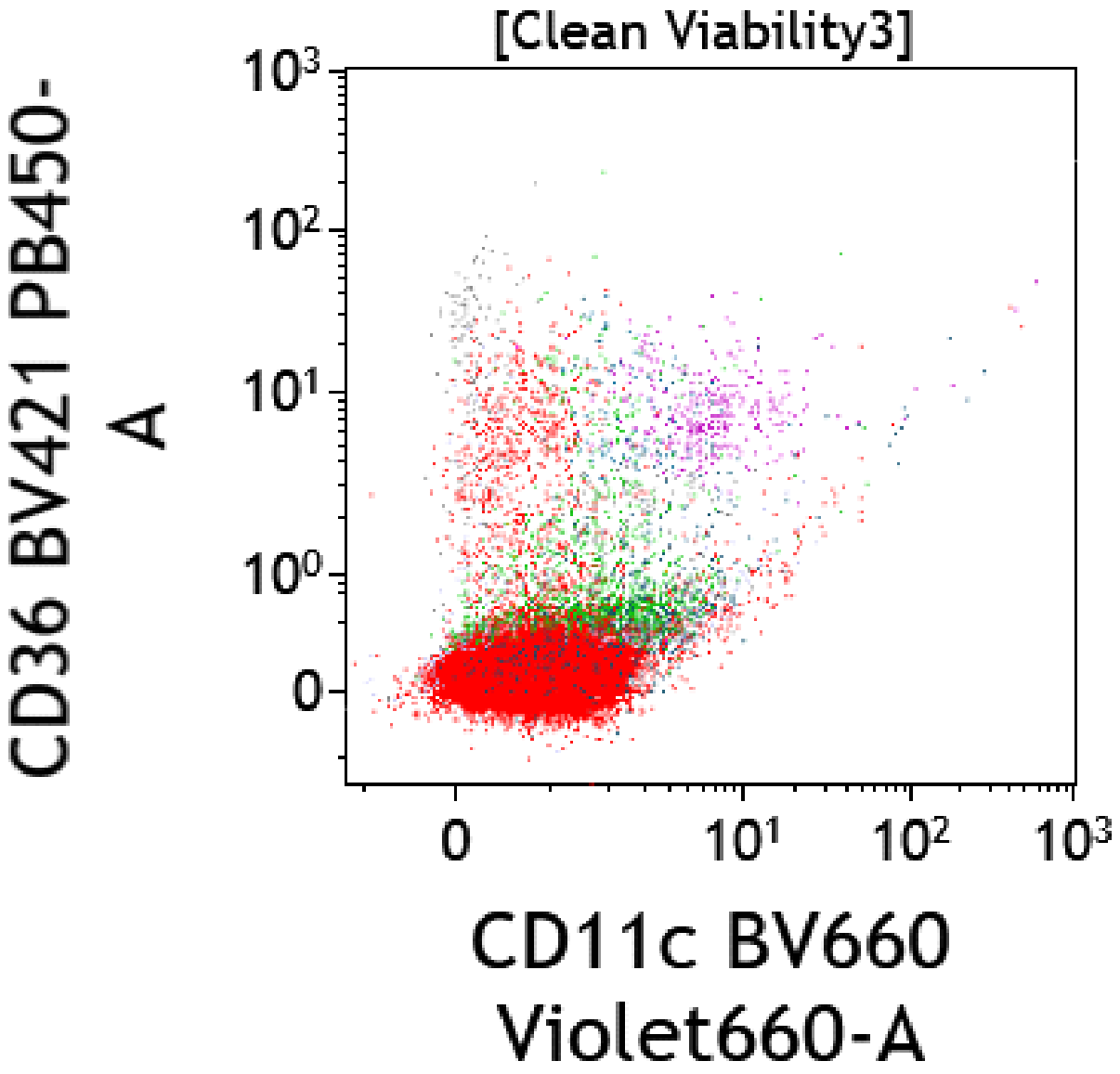
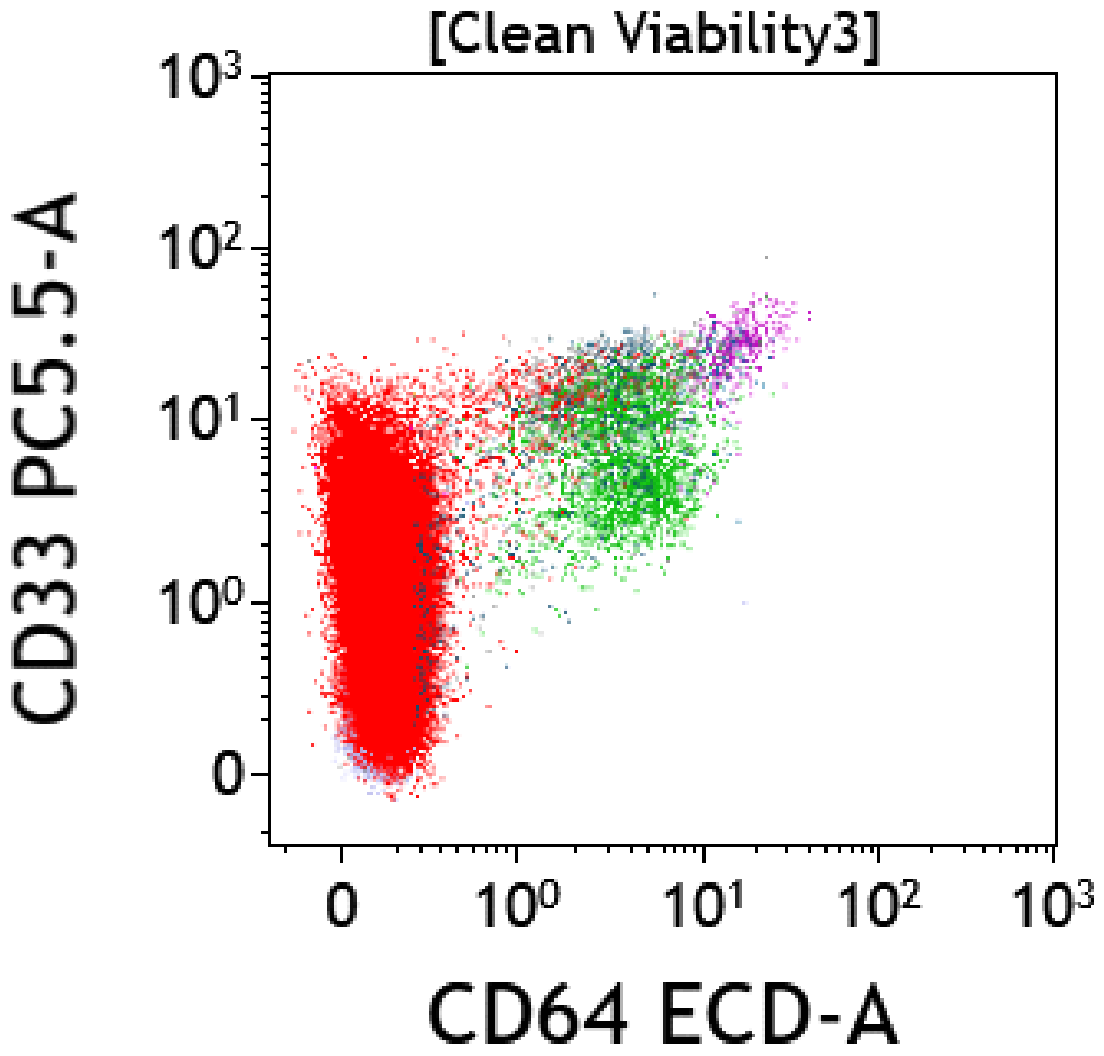
CD123 BV421 PB450-A
10^3
10^2
10^1
10^0
0
0
10^1
10^2
10^3
CD20 BV510 KO525-A

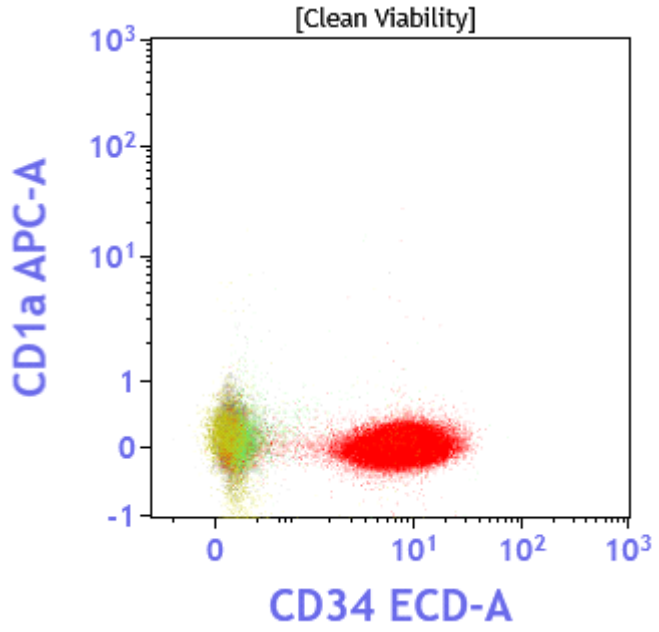

[clean38MNC]
CD34 PerCPVio700 PC5.5-A
10^3
10^2
10^1
10^0
0
0
10^1
10^2
10^3
CD25 BV786 Violet780-A

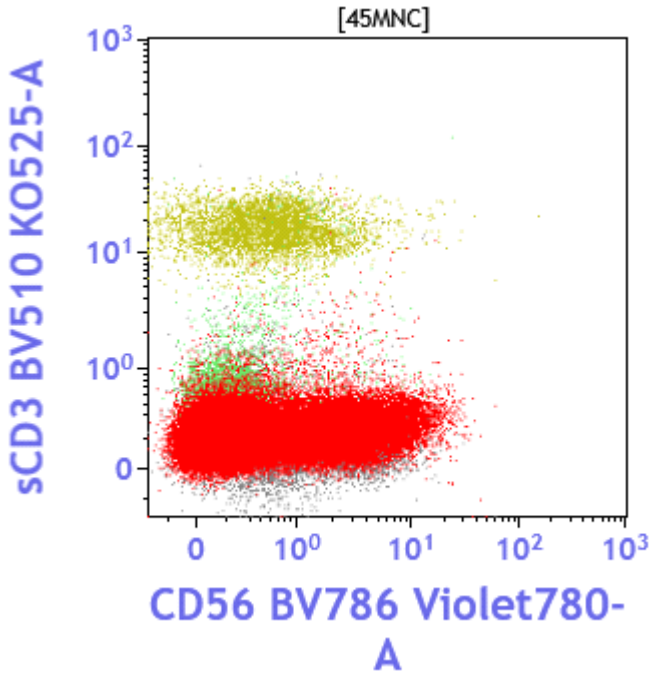

[PURE BLASTS]
CD15 FITC-A
HLADR BV510
KO525-A
10^3
10^2
10^1
10^0
0
0
10^1
10^2
10^3


[Viability]
CD117 BV421
PB450-A
CD13 PE-A
10^3
10^2
10^1
0
0
10^0
10^1
10^2
10^3


[Clean Viability3]
CD36 BV421 PB450-A
10^3
10^2
10^1
10^0
0
0
10^1
10^2
10^3
CD11c BV660 Violet660-A


[Clean Viability3]
CD33 PC5.5-A
10^3
10^2
10^1
10^0
0
0
10^0
10^1
10^2
10^3
CD64 ECD-A


[Clean Viability]
CD1a APC-A
CD34 ECD-A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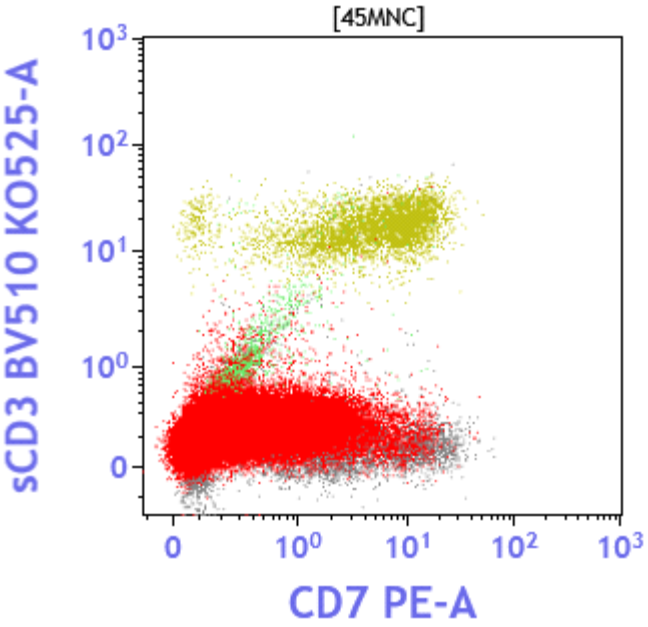
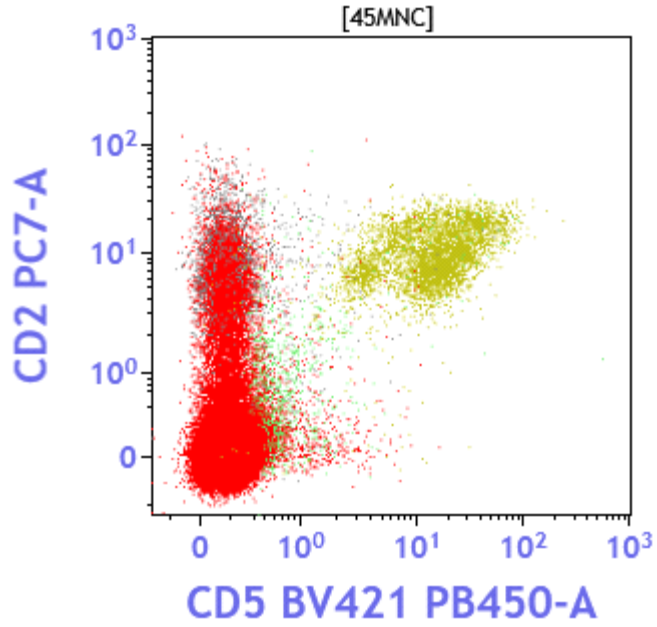
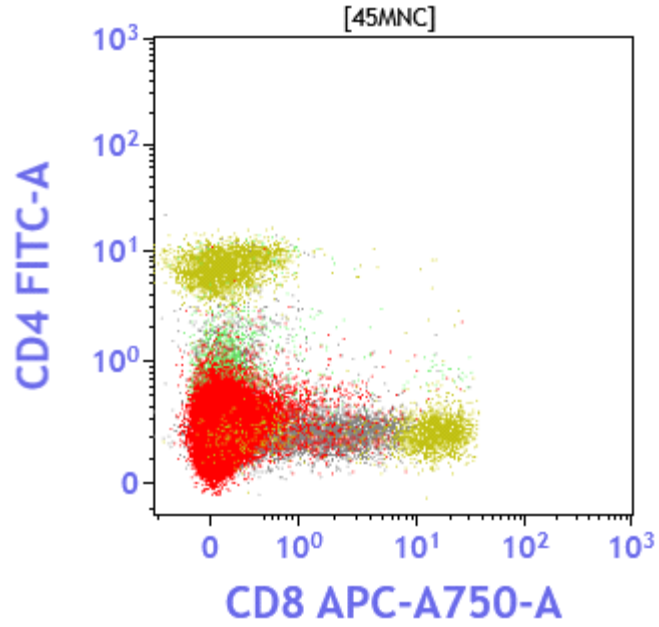
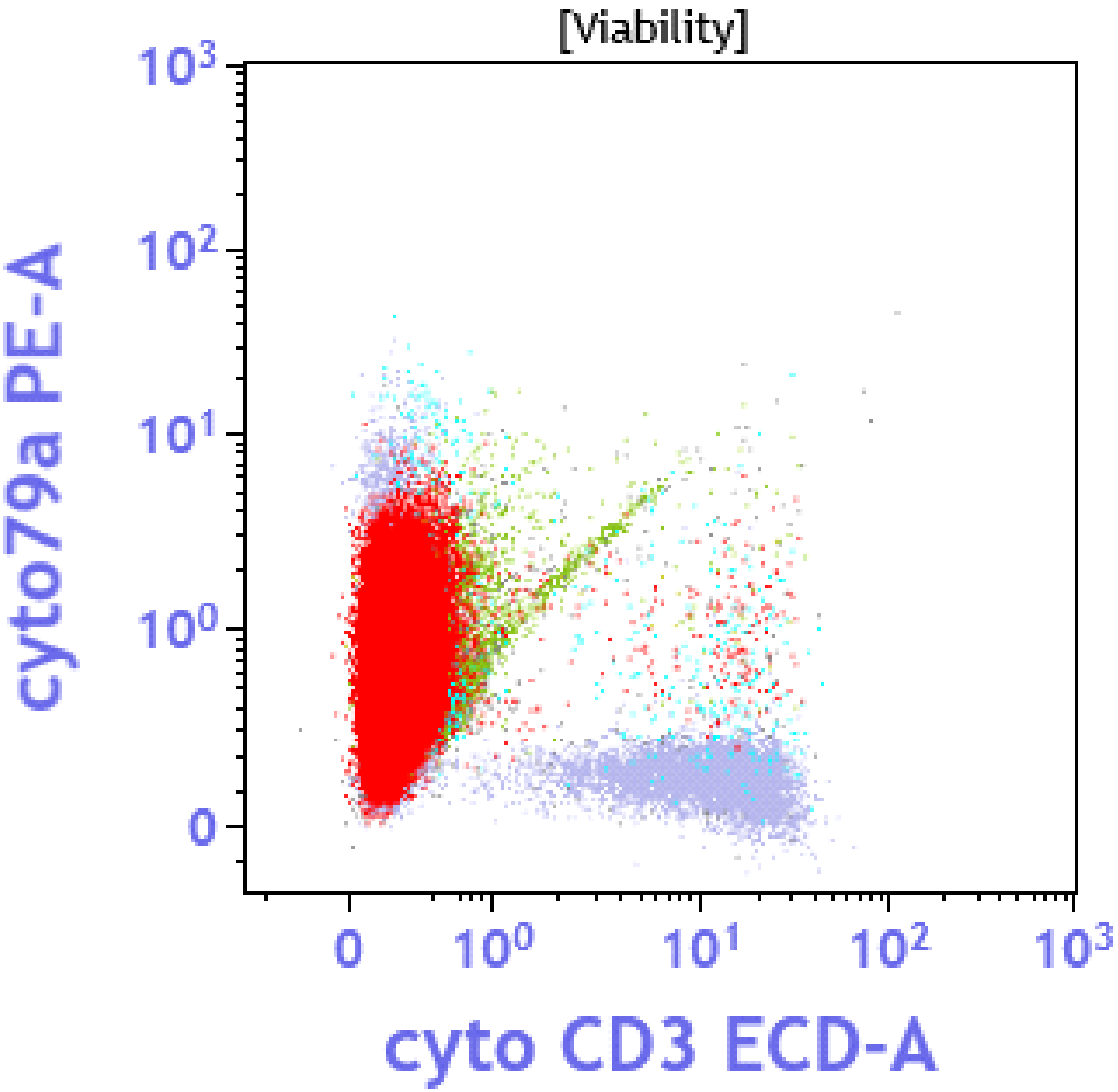
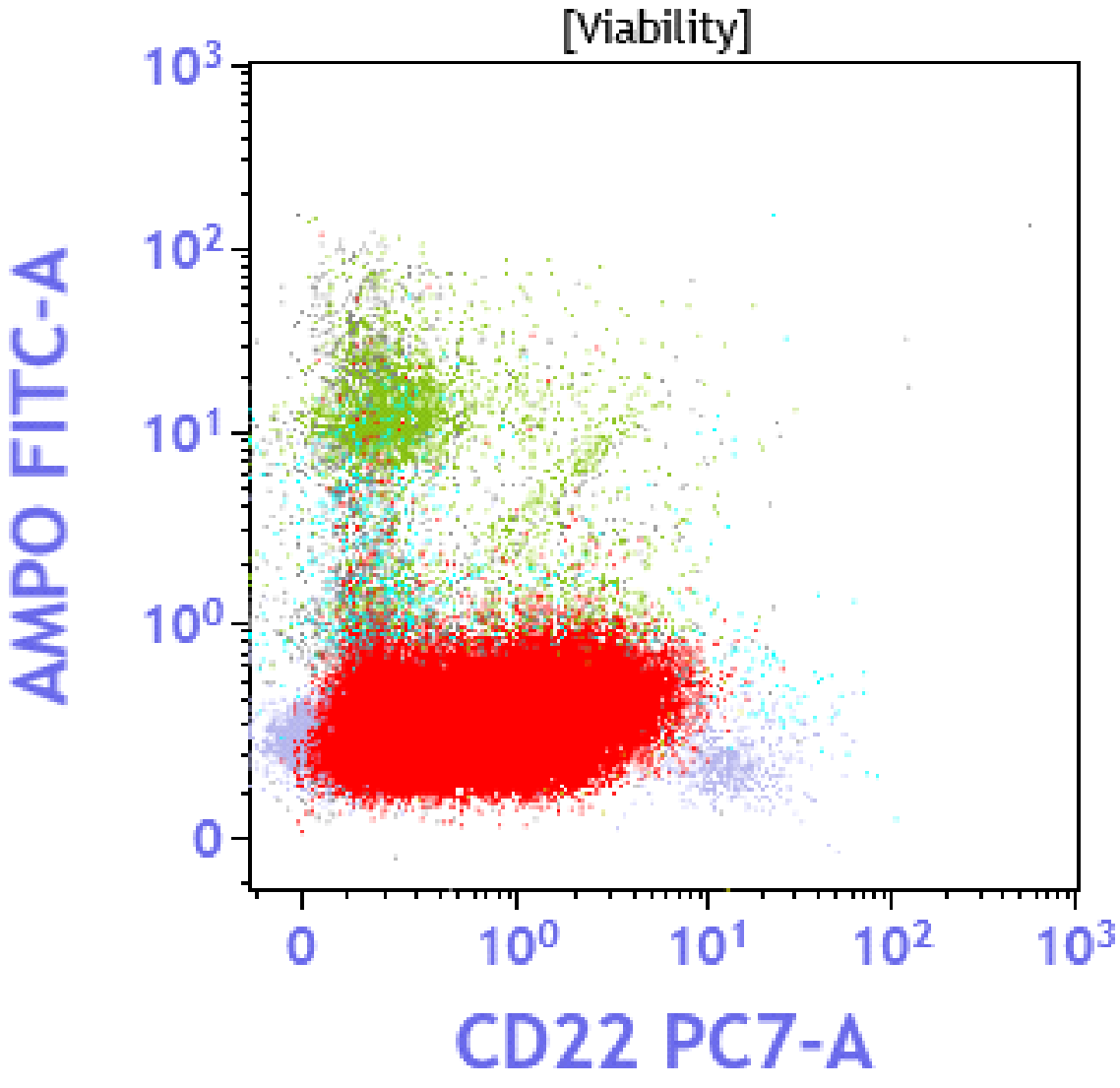
sCD3 BV510 KO525-A
CD56 BV786 Violet780-A


[45MNC]
sCD3 BV510 KO525-A
CD7 PE-A


[45MNC]
CD2 PC7-A
CD5 BV421 PB450-A


[45MNC]
CD4 FITC-A
CD8 APC-A750-A


[Viability]
cyto79a PE-A
10^3
10^2
10^1
10^0
0
0
10^0
10^1
10^2
10^3
cyto CD3 ECD-A


[Viability]
AMPO FITC-A
10^3
10^2
10^1
10^0
0
0
10^0
10^1
10^2
10^3
CD22 PC7-A

Diagnosis

B-ALL with CD25+, CD13+, CD33+ expression

BCR-ABL1+ ALL (BCR-ABL1 translocation with e1a2 p190 transcript)

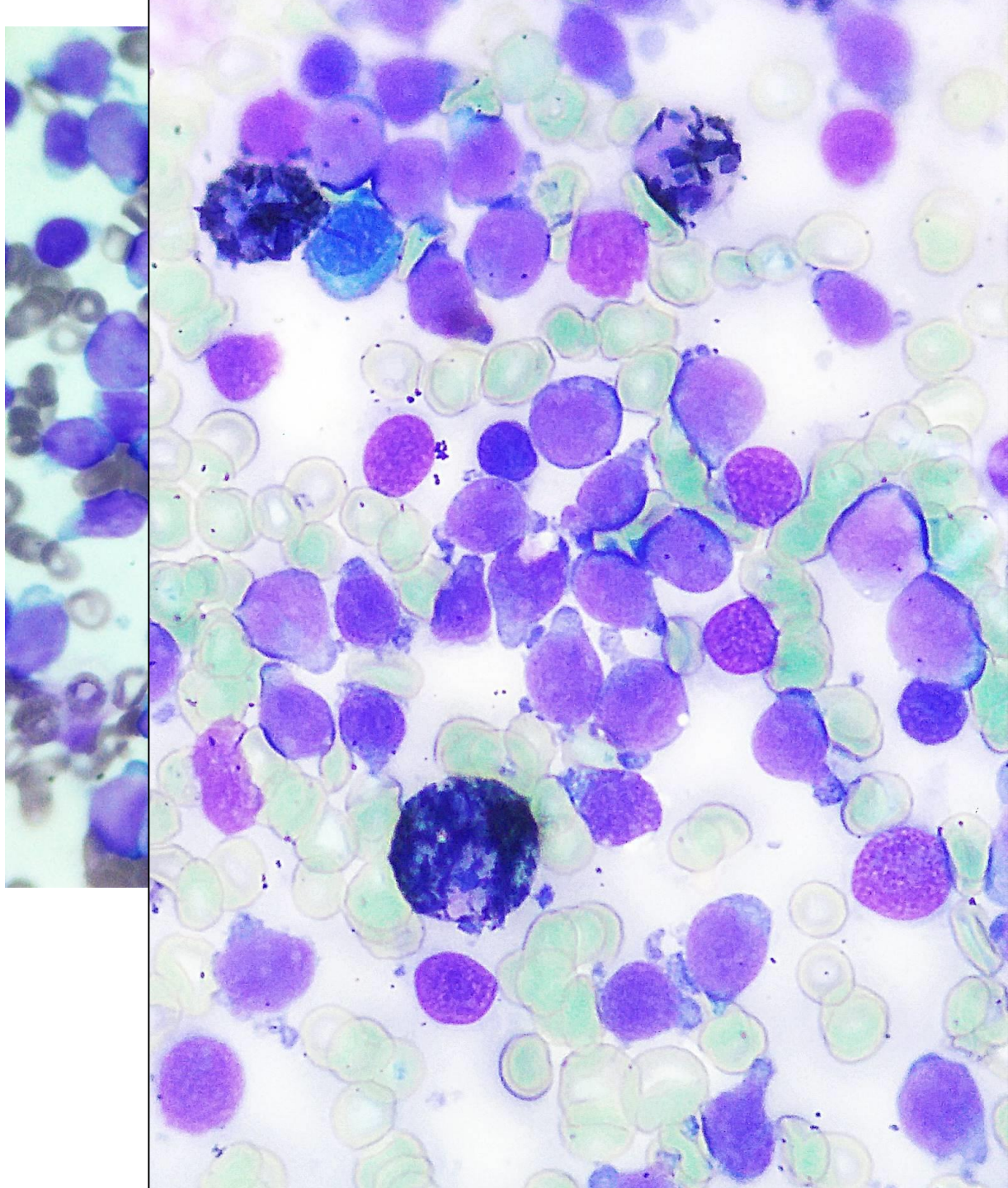
- **Learning points:**
- **B-ALLs may express aberrant myeloid markers.**
- **Clue towards underlying genomic abnormalities**

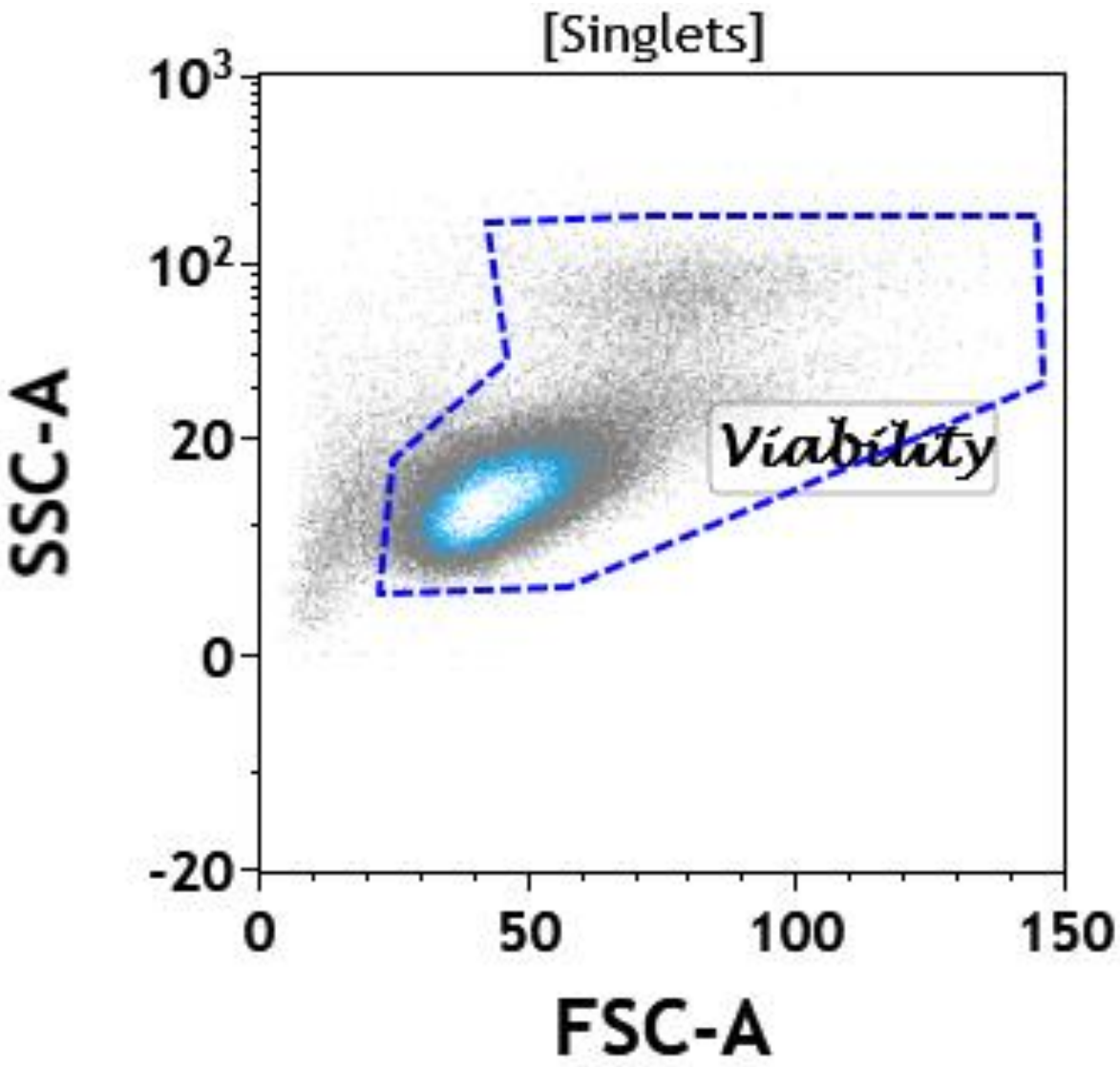
Case 3 -

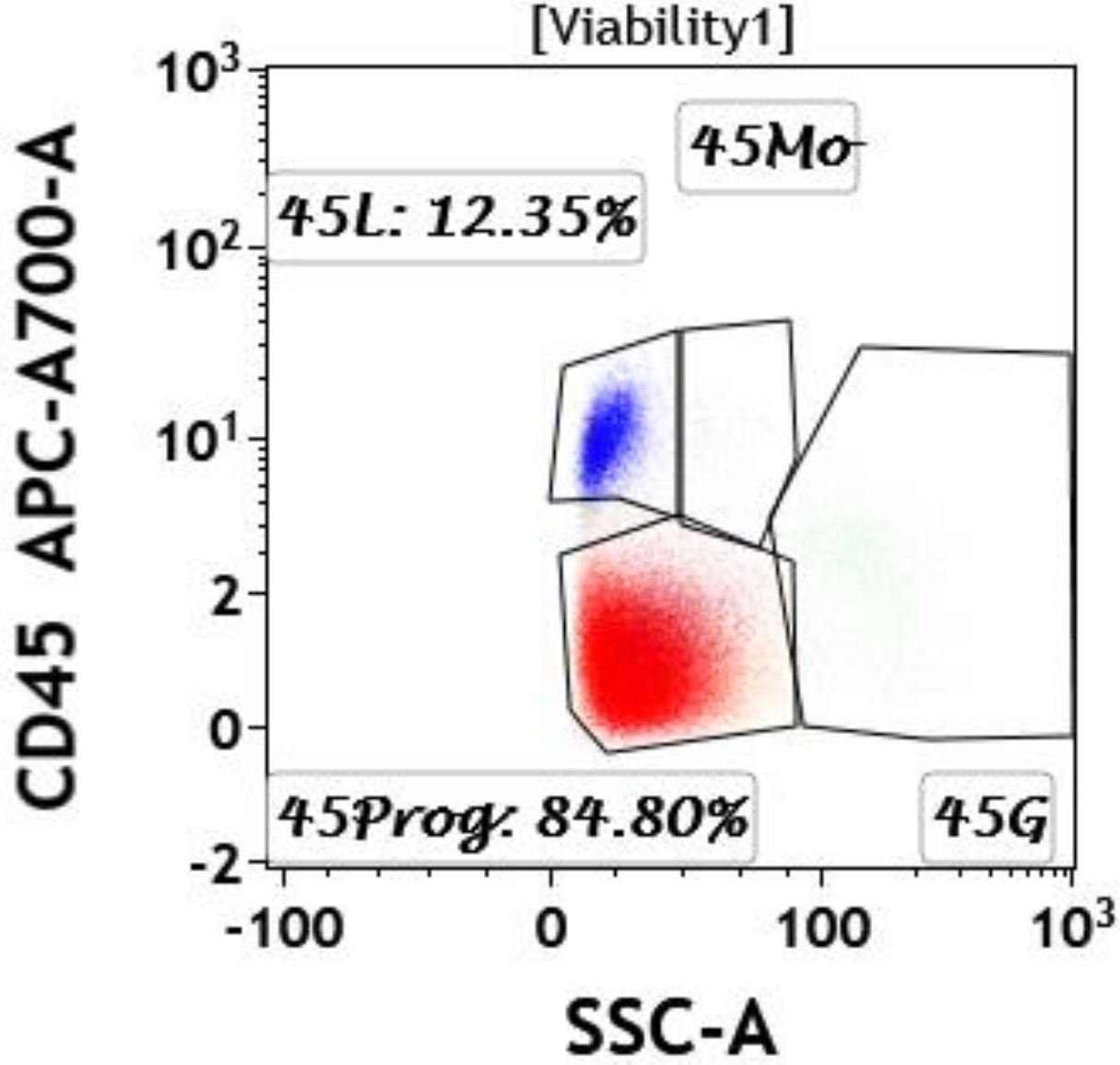
6-month-old boy
fever x 6 weeks
a/w joint pains
No h/o bleeding

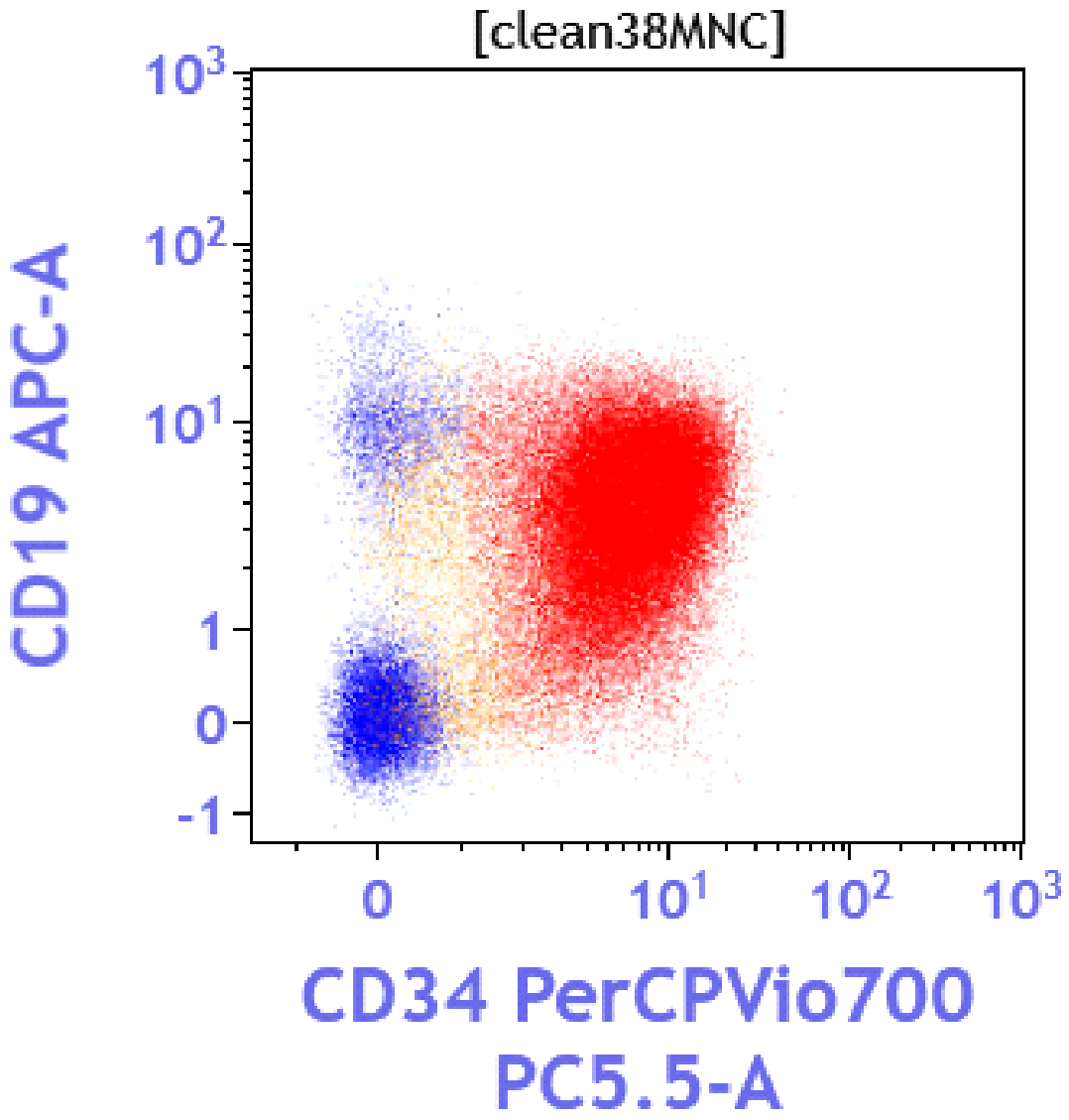
CBC > Hb-6.8
TLC-8600
Plt-20000 ;
PBS - DC- N20 L8 M2 Blast 70-%

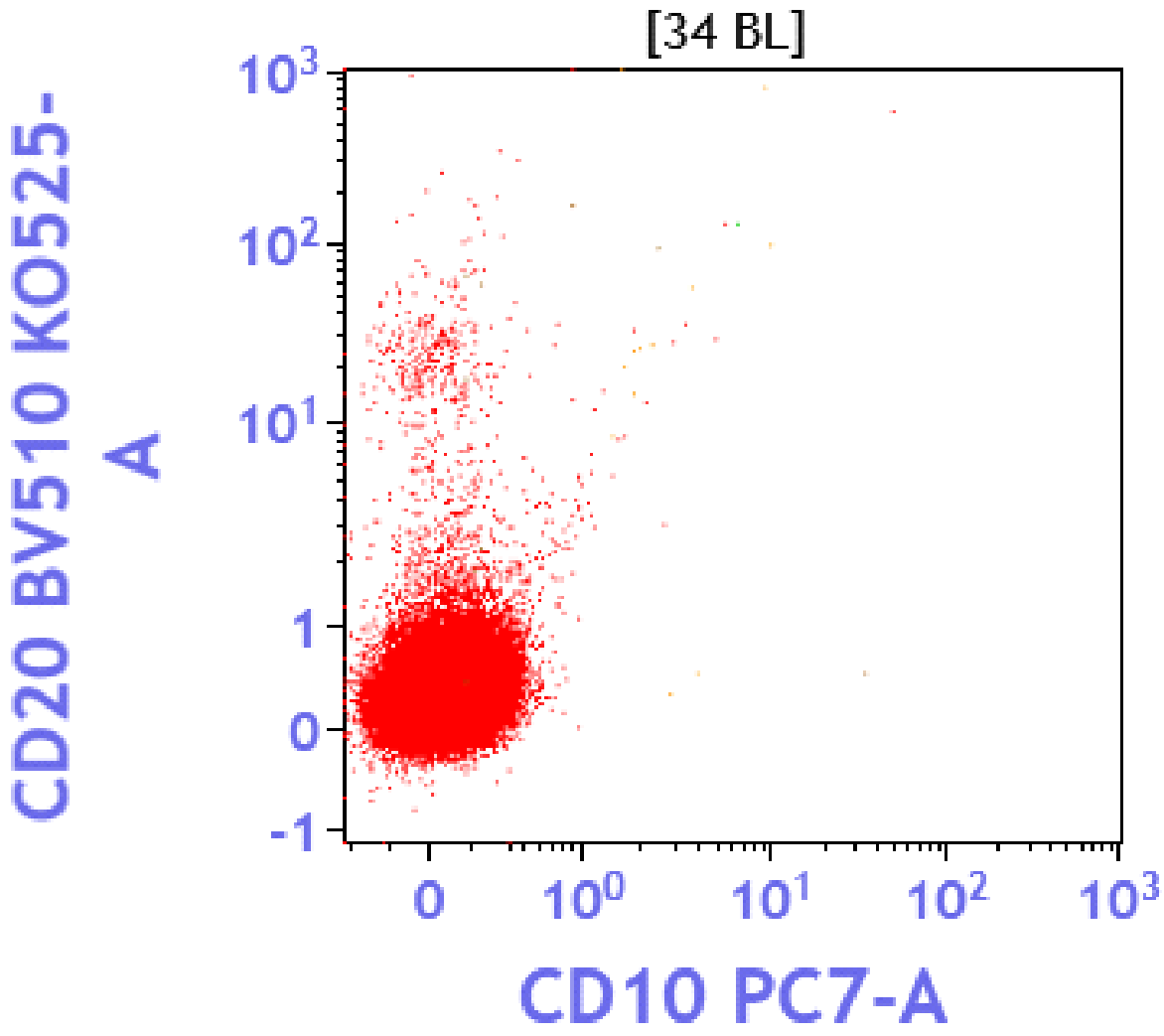
BMA(13/5/19)---> 90% Blasts

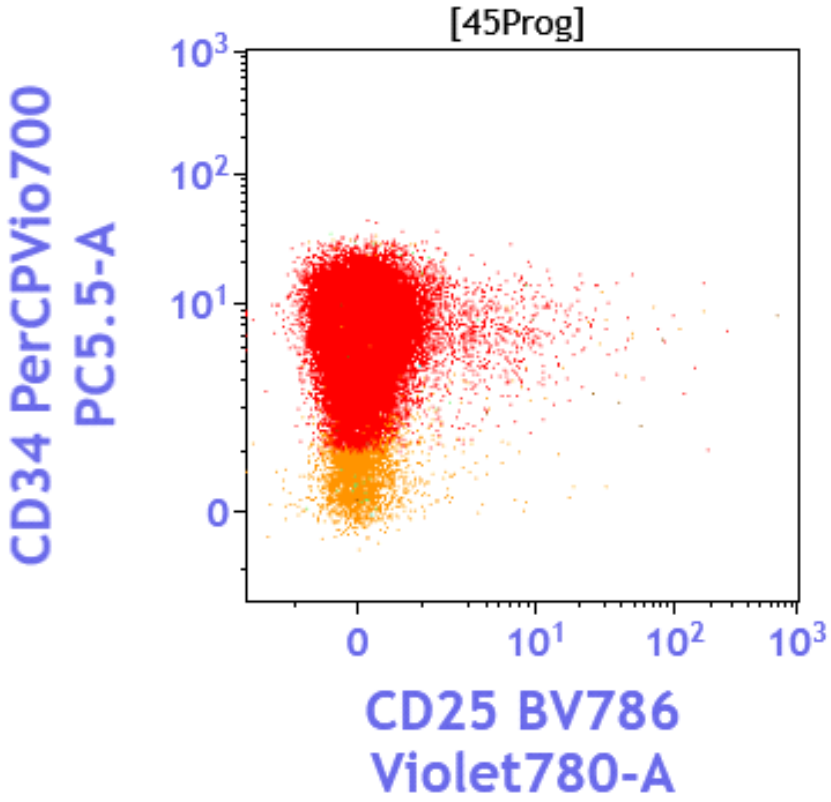


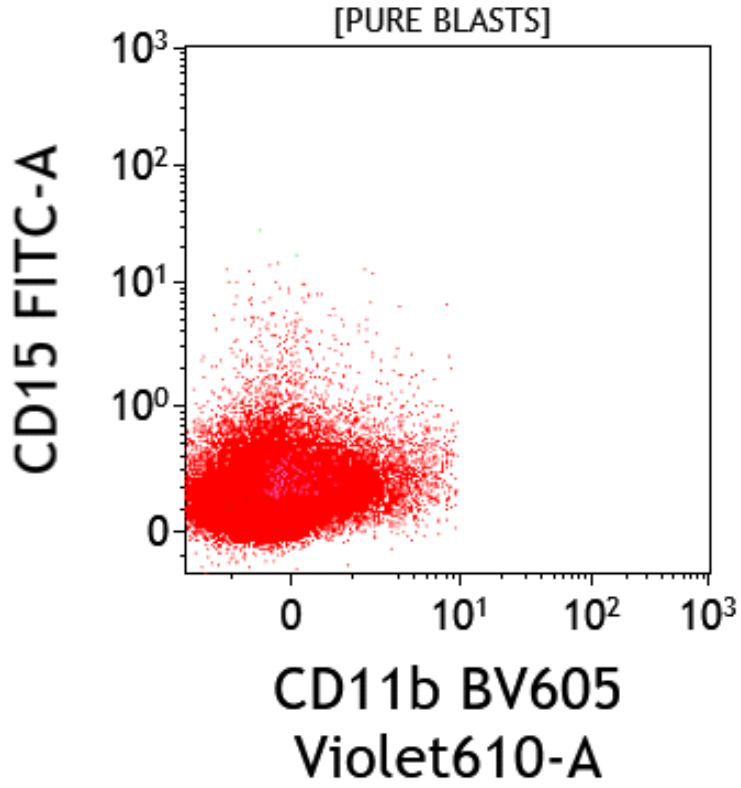

[Singlets]
SSC-A
FSC-A
Viability
10^3
10^2
20
0
-20
0
50
100
150


[Viability1]
CD45 APC-A700-A
SSC-A
45Mo
45L: 12.35%
45Prog: 84.80%
45G
10^3
10^2
10^1
2
0
-2
-100
0
100
10^3


[clean38MNC]
CD19 APC-A
10^3
10^2
10^1
1
0
-1
0
10^1
10^2
10^3
CD34 PerCPVio700
PC5.5-A


[34 BL]
CD20 BV510 KO525-A
10^3
10^2
10^1
1
0
-1
0
10^0
10^1
10^2
10^3
CD10 PC7-A


[45Prog]
CD34 PerCPVio700
PC5.5-A
CD25 BV786
Violet780-A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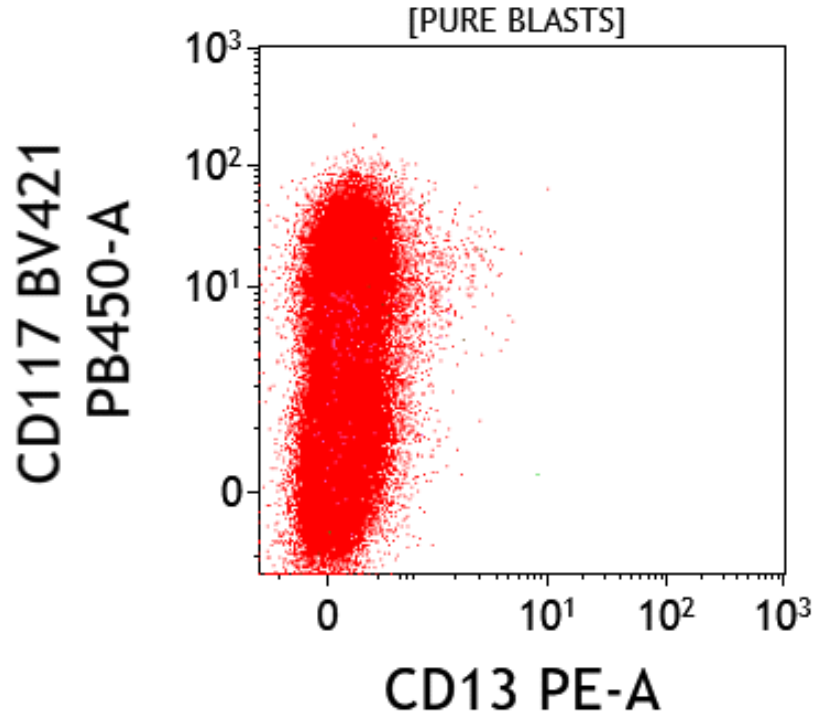
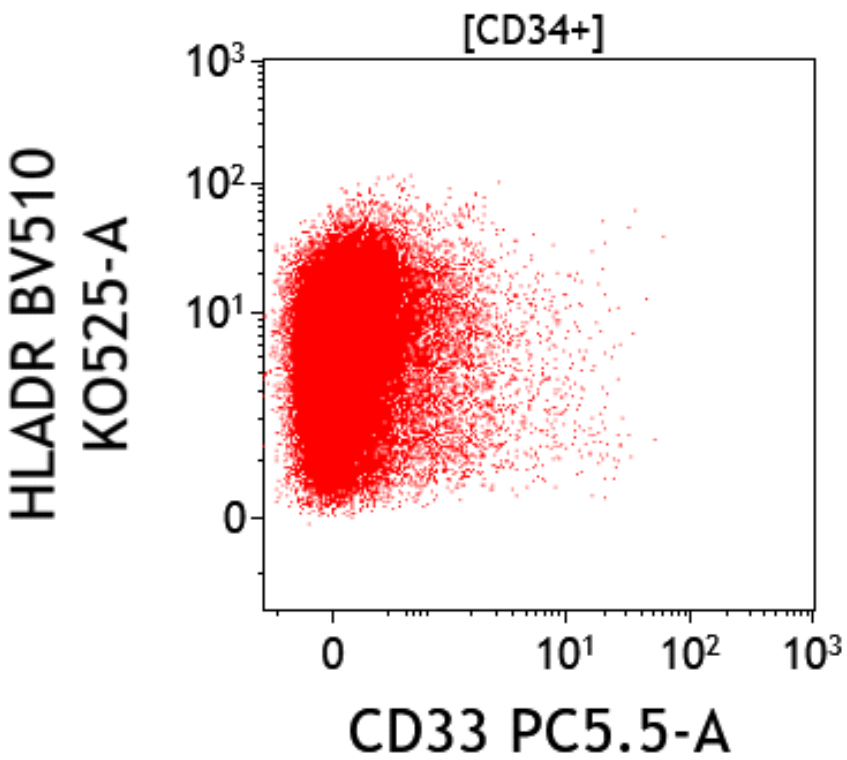
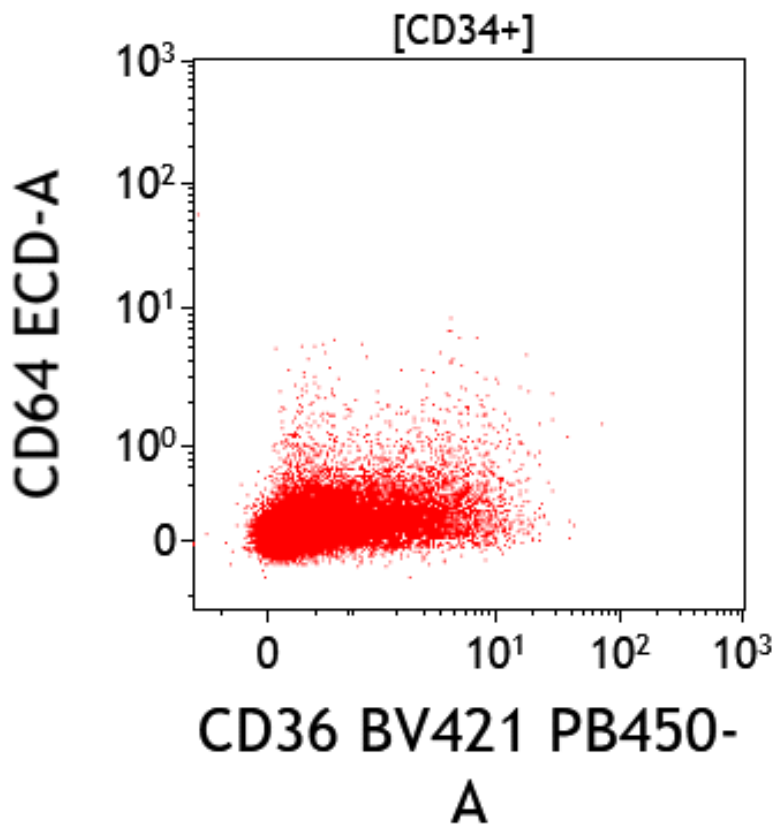
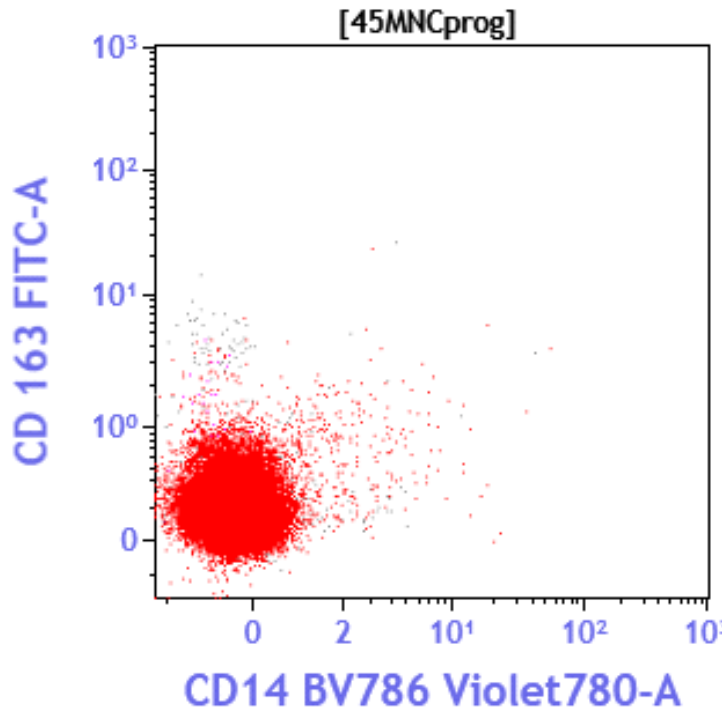
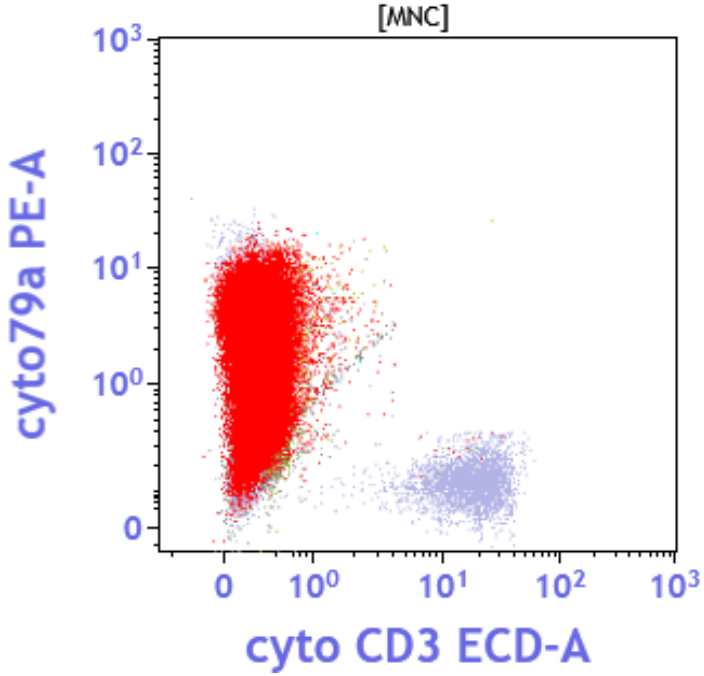
CD15 FITC-A
CD11b BV605
Violet610-A


[PURE BLASTS]
CD117 BV421
PB450-A
CD13 PE-A


[CD34+]
HLADR BV510 KO525-A
CD33 PC5.5-A


[CD34+]
CD64 ECD-A
CD36 BV421 PB450-A


[45MNCprog]
CD 163 FITC-A
CD14 BV786 Violet780-A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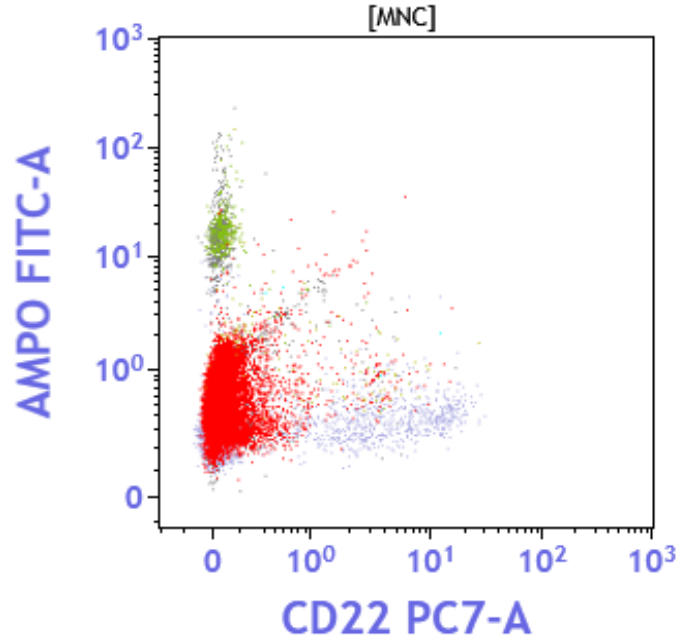
cyto79a PE-A
cyto CD3 ECD-A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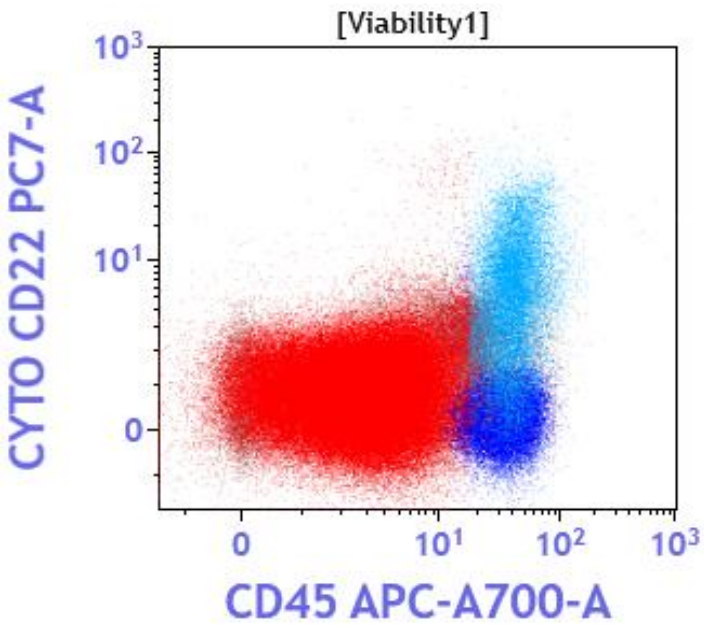
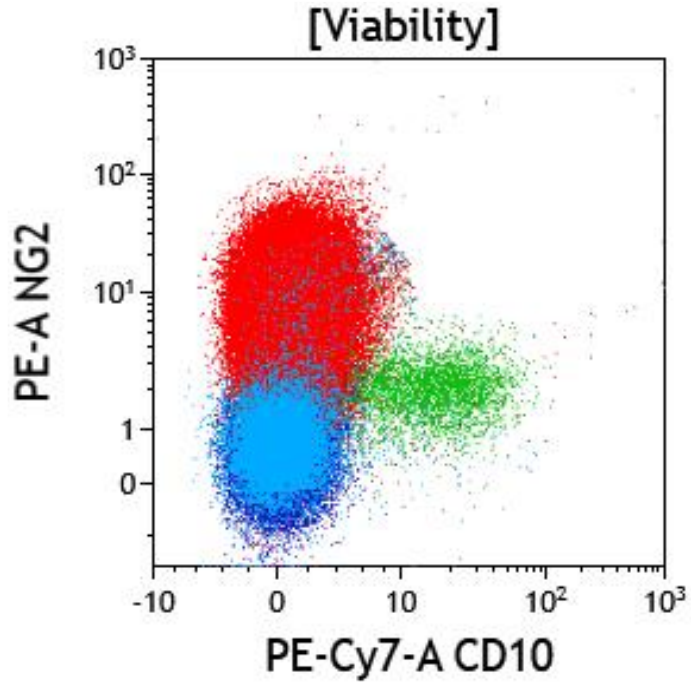
AMPO FITC-A
CD22 PC7-A


[Viability1]
CYTO CD22 PC7-A
CD45 APC-A700-A


[Viability]
PE-A NG2
PE-Cy7-A CD10

Diagnosis

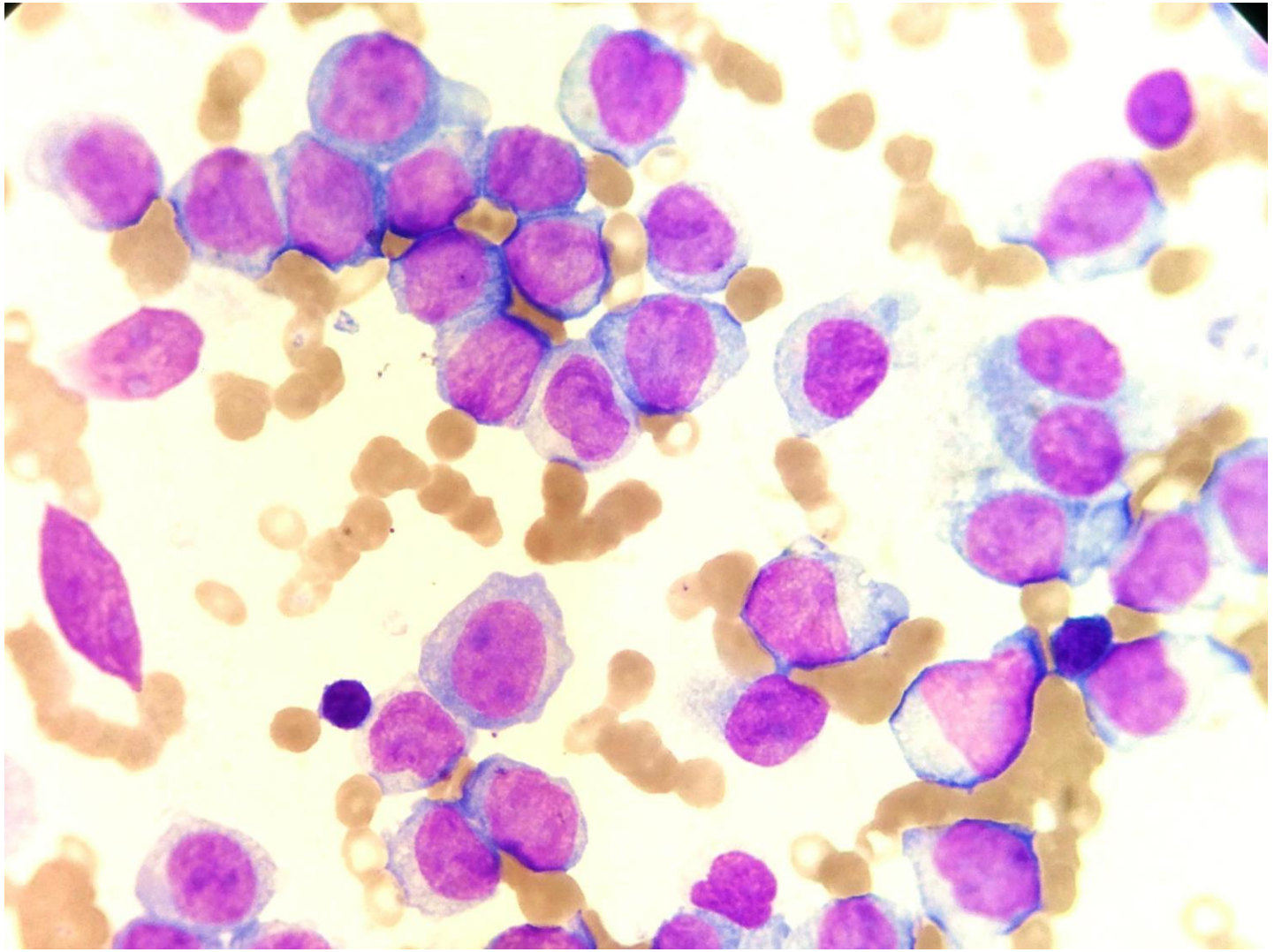
B-ALL with CD15+CD10-NG2+

KMT2A-AFF1 t(4;11) rearranged B-ALL

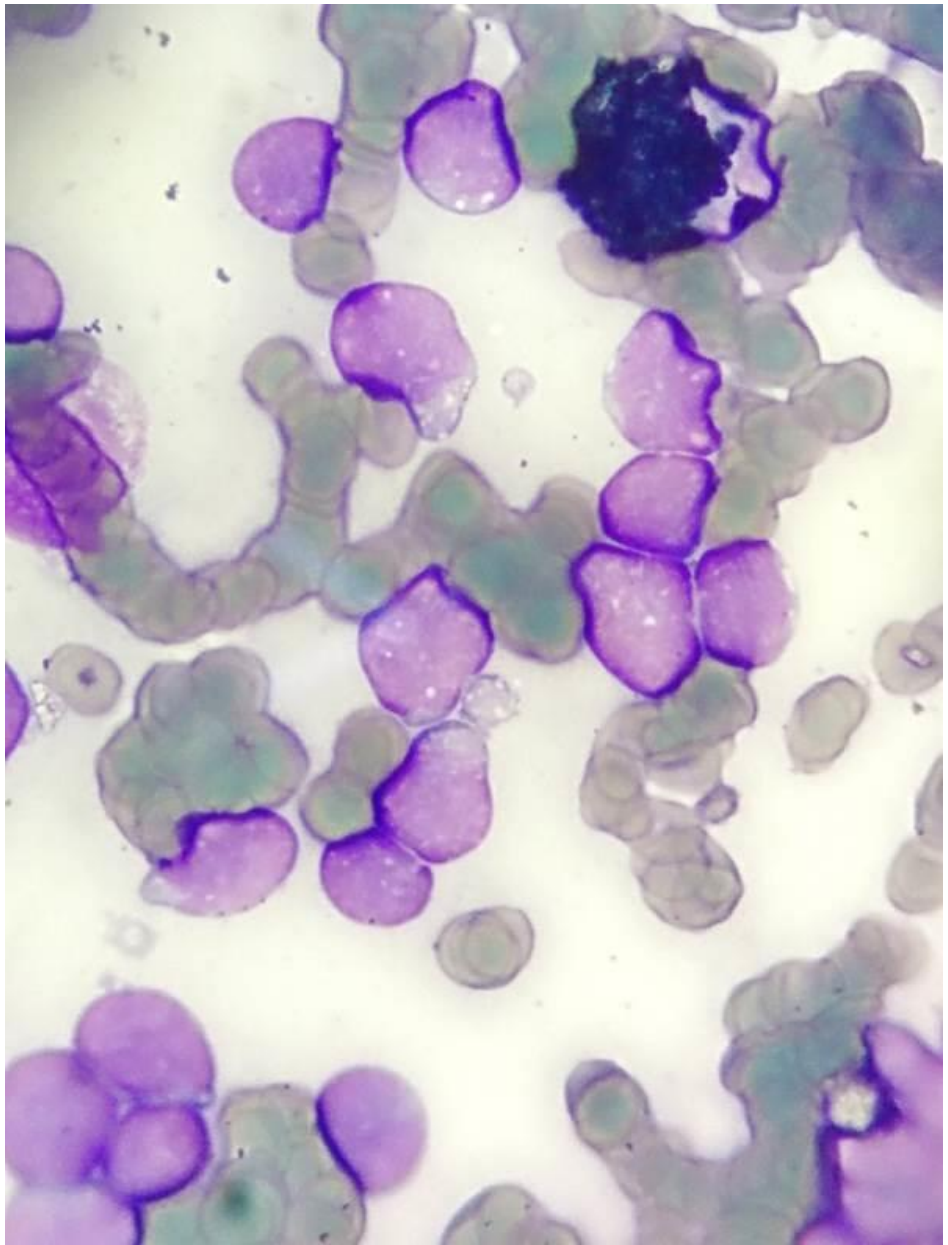
- **Learning points:**
- **Not all lineage markers may be present in a particular case.**
- **Additional markers are helpful in context.**

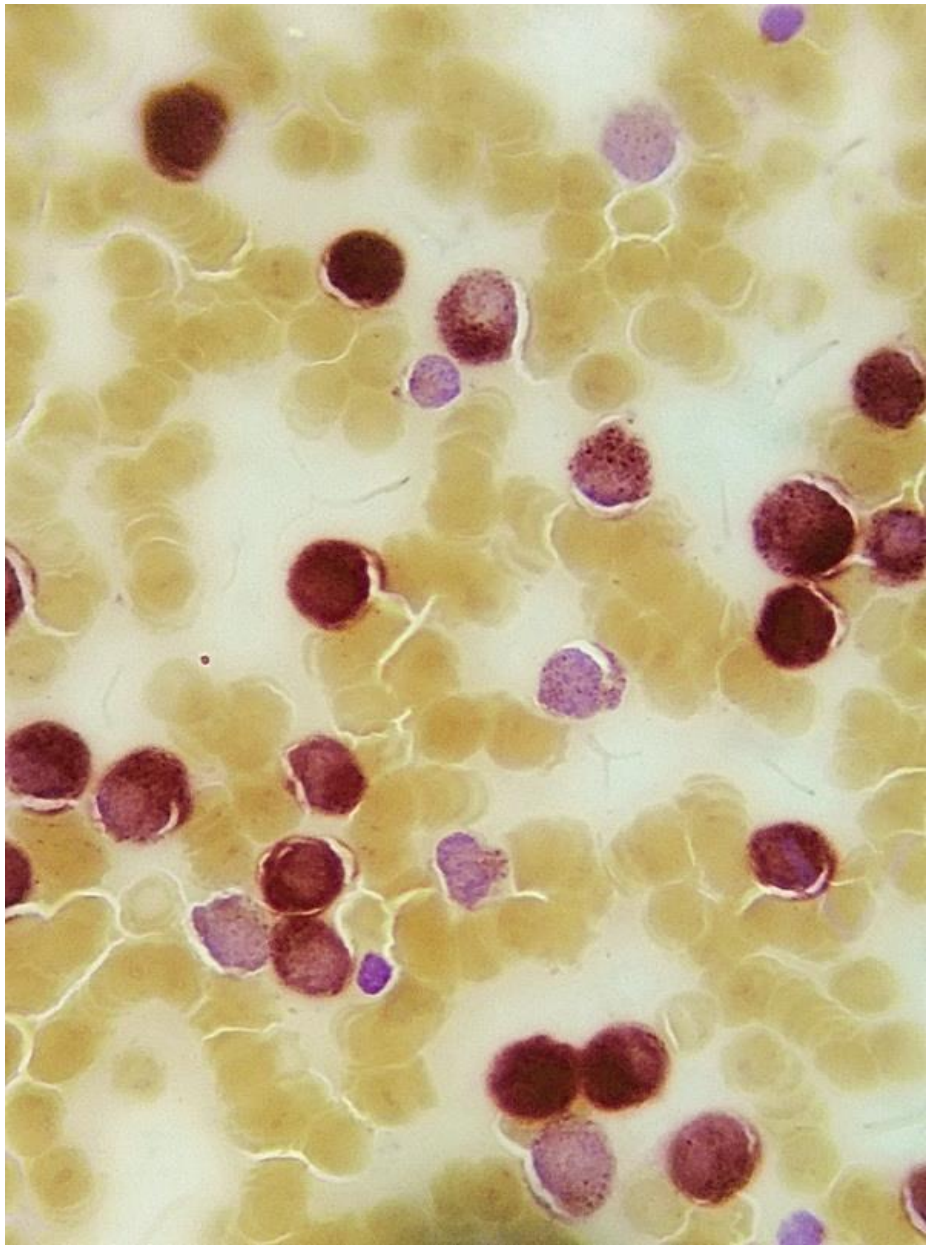
Case 4

- 9 yrs male
- High count – $80x10^3$/ul
- Platelet - $27x10^3$/ul
- Peripheral Smear - 92% blasts

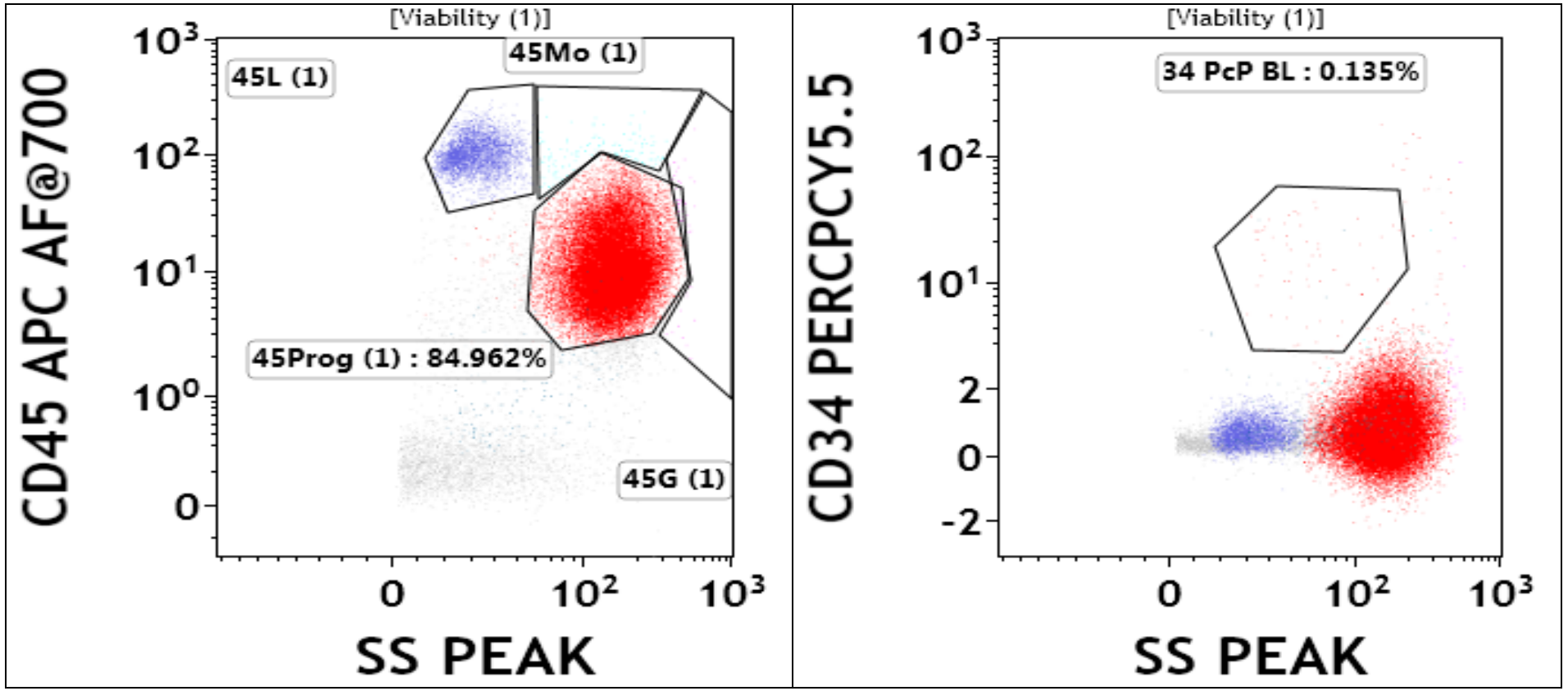


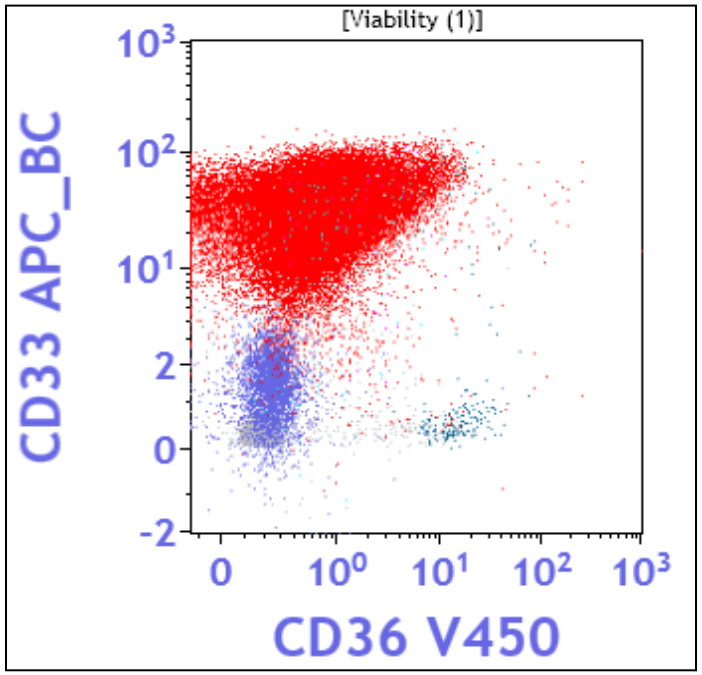
Cytochemistry

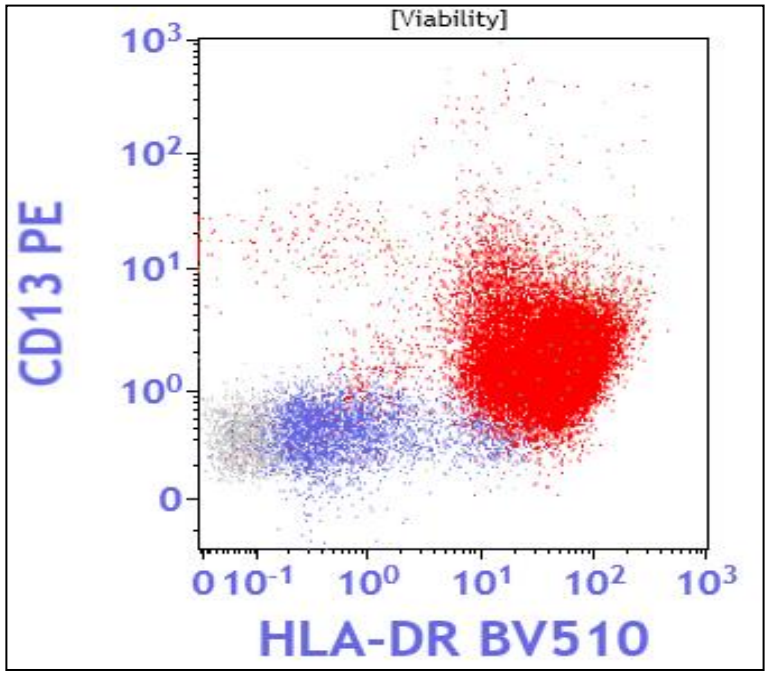


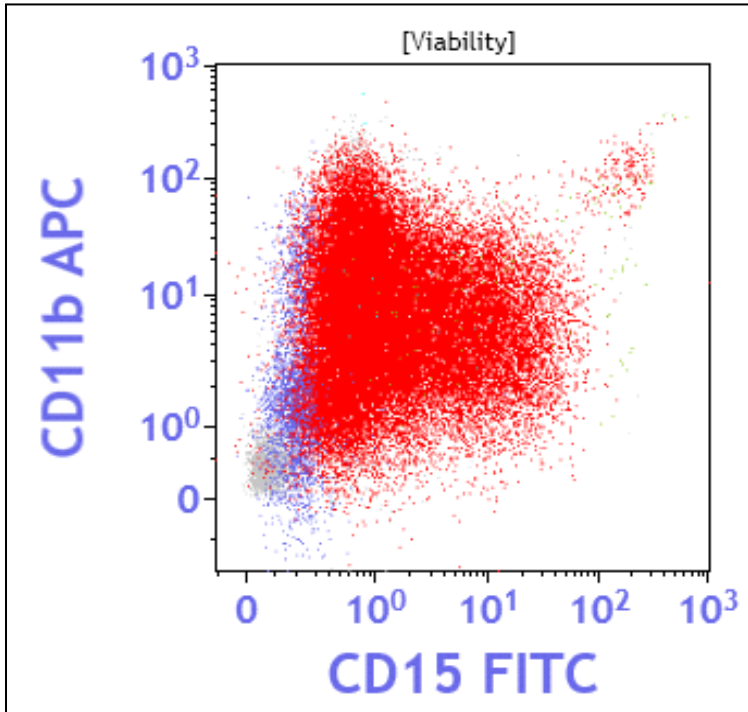


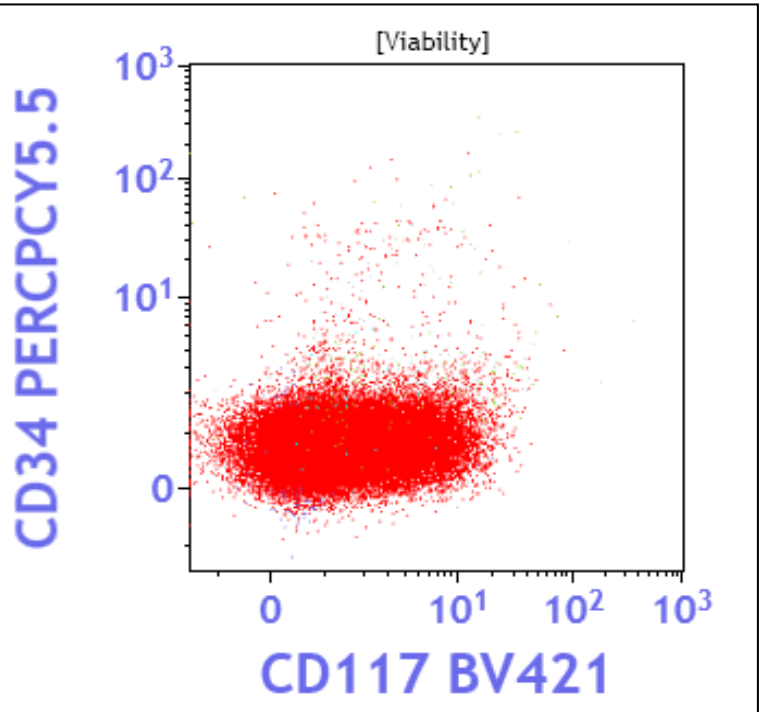
Immunophenotyping

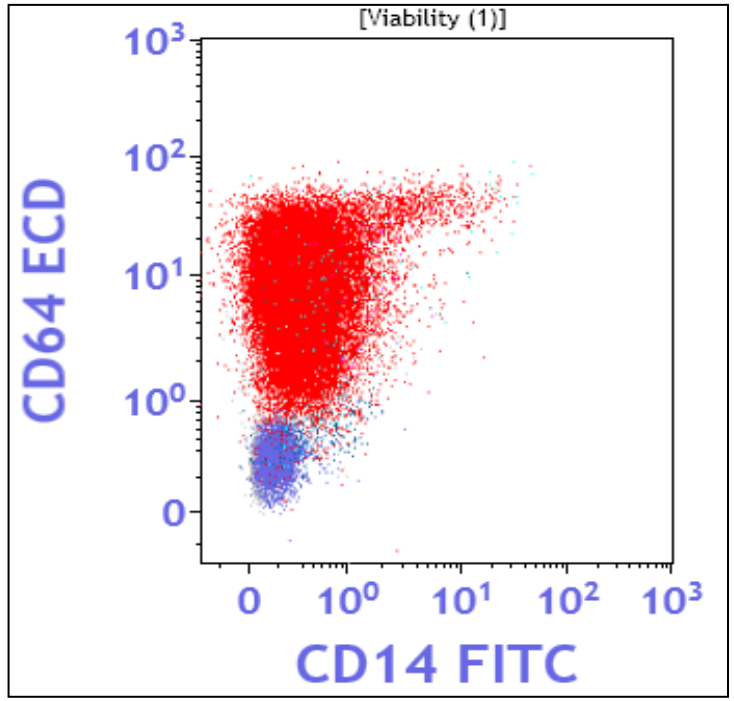


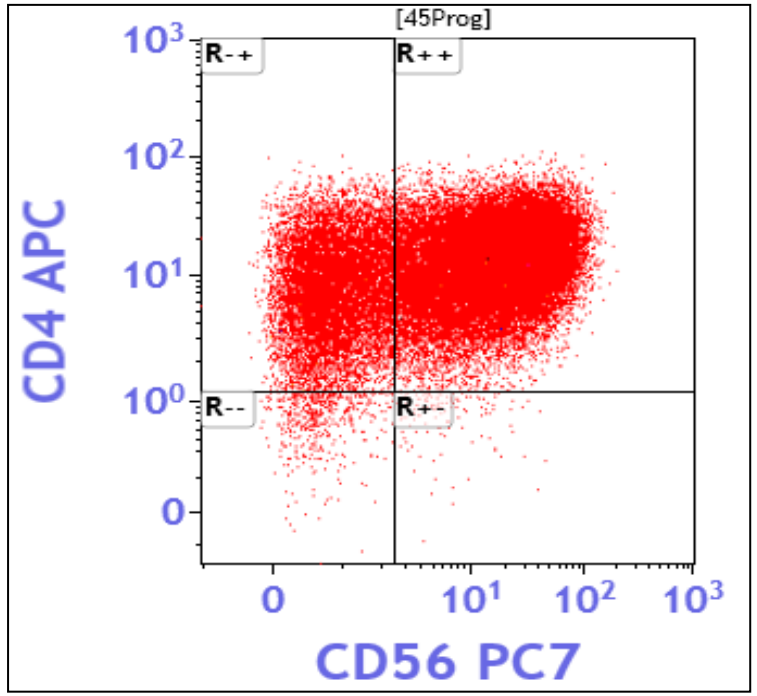

[Viability (1)]
CD33 APC_BC
CD36 V450

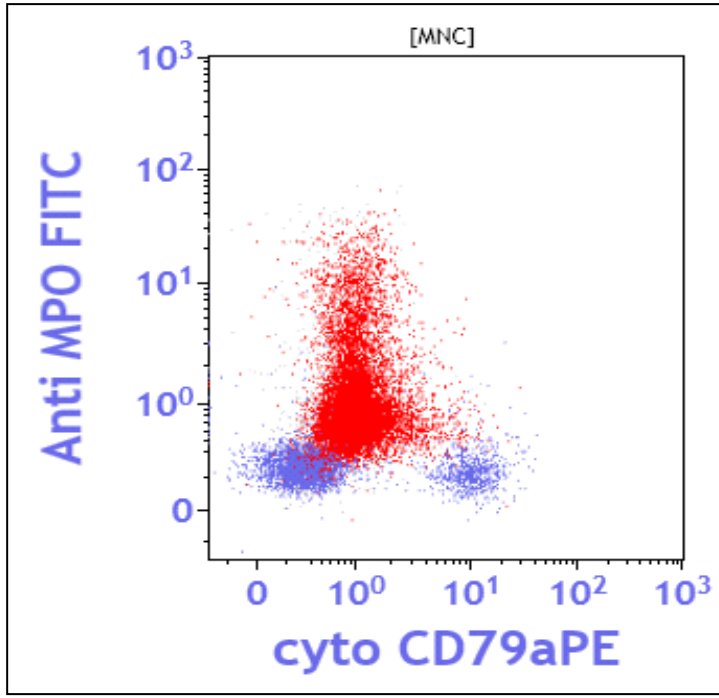

[Viability]
CD13 PE
HLA-DR BV510

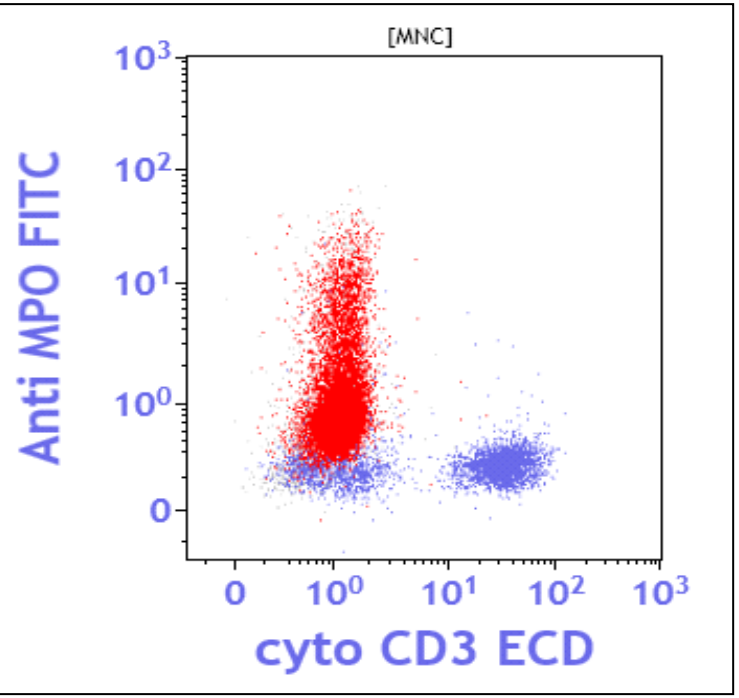

[Viability]
CD11b APC
CD15 FITC


[Viability]
CD34 PERCPCY5.5
CD117 BV421


[Viability (1)]
CD64 ECD
CD14 FITC
10^3
10^2
10^1
10^0
0


[45Prog]
R- +
R+ +
R- -
R+ -
CD4 APC
CD56 PC7
10^3
10^2
10^1
10^0
0


[MNC]
Anti MPO FITC
cyto CD79aPE
10^3
10^2
10^1
10^0
0


[MNC]
Anti MPO FITC
cyto CD3 ECD
10^3
10^2
10^1
10^0
0

Diagnosis

AML with monoblastic differentiation

KMT2A-MLLT1 t(11;19) rearranged AML

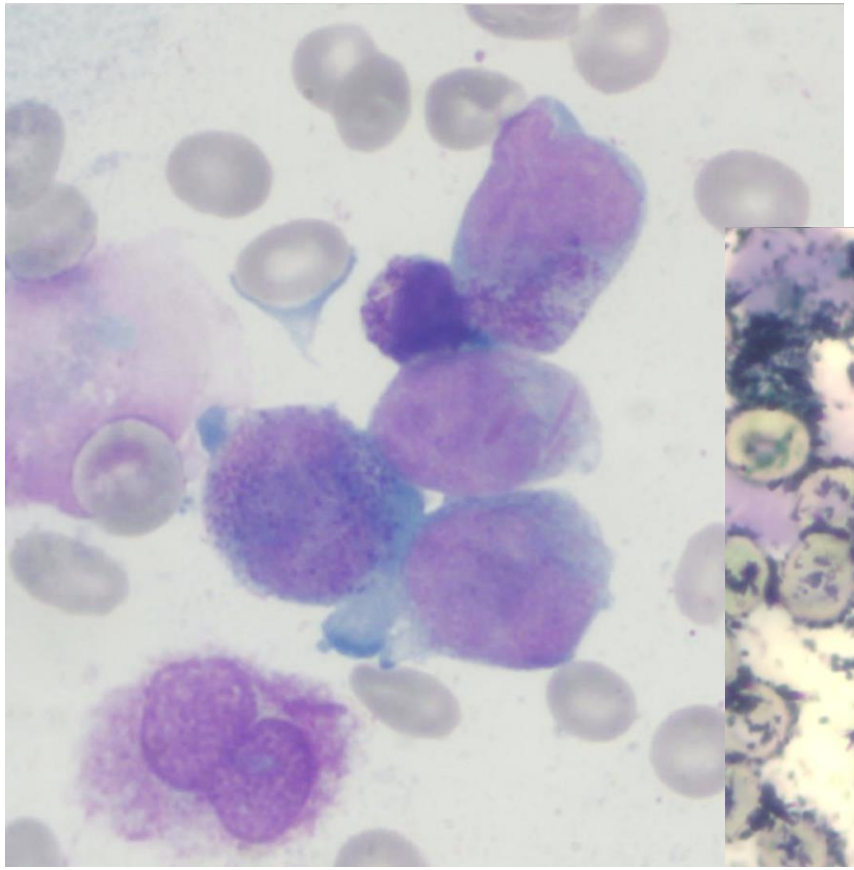
- **Learning points:**
- **All blasts may not express markers of immaturity**

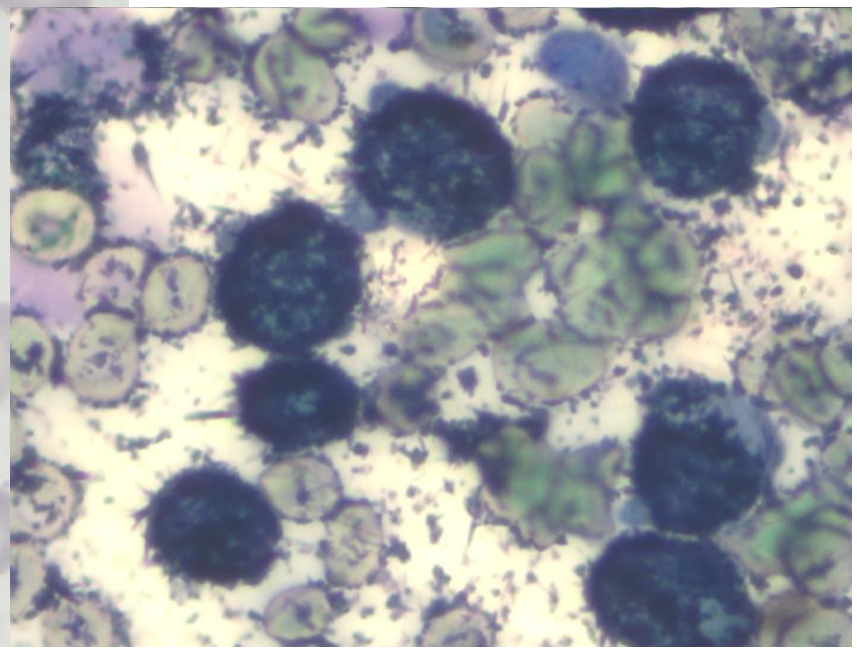
Case 5

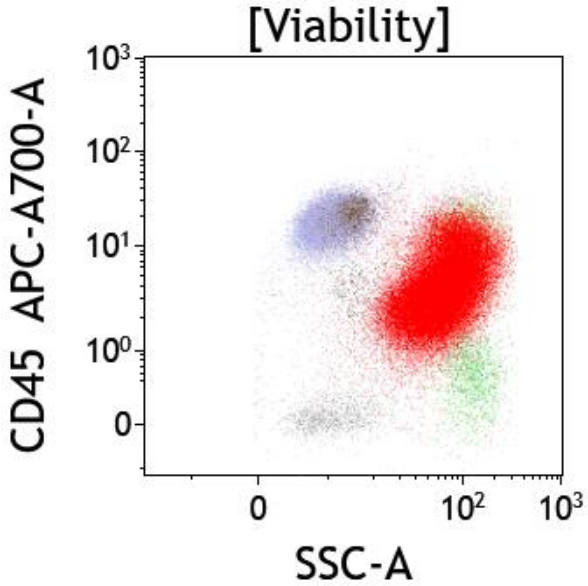
- 15/m
- No comorbidities

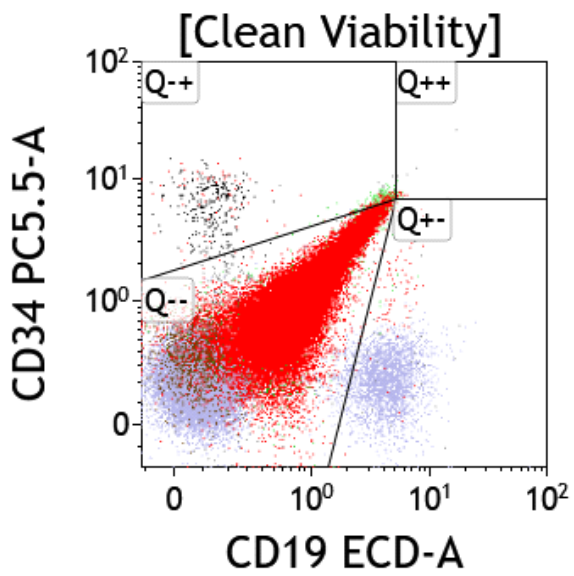
- **CBC 22/7/2020**
- Hb 5.9 TLC 2.1 Plat 46

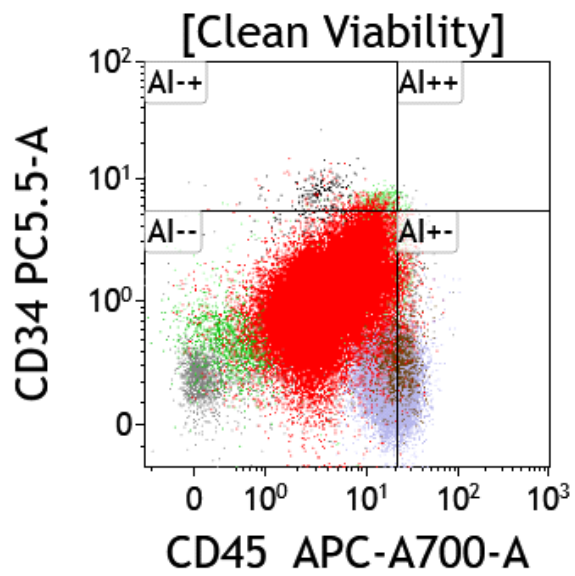
- **BM 24/7/2020**
- AML **CD34+**
- Molecular- FLT3 -ITD - Low allelic ratio

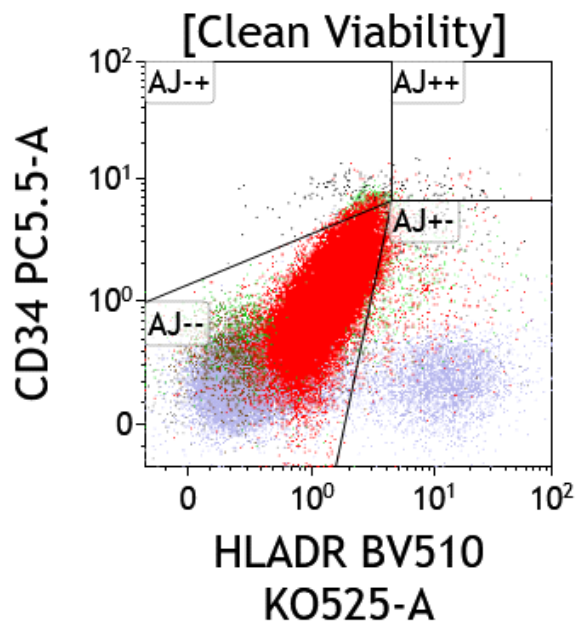




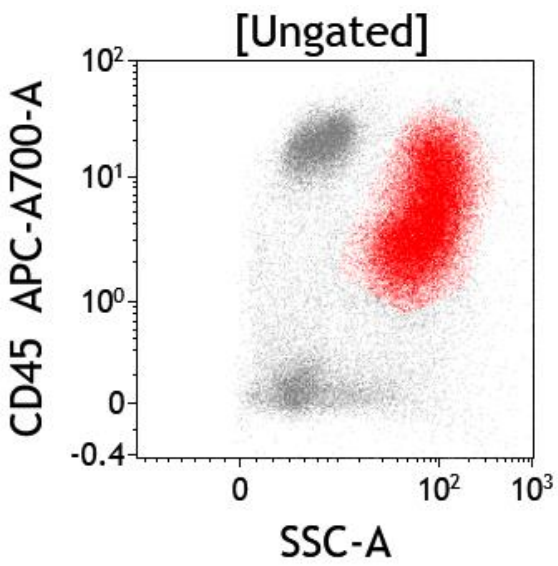

[Viability]
CD45 APC-A700-A
SSC-A

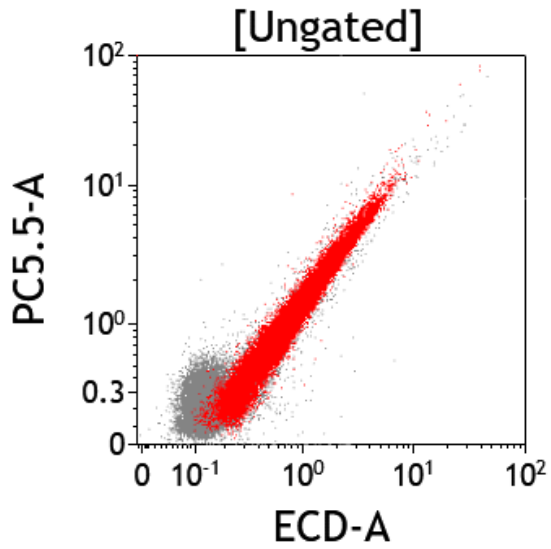

[Clean Viability]
Q-+
Q++
Q+-
Q--
CD34 PC5.5-A
CD19 ECD-A

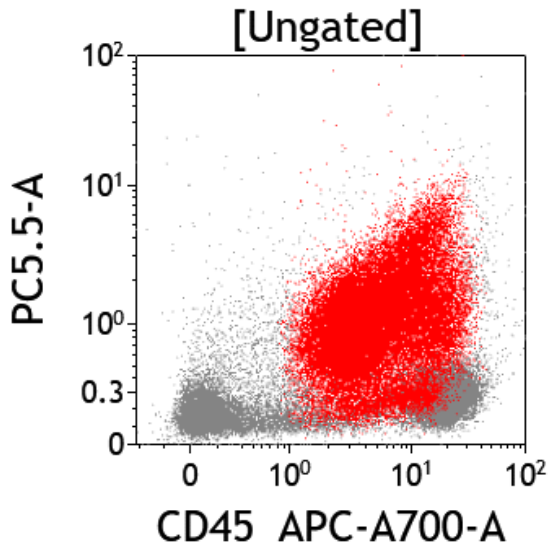

[Clean Viability]
AI-+
AI++
AI--
AI+-
CD34 PC5.5-A
CD45 APC-A700-A


[Clean Viability]
AJ-+
AJ++
AJ+-
AJ--
CD34 PC5.5-A
HLADR BV510
KO525-A

Only CD45


[Ungated]
CD45 APC-A700-A
SSC-A


[Ungated]
PC5.5-A
ECD-A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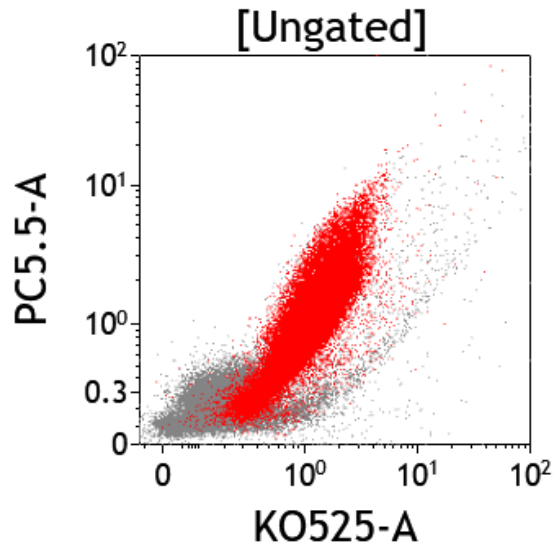
PC5.5-A
CD45 APC-A700-A


[Ungated]
PC5.5-A
KO525-A

Diagnosis

Acute promyelocytic leukemia, FLT3-ITD+

PML-RARA bcr1 transcript

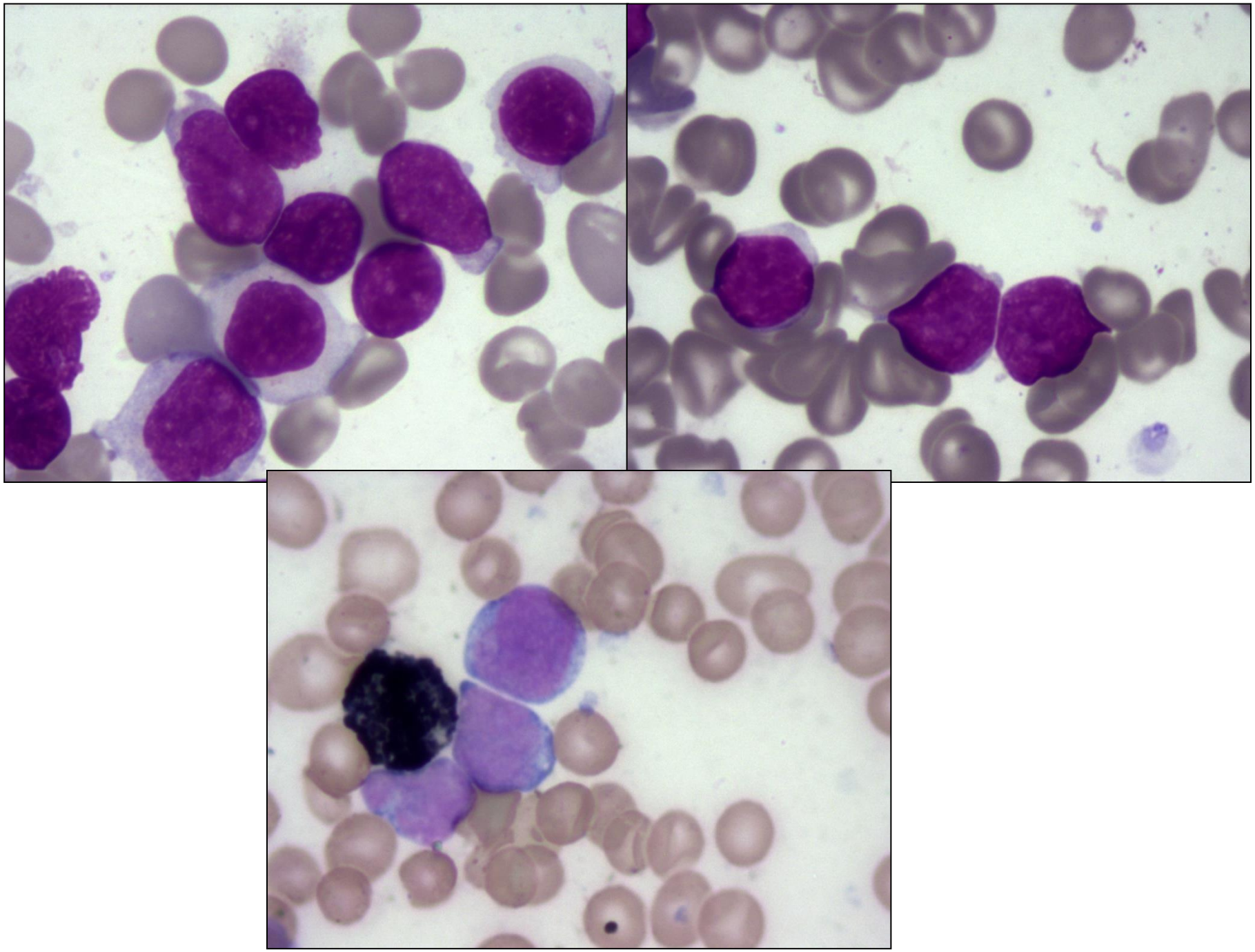
- **Learning points:**
- **Awareness about autofluorescence**
- **Unstained/only CD45 tube**

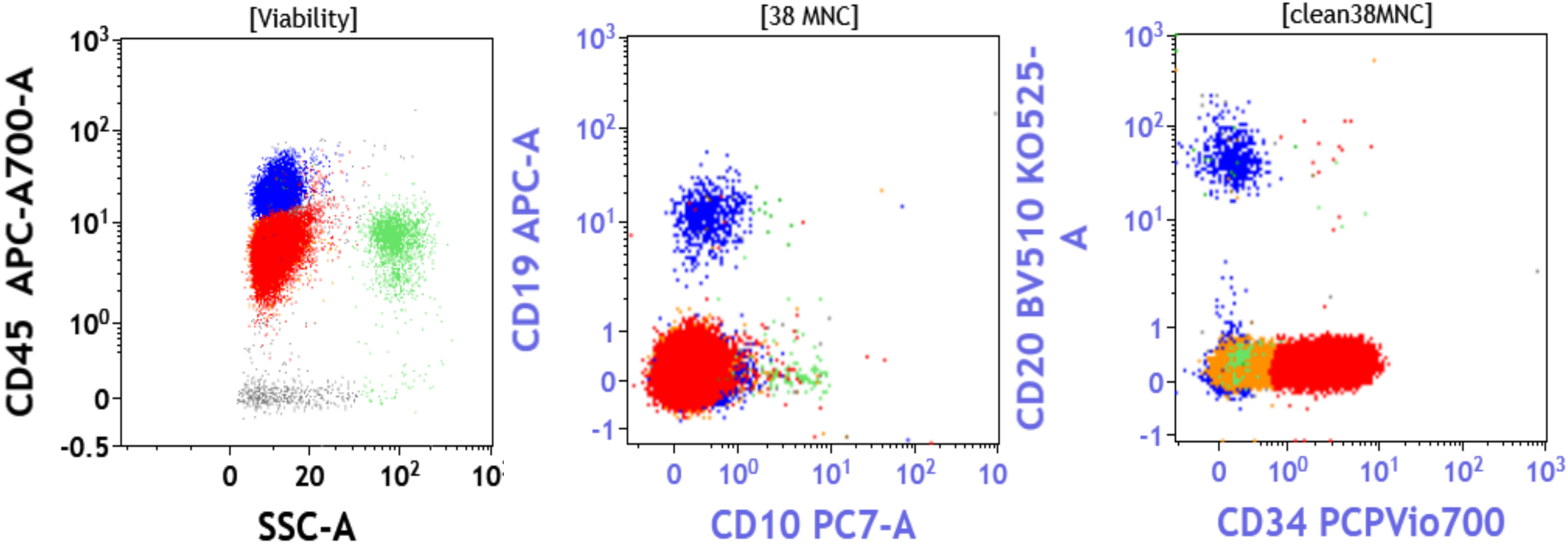
Case 6

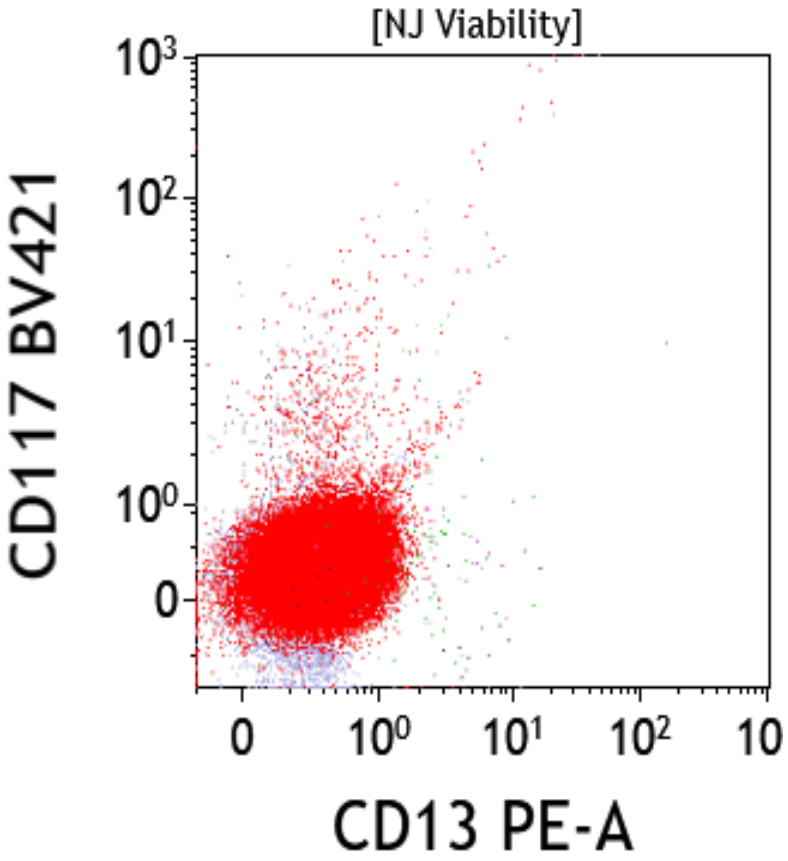
11 yr male

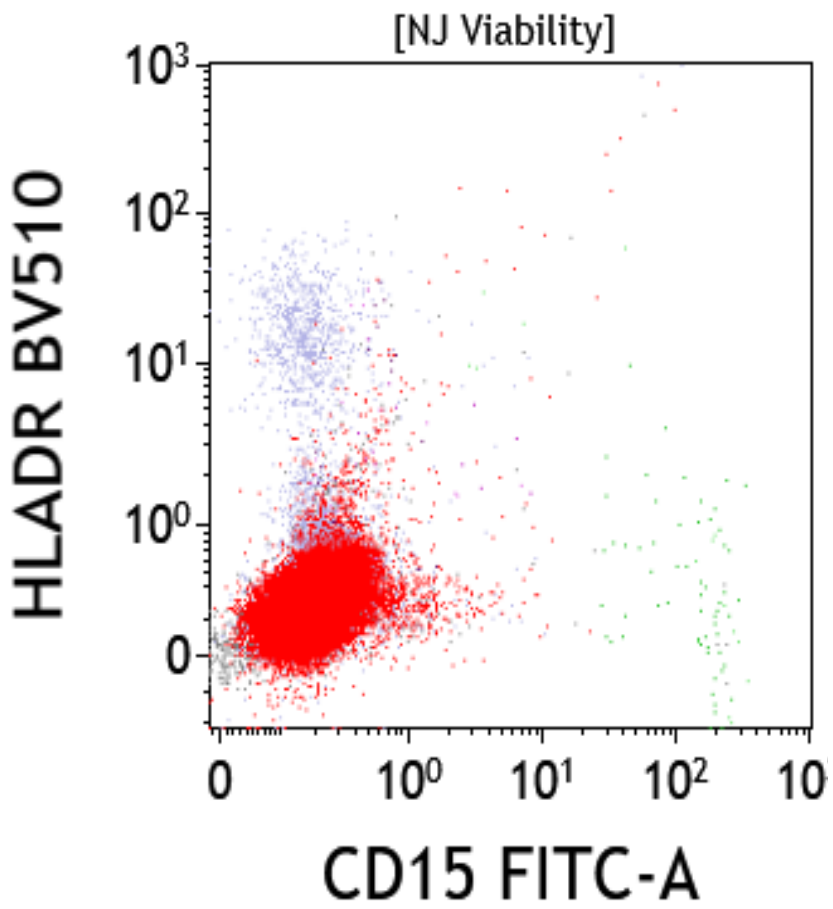
- Fever - 2 month
- Hepatosplenomegaly

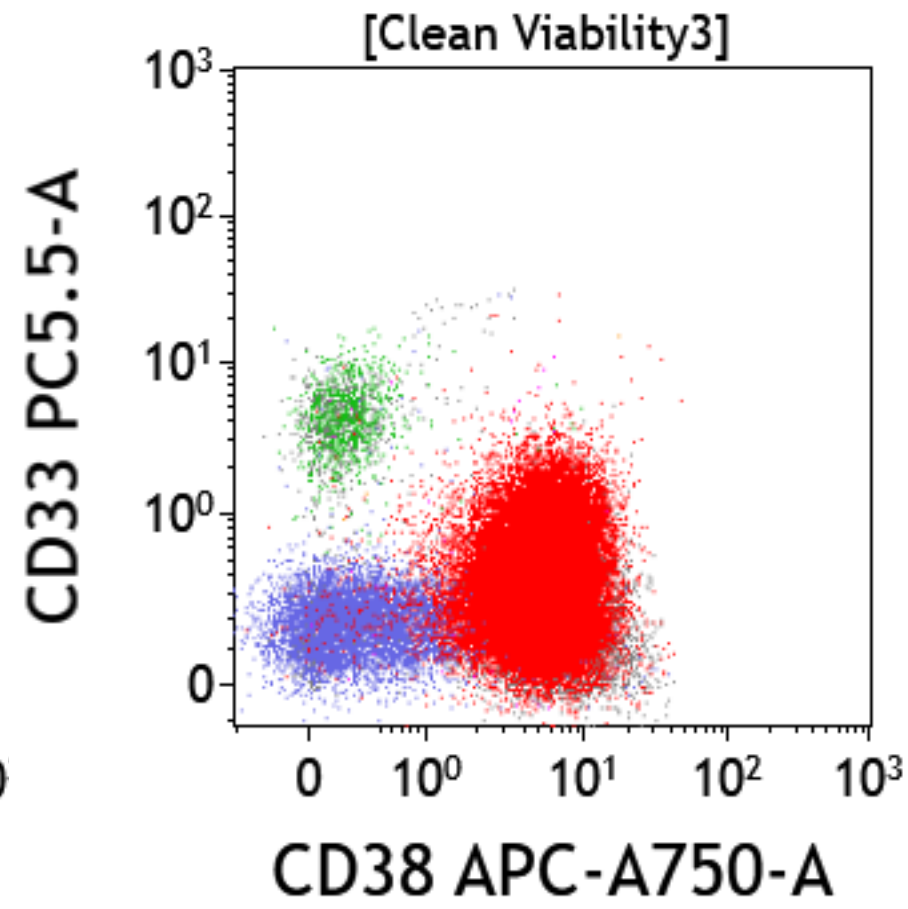
- HB- 9.7 gm/dl
- Platelets – 250 *10^9/L
- WBC – 2.3 * 10^9/L

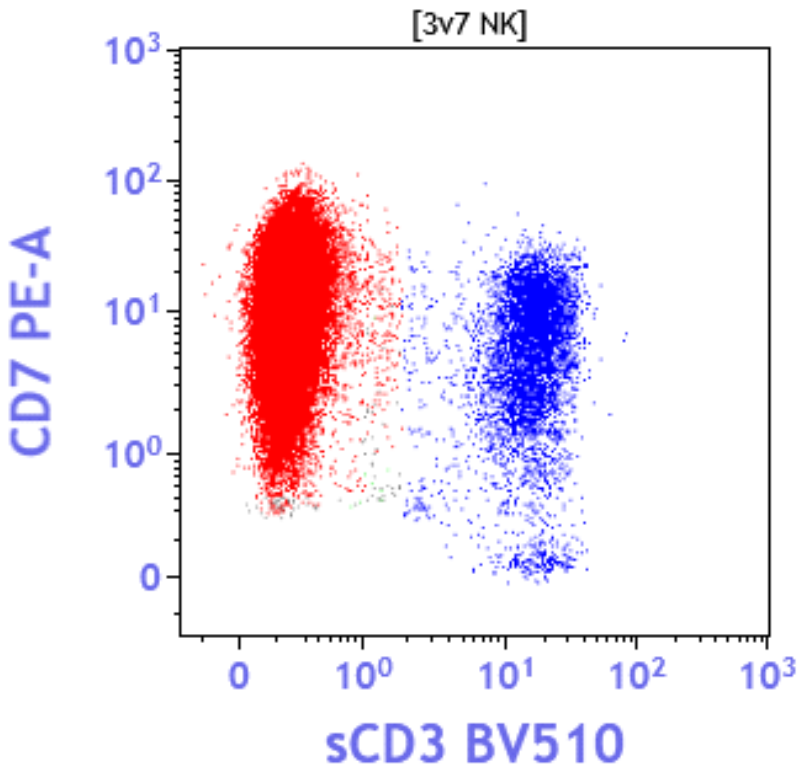


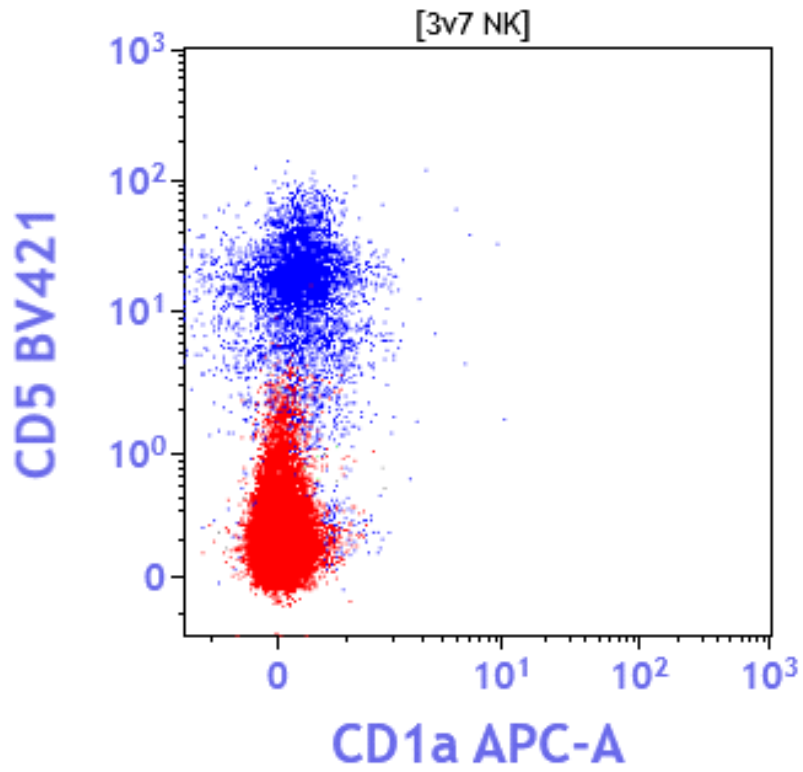

[Viability]
CD45 APC-A700-A
SSC-A
[38 MNC]
CD19 APC-A
CD10 PC7-A
CD20 BV510 KO525-A
[clean38MNC]
CD34 PCPVio700

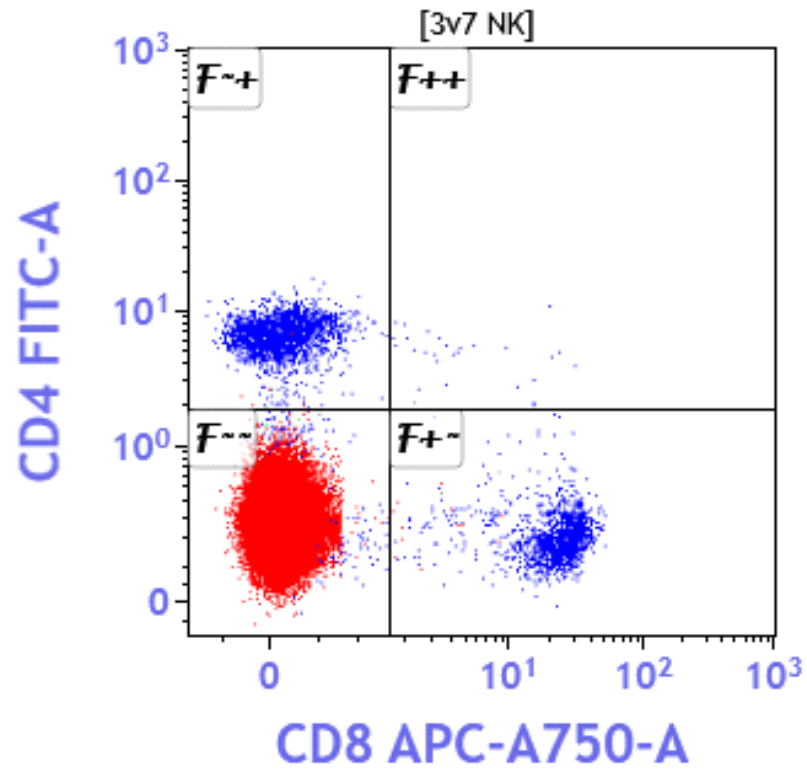

[NJ Viability]
CD117 BV421
CD13 PE-A

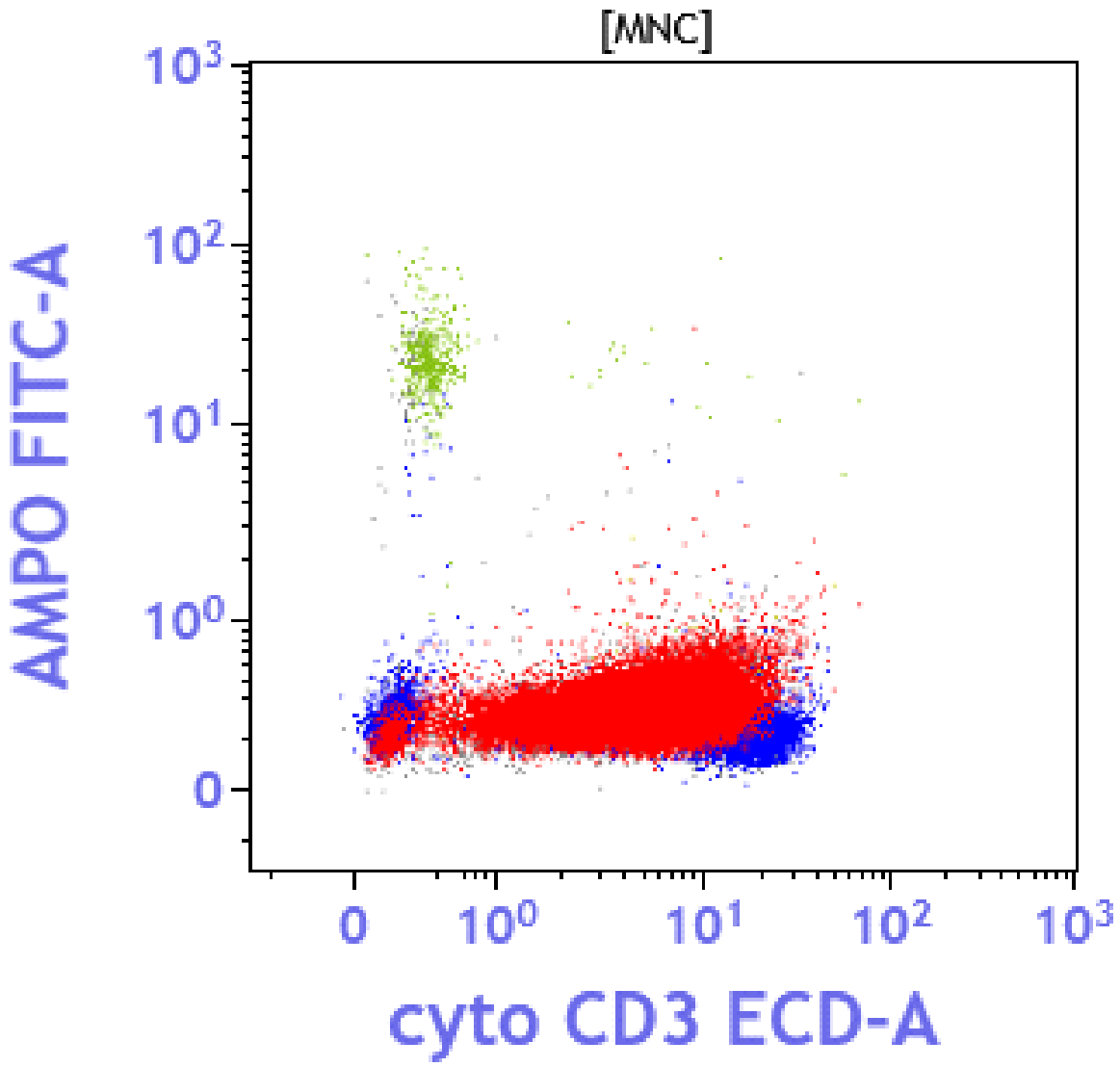

[NJ Viability]
HLADR BV510
CD15 FITC-A

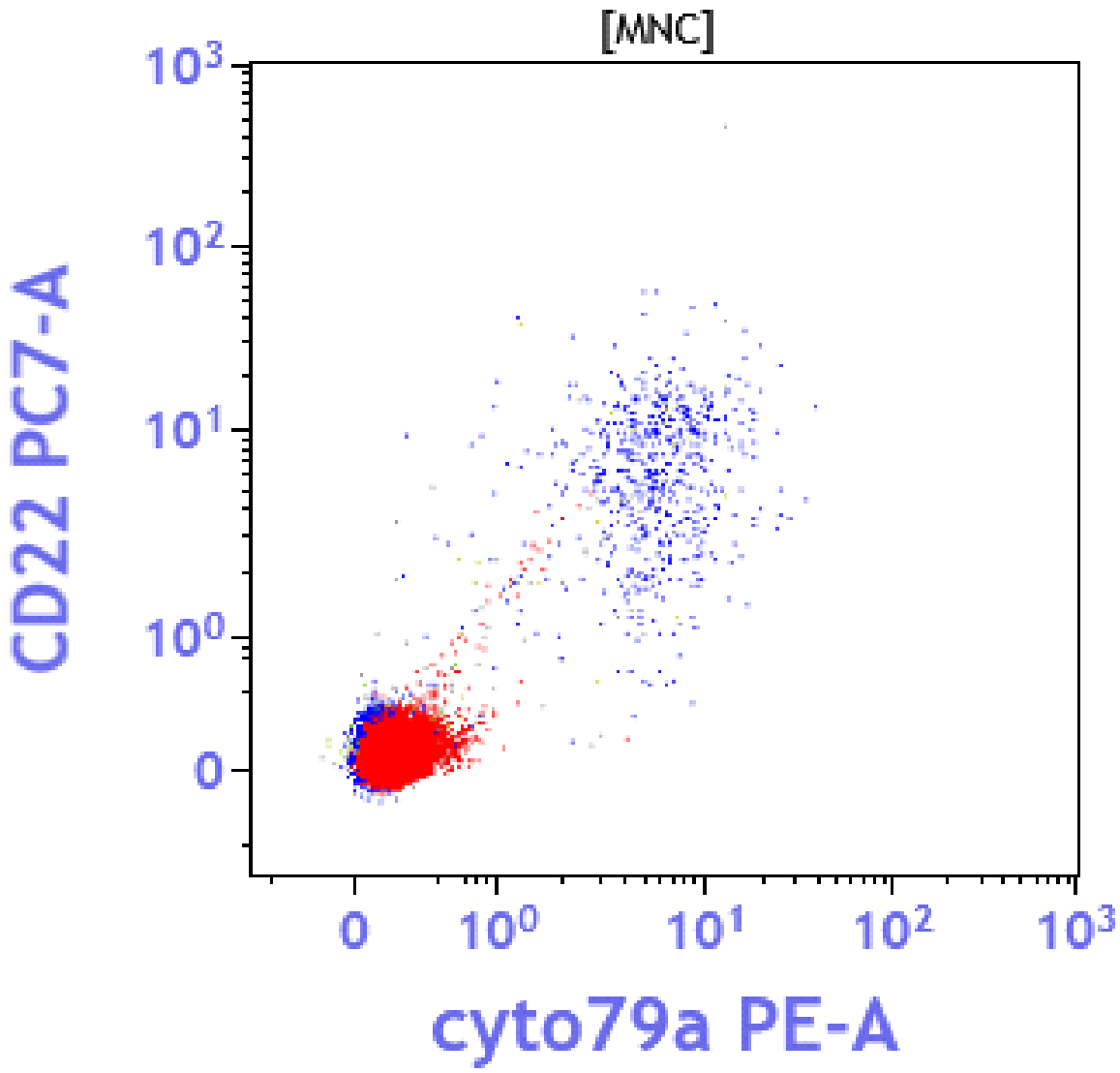

[Clean Viability3]
CD33 PC5.5-A
CD38 APC-A750-A


[3v7 NK]
CD7 PE-A
sCD3 BV510


[3v7 NK]
CD5 BV421
CD1a APC-A

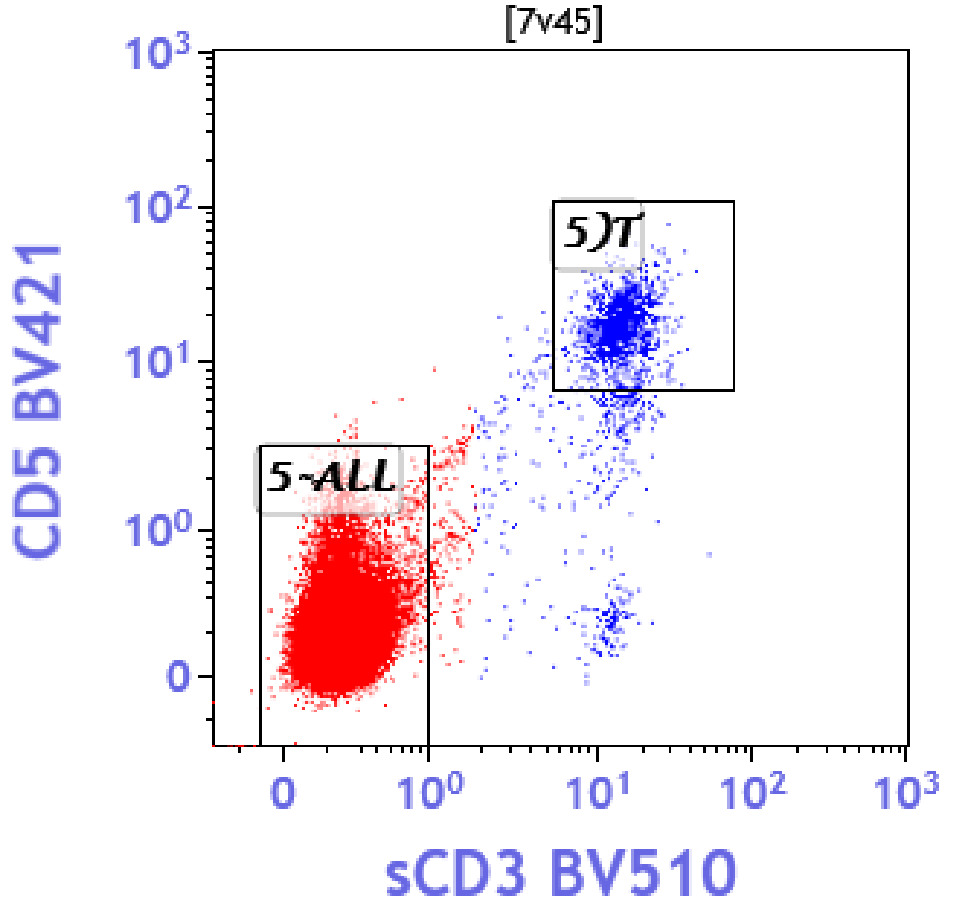

[3v7 NK]
F-+
F++
F--
F+-
CD4 FITC-A
CD8 APC-A750-A


[MNC]
AMPO FITC-A
cyto CD3 ECD-A
0
10^0
10^1
10^2
10^3


[MNC]
CD22 PC7-A
cyto79a PE-A
0
10^0
10^1
10^2
10^3

Blasts

- Express Mod CD34, dim CD33,
- Express moderate CD7, variable CD2, partial CD56
- Express Cytoplasmic CD3
- Negative for CD8, CD5, CD1a
- Negative for B lymphoid, and other myeloid markers
- Negative for cytoplasmic MPO and CD79a

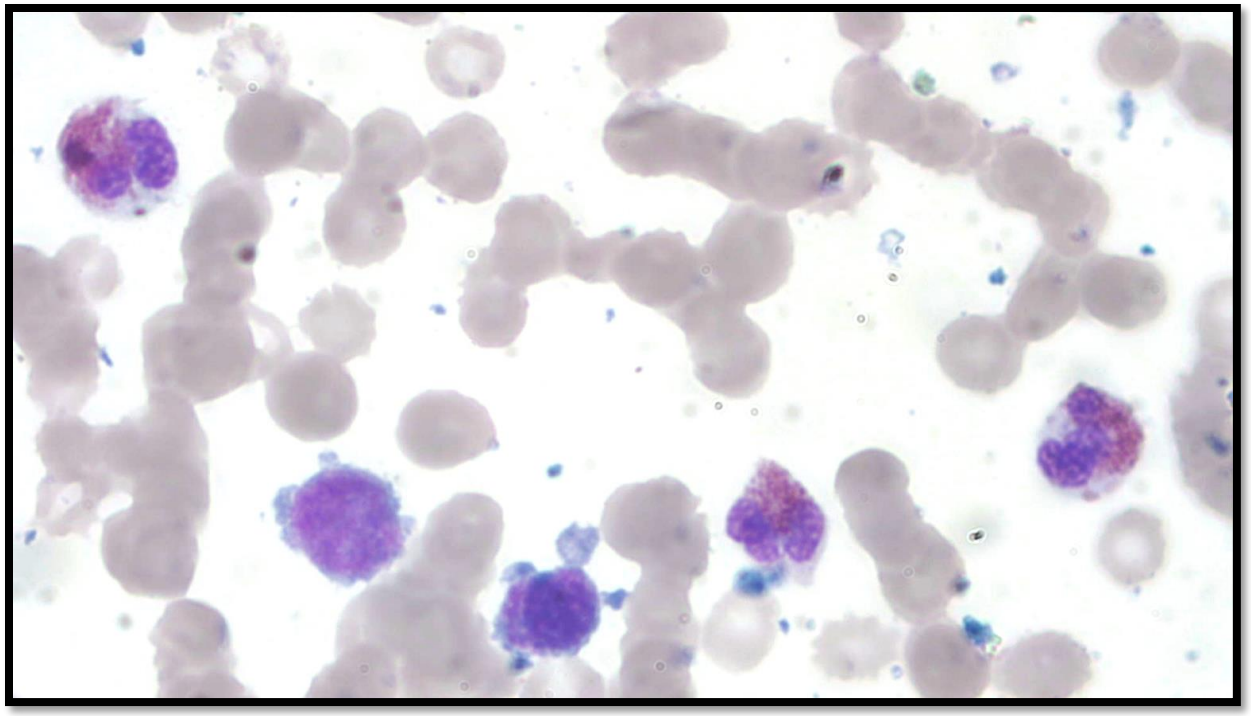


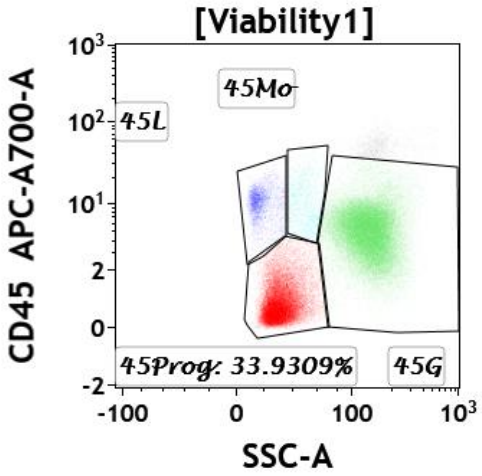
Gate	%Gated	Y-GMean
All	100.0000	0.2160
5-ALL	97.7973	0.1889
5)T	1.4742	17.1968

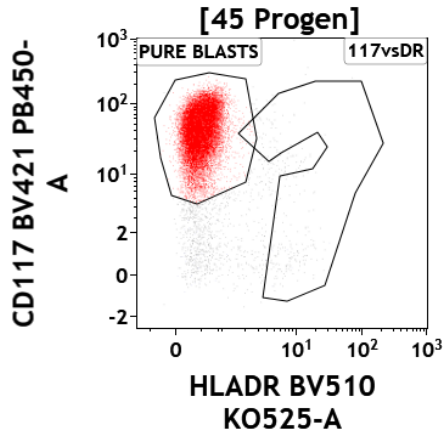
Early thymic precursor TALL (ETPALL)

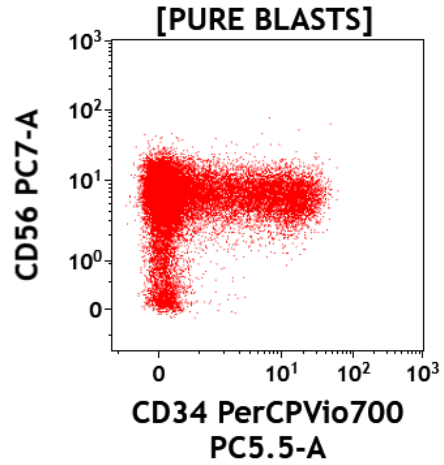
Case 7

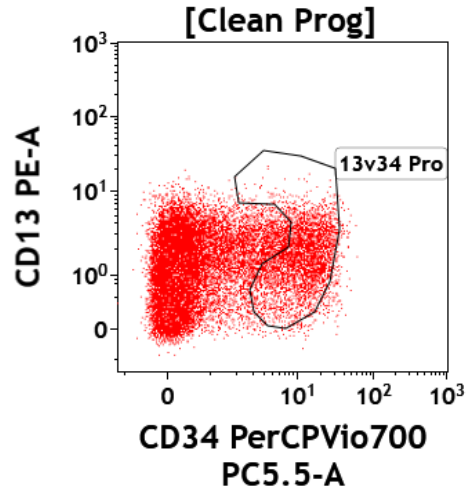
- 1 year 5-month-old boy
- Presented with complaints of fever on-off for 15 days and poor appetite
- No organomegaly or lymphadenopathy
- CBC(29/5): Hb 2.2TLC 80.6 Plt 12

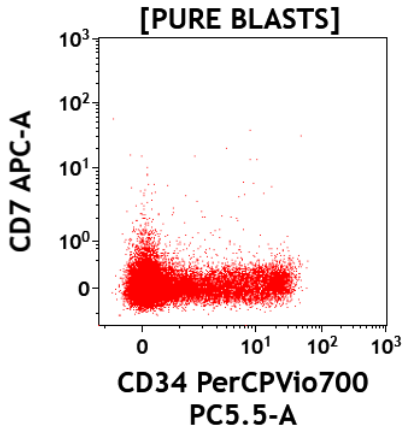


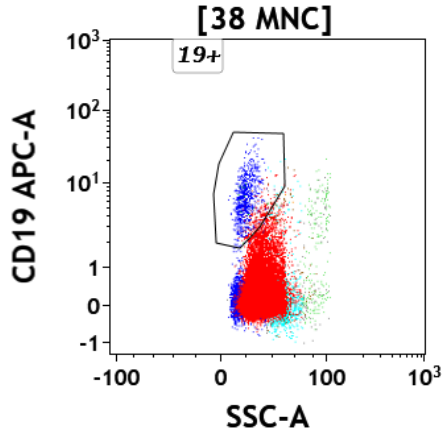

[Viability1]
45Mo
45L
45Prog: 33.9309%
45G
CD45 APC-A700-A
SSC-A

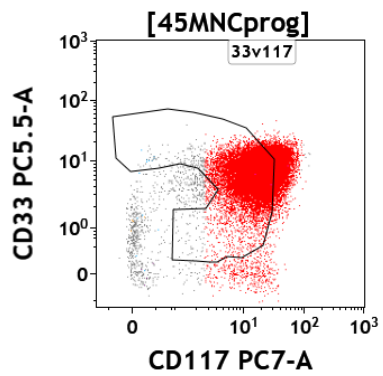

[45 Progen]
PURE BLASTS
117vsDR
CD117 BV421 PB450-A
HLADR BV510 KO525-A

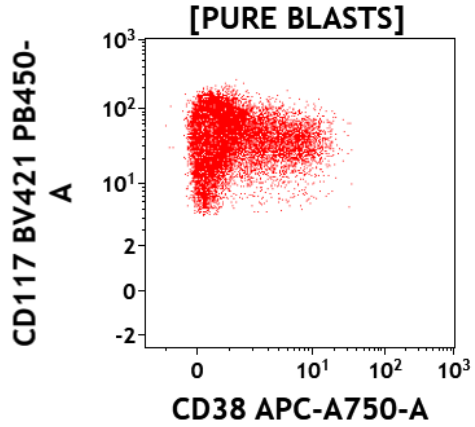

[PURE BLASTS]
CD56 PC7-A
CD34 PerCPVio700 PC5.5-A

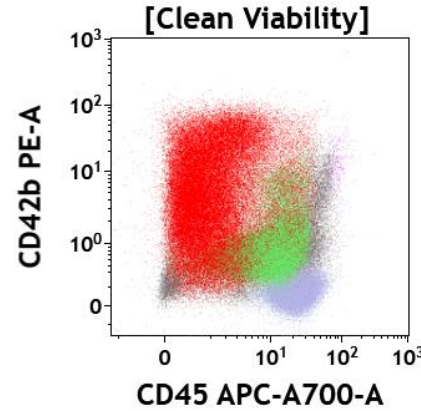

[Clean Prog]
13v34 Pro
CD13 PE-A
CD34 PerCPVio700 PC5.5-A

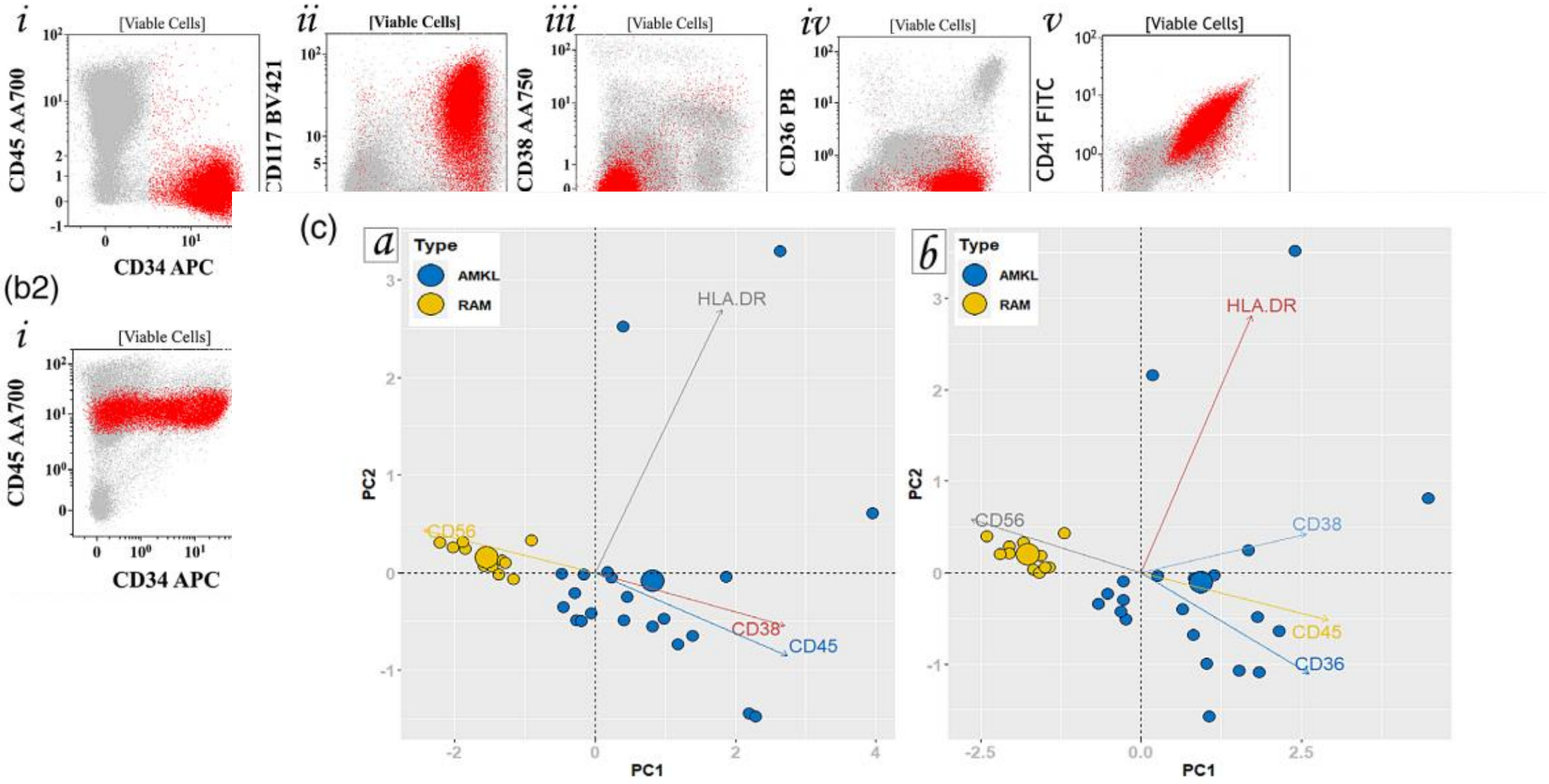

[PURE BLASTS]
CD7 APC-A
CD34 PerCPVio700 PC5.5-A


[38 MNC]
19+
CD19 APC-A
SSC-A


[45MNCprog]
33v117
CD33 PC5.5-A
CD117 PC7-A

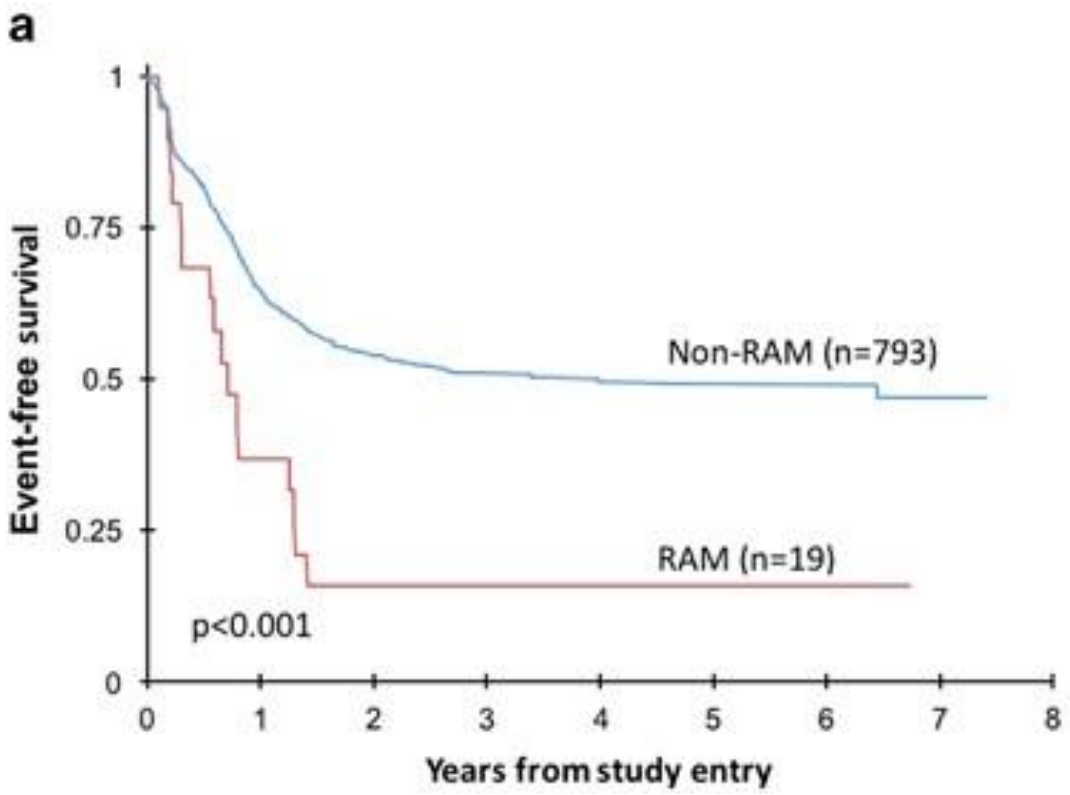

[PURE BLASTS]
CD117 BV421 PB450-A
CD38 APC-A750-A

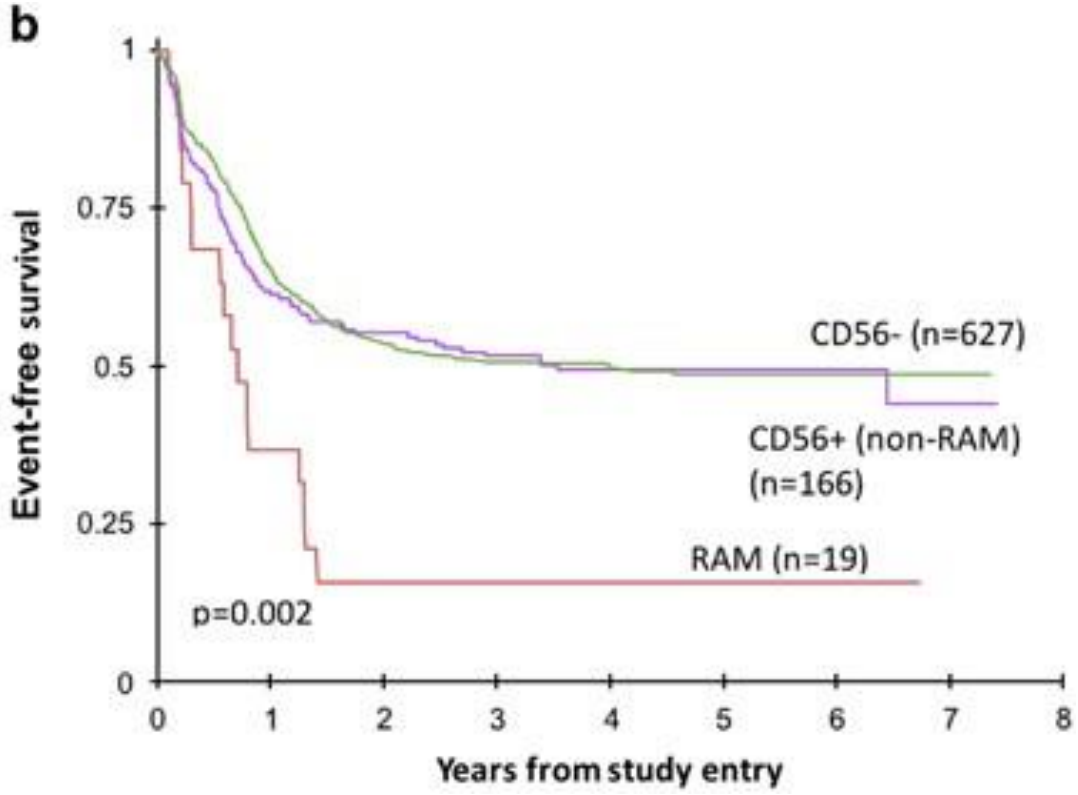

[Clean Viability]
CD42b PE-A
CD45 APC-A700-A



Panda et al. Clinical Cytometry doi.org/10.1002/cyto.b.21943

RAM phenotype independently predicts poor prognosis in pediatric AML



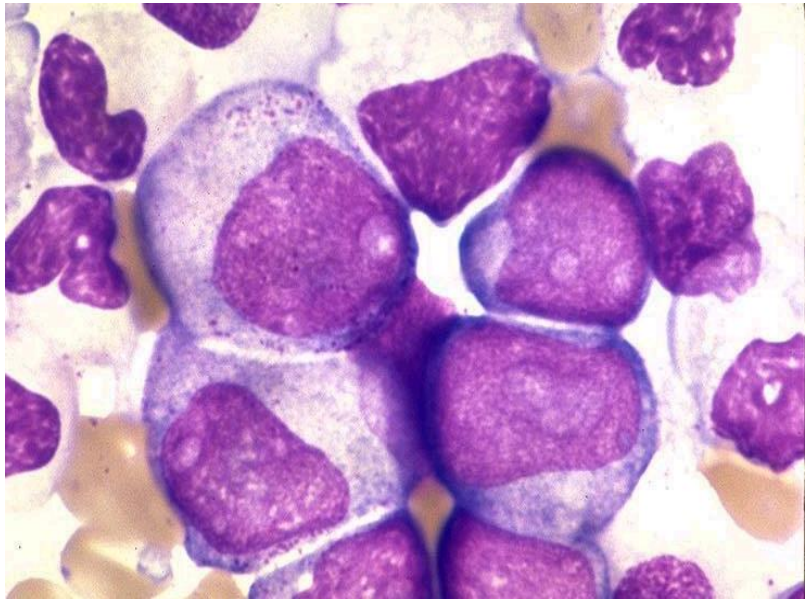


- **Learning points:**
- **FCM can help in picking up clinically relevant subtypes of Acute Leukemia**

Brodersen et al. Leukemia DOI: 10.1038/leu.2016.119

Role of FCM in AL

- **Confirmation** of presence of **abnormal** blasts
- Accurate **quantification** of blasts
- **Lineage determination** and **characterization** of abnormal blasts.

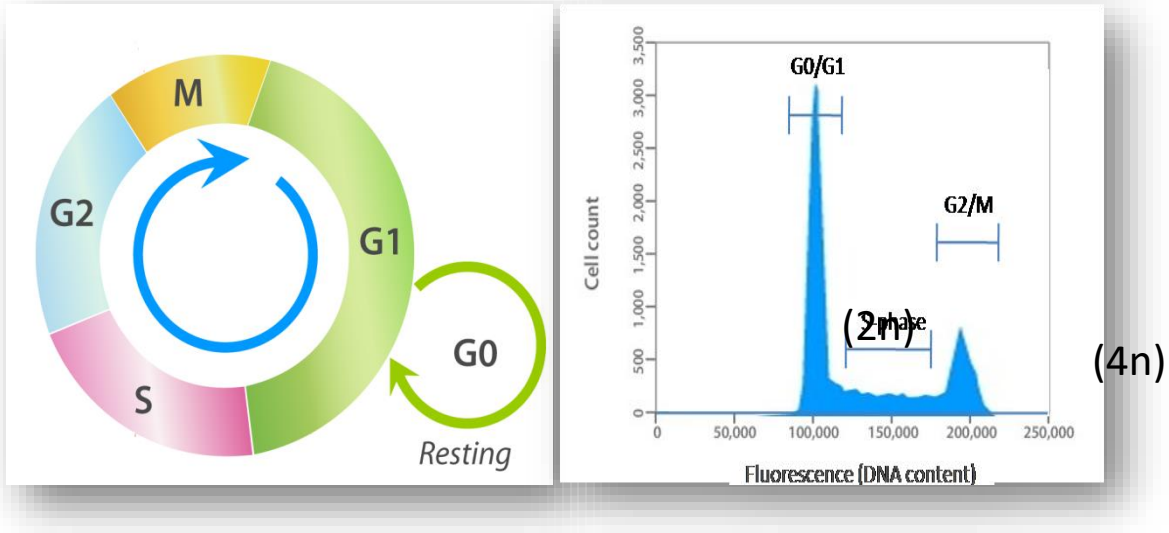


- FCM can provide reliable **prognostic information**
- Typical FCM marker expression can **predict disease genotype**.
- FCM evaluation of biomarkers can predict **response to targeted therapy**
- FCM based **pattern-analysis** identifies aberrant maturation

- FCM is a practically useful, **highly sensitive** technique to **monitor treatment response** and quantitate measurable residual disease (MRD).

Ploidy analysis in B-ALL

- Hyperdiploidy - **good** prognosis
- Hypodiploidy - **poor** prognosis



DNA index (DI) = MFI G1 Blasts / MFI G1 Lymph

(Mean DNA immunofluorescence of G1 peak)

Cytometry PART A
Cytometry Part A
Volume 89, Issue 3, pages 281–291, March 2016

Original Article

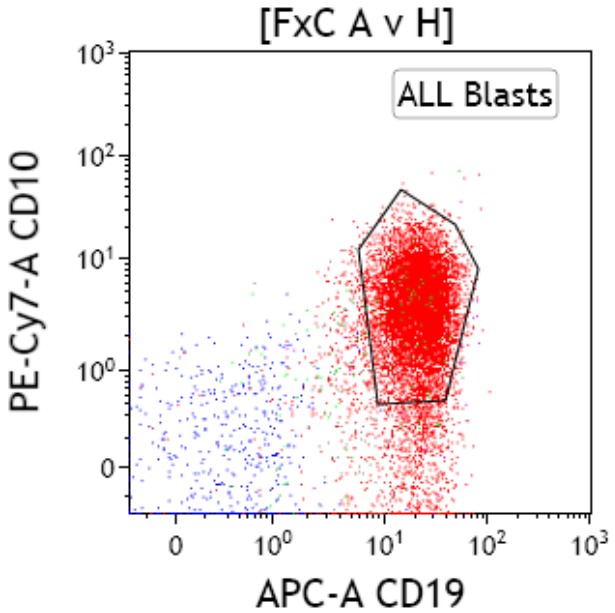
A novel and easy FxCycle™ violet based flow cytometric method for simultaneous assessment of DNA ploidy and six-color immunophenotyping

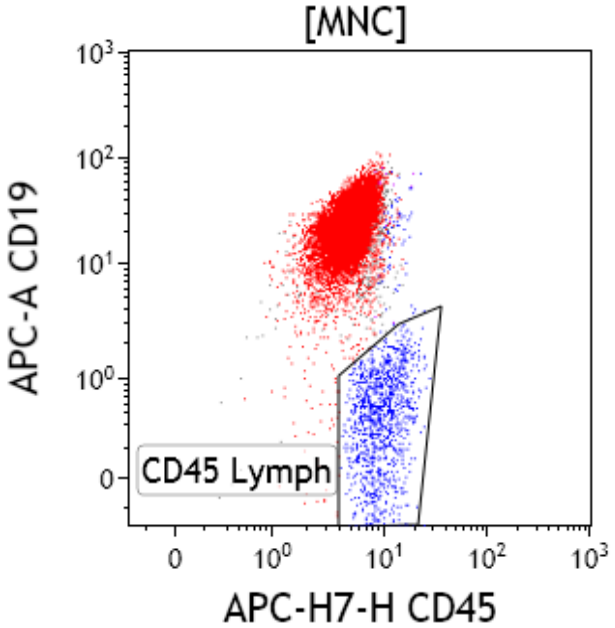
Prashant Tembhare, Yajamanam

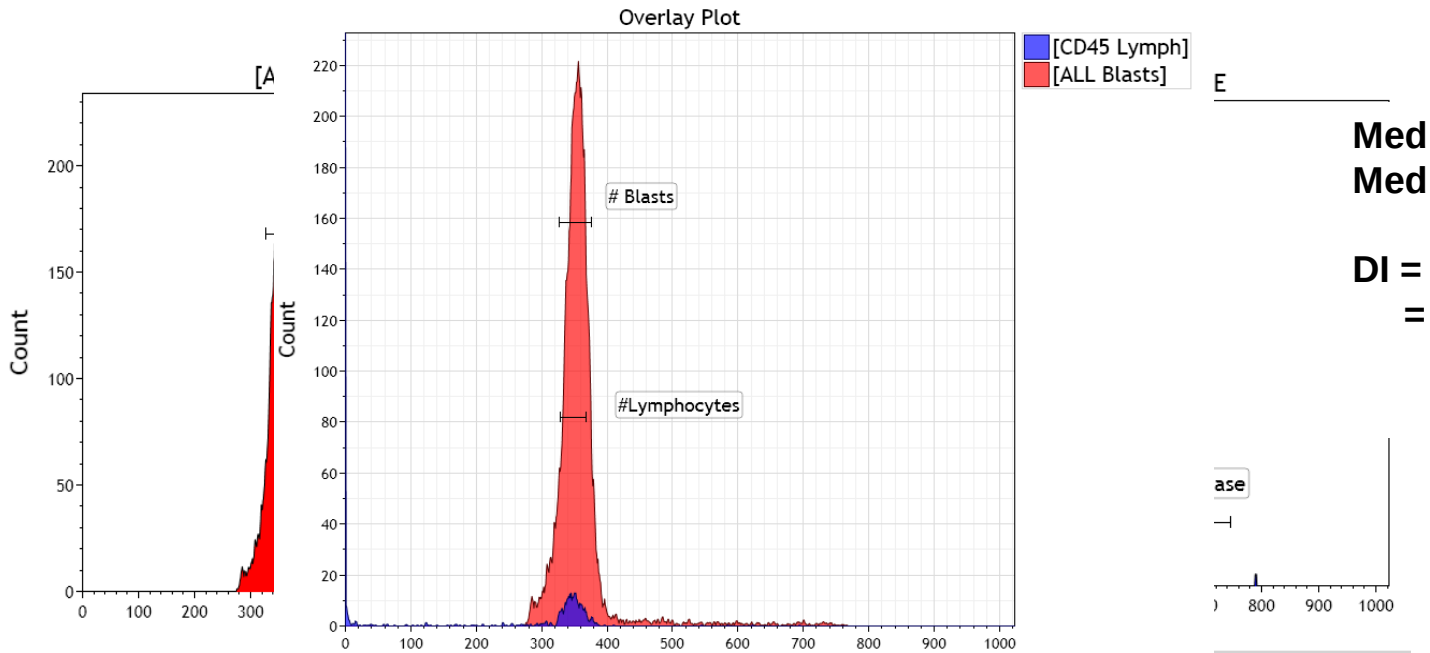
Issue

ıl aspects in analysis of cellular DNA content. Curr Protoc Cytom. 2011
San Miguel JF et al. Blood, Vol 85, No 2 (January 15), 1995:448-455.
Lima M et al. Blood Cells, Molecules, and Diseases (2000) 26(6): 634–645.
Smets LA et al. Med Pediatr Oncol. 1995 Dec;25(6):437-44.

B ALL Case - Diploid







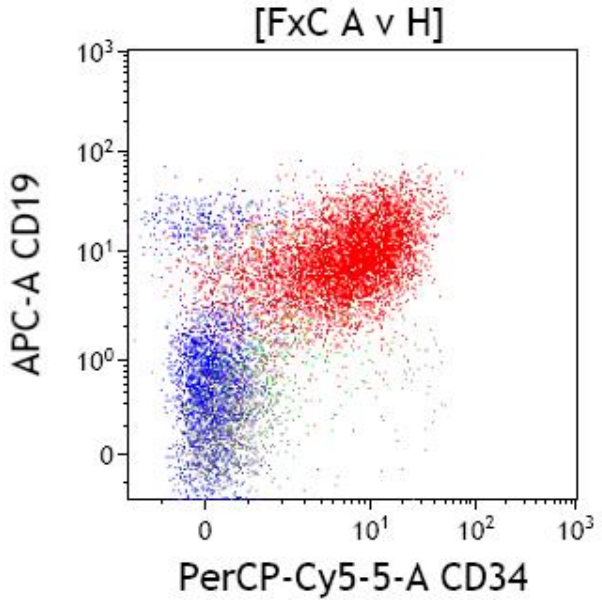
Gate	Number	X-CV	X-GMean
All	9,592	13.5	356.6
G1	7,870	3.6	353.7
G2	75	3.5	713.2
S Phase	333	14.3	502.7

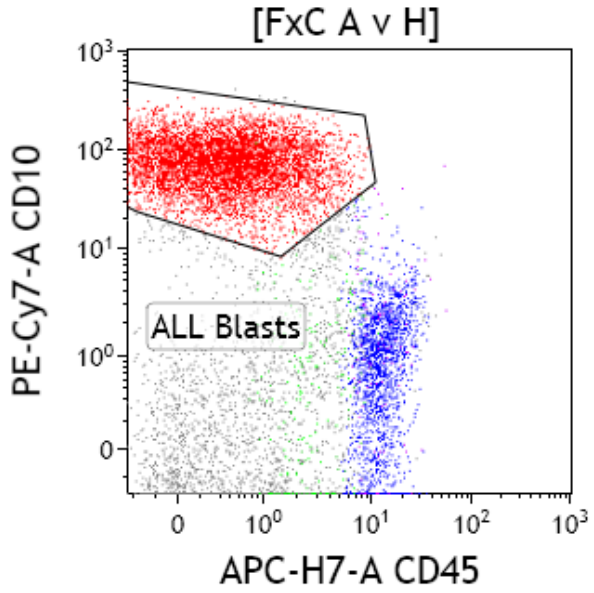
Marker	X-GMean
All	13.3
All	356.6
# Blasts	348.3
# Blasts	352.5
#Lymphocytes	347.4
#Lymphocytes	350.1

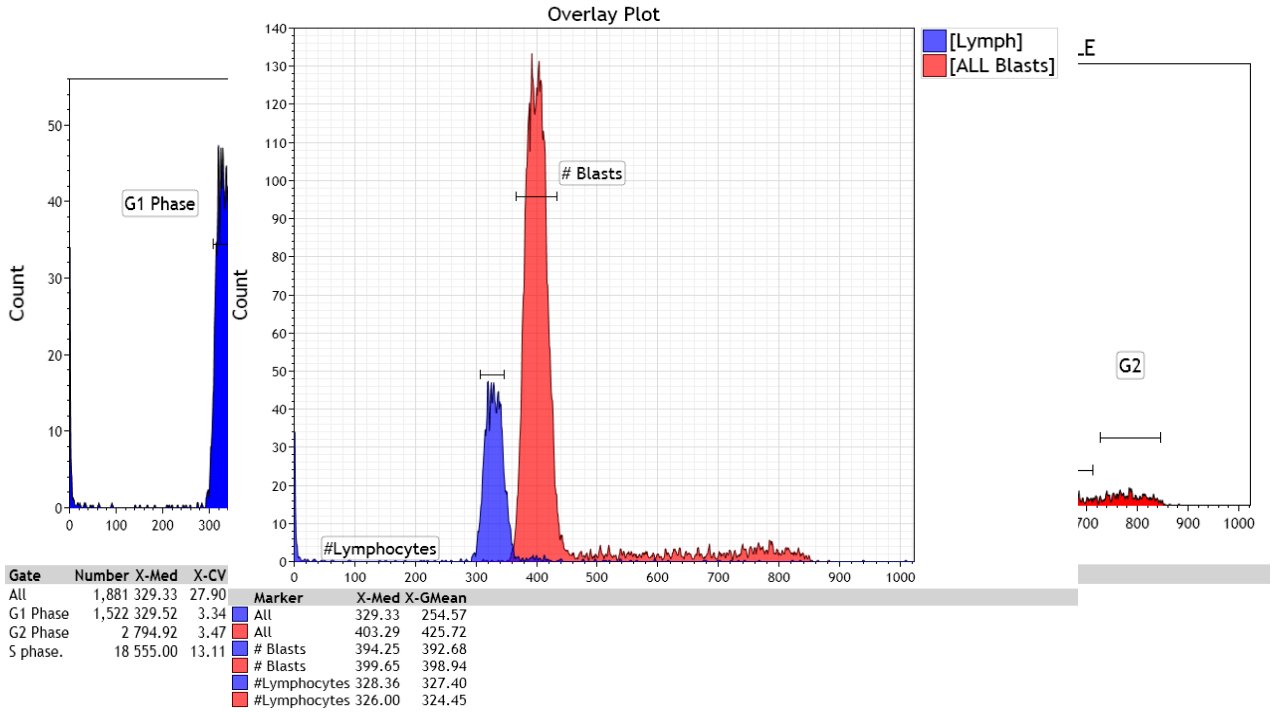
Med G1 BL = 353.7
Med G1 Ly = 345.3

DI = 353.7/345.3
= 1.02

B ALL Case - Hyperdiploidy



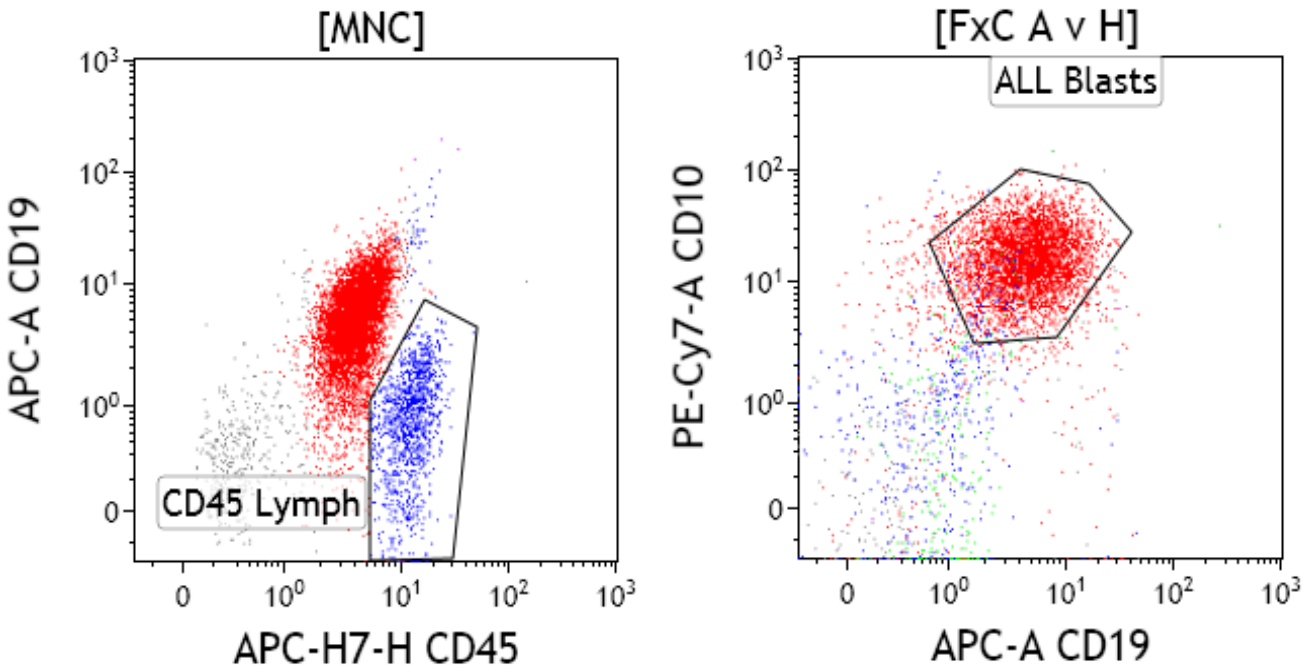


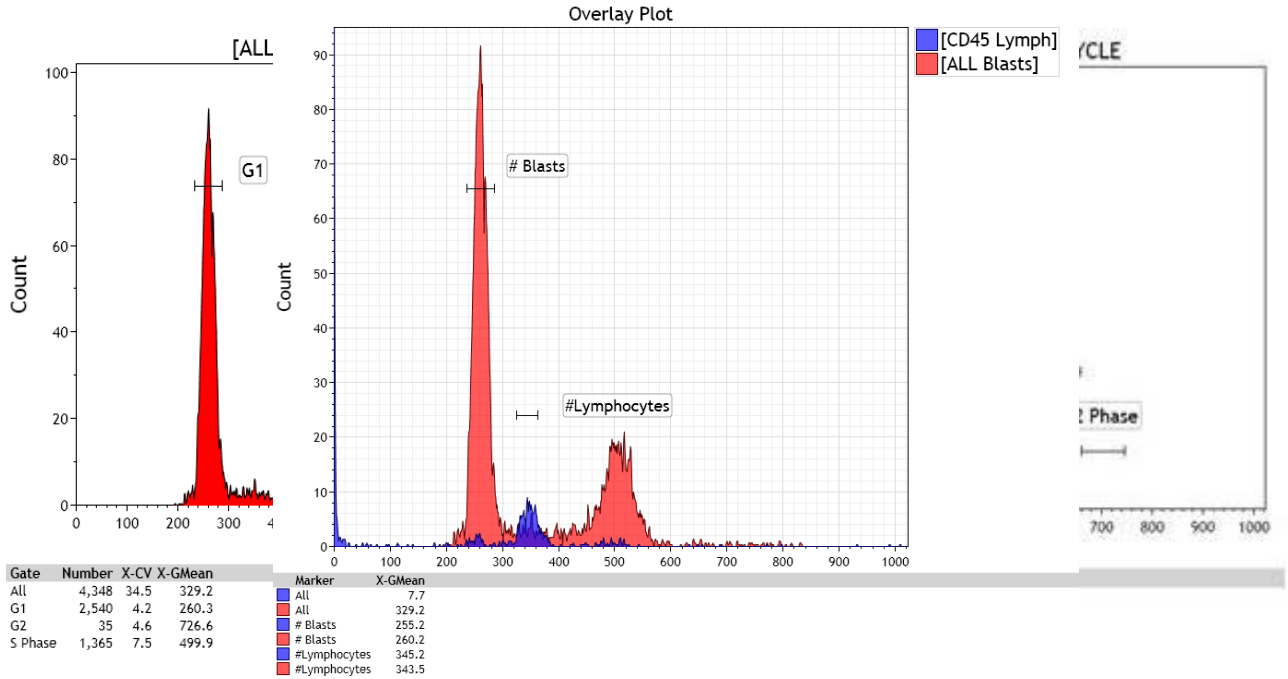


Med G1 BL = 400.2
Med G1 Ly = 329.5

DI = 400.2/329.5
= 1.21

B ALL Case - Hypodiploidy



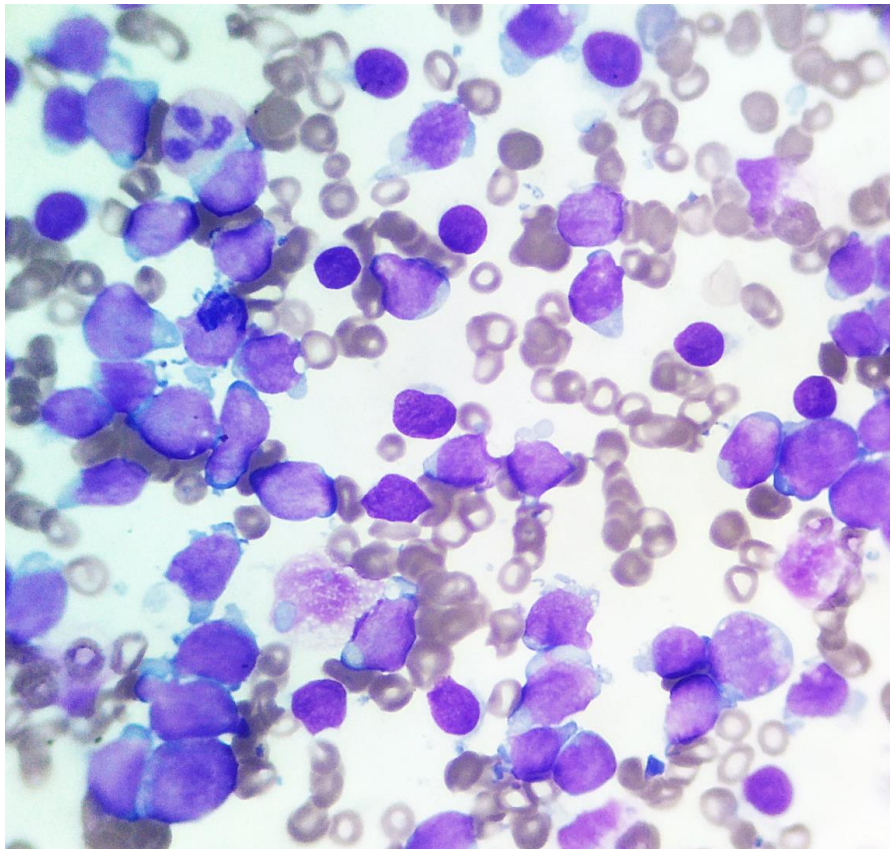


Med G1 BL = 260.3
Med G1 Ly = 349.5

DI = 260.3/349.5
= 0.72

Case 8

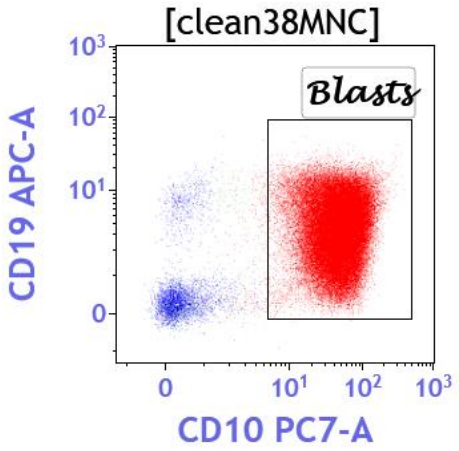
- 12/M, fever for 1 wk.
- Maculopaular rash over trunk and arms
- O/e: No palpable lymphadenopathy.
  P/a soft
- Hb 13.2 TLC 17.7 Plt 34

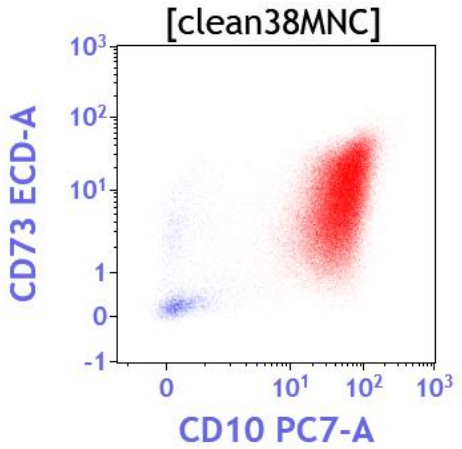


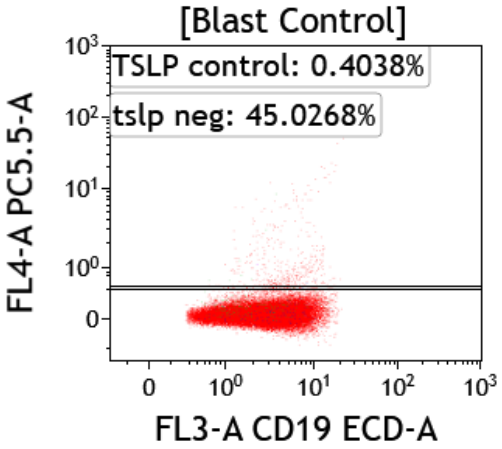
B-lymphoblastic leukemia

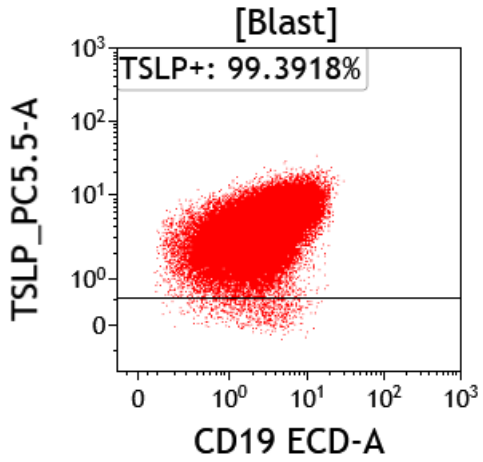
Negative for ETV6-RUNX1, BCR-ABL1, MLL gene rearrangements

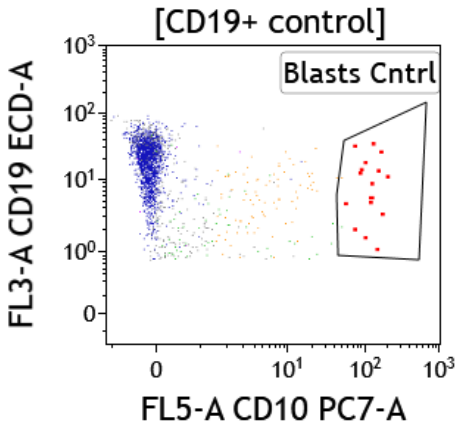
No trisomy 4,10,18

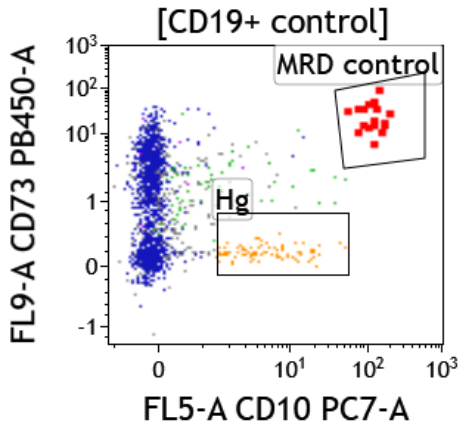


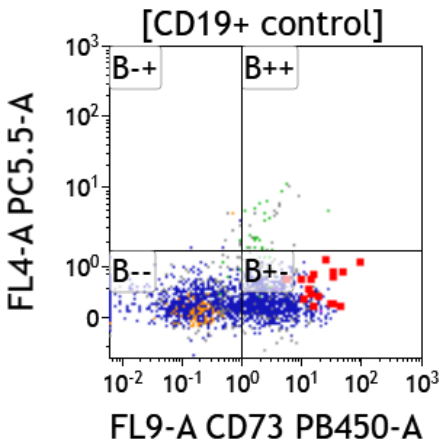




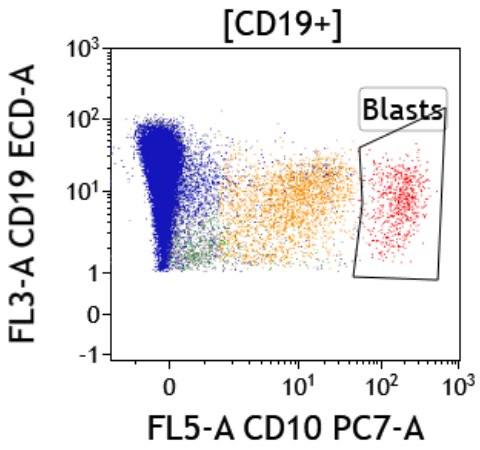


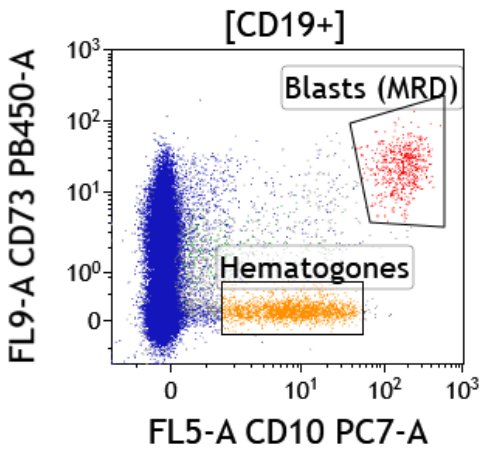


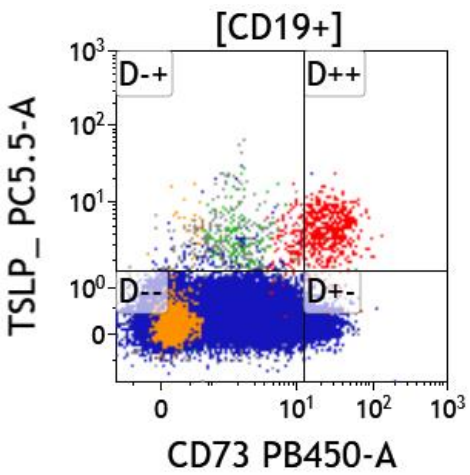




TSLP (MRD) Control tube

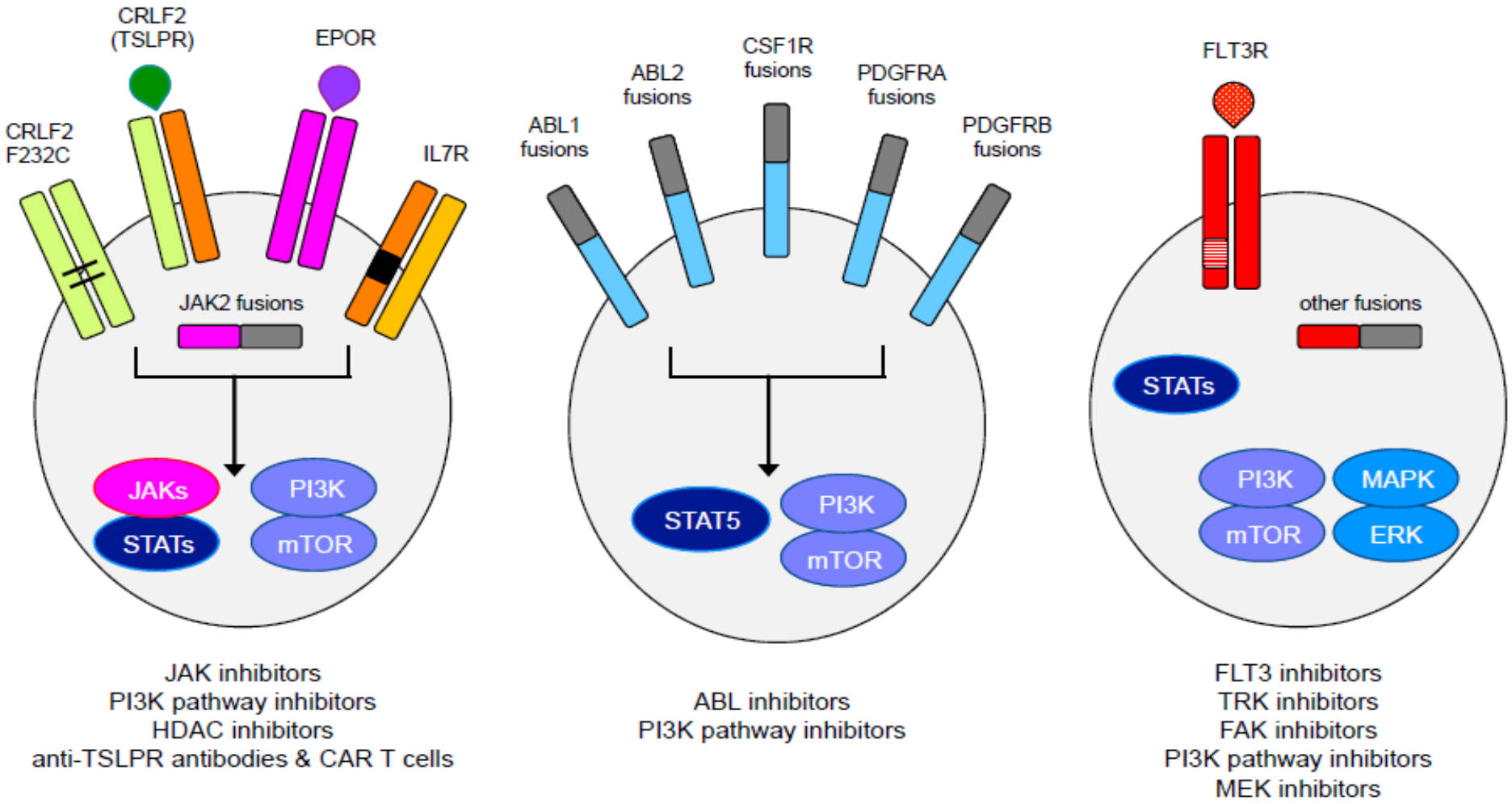






TSLP (MRD) Main tube

Ph-like B-ALL



JAK inhibitors
PI3K pathway inhibitors
HDAC inhibitors
anti-TSLPR antibodies & CAR T cells

ABL inhibitors
PI3K pathway inhibitors

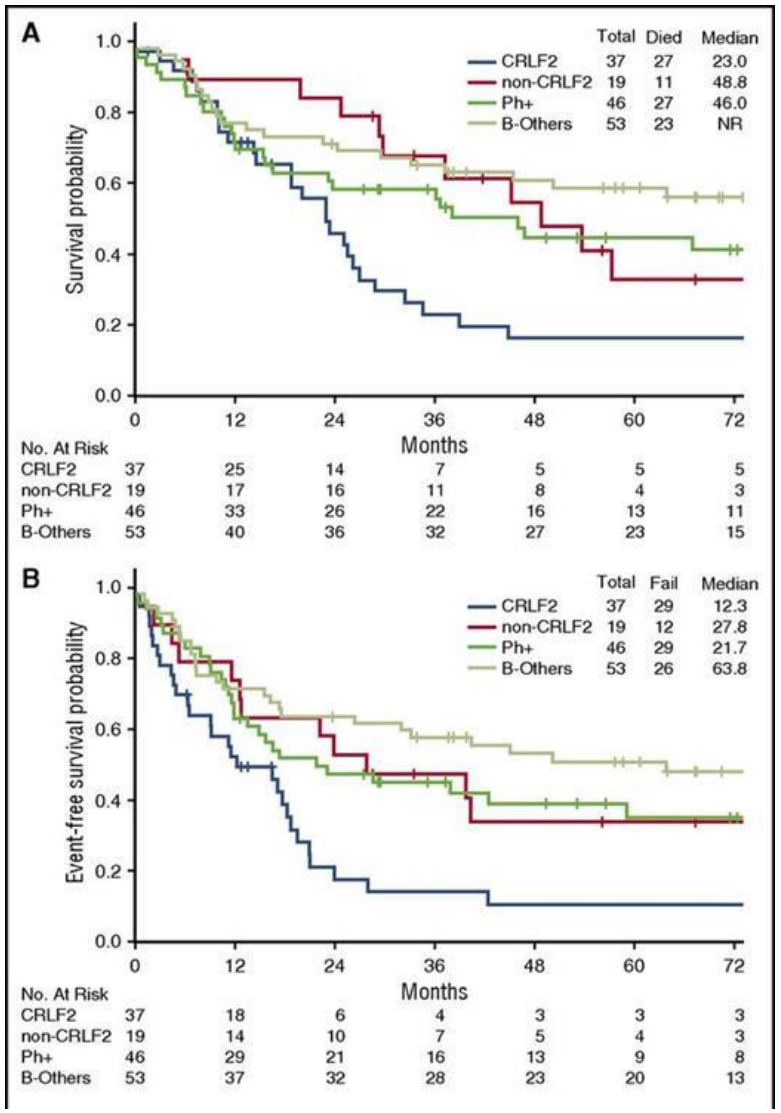
FLT3 inhibitors
TRK inhibitors
FAK inhibitors
PI3K pathway inhibitors
MEK inhibitors

N Engl J Med 2014;371:1005-15.

Currently available modalities to diagnose Ph-like B-ALL

- Flow Cytometry – CRLF2 (TSLP-R) expression
- Easiest and fastest
- 50-60% cases

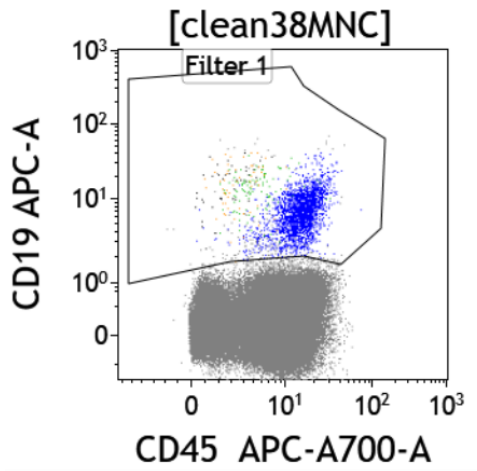
- Molecular targeted approaches or comprehensive sequencing

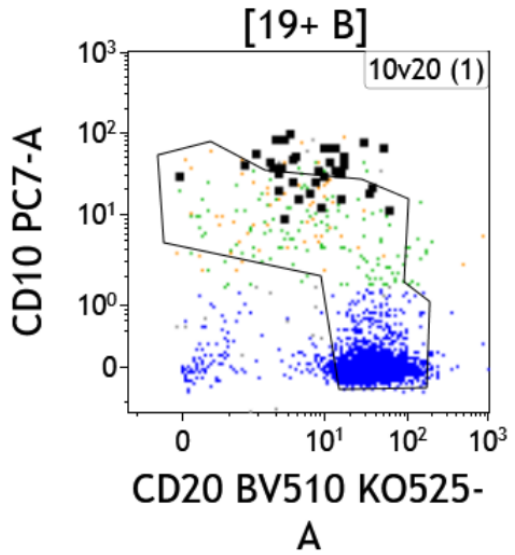


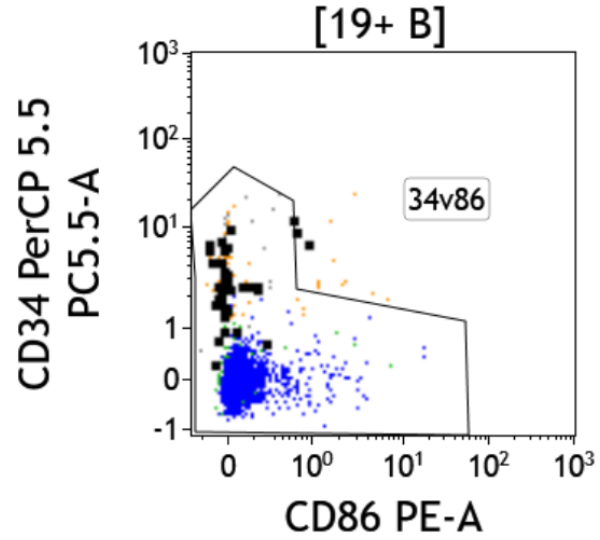
Jain et al. Blood (2017) 129 (5): 572–581.

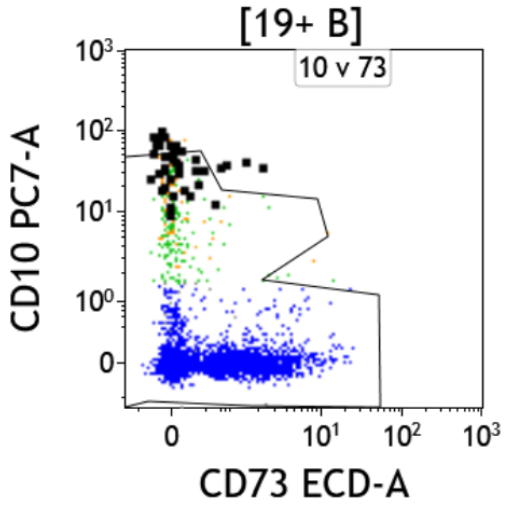
Case 9

- 48 Y/M
- Presented with huge splenomegaly and high TLC
- Hb 7.8 TLC 125 Plt 350
- Pb Bl 03 PM 10 My 25 MM 20 Eo 01 N 35 L 02 Ba 03
- BMA
- Diagnosed as CML CP


[clean38MNC]
Filter 1
CD19 APC-A
CD45 APC-A700-A


[19+ B]
10v20 (1)
CD10 PC7-A
CD20 BV510 KO525-A


[19+ B]
34v86
CD34 PerCP 5.5 PC5.5-A
CD86 PE-A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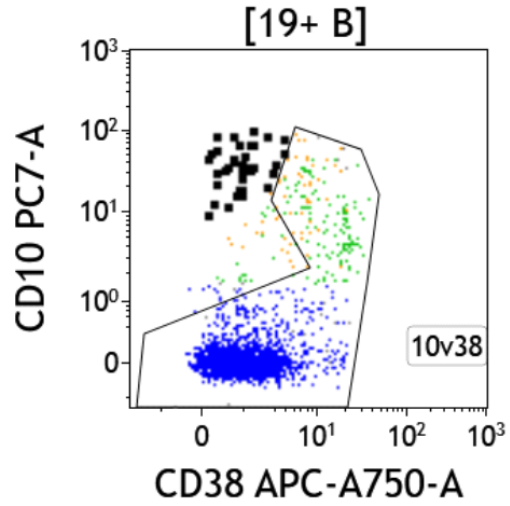
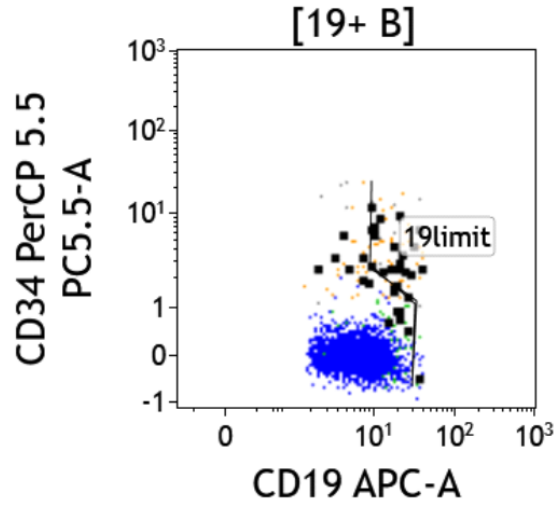
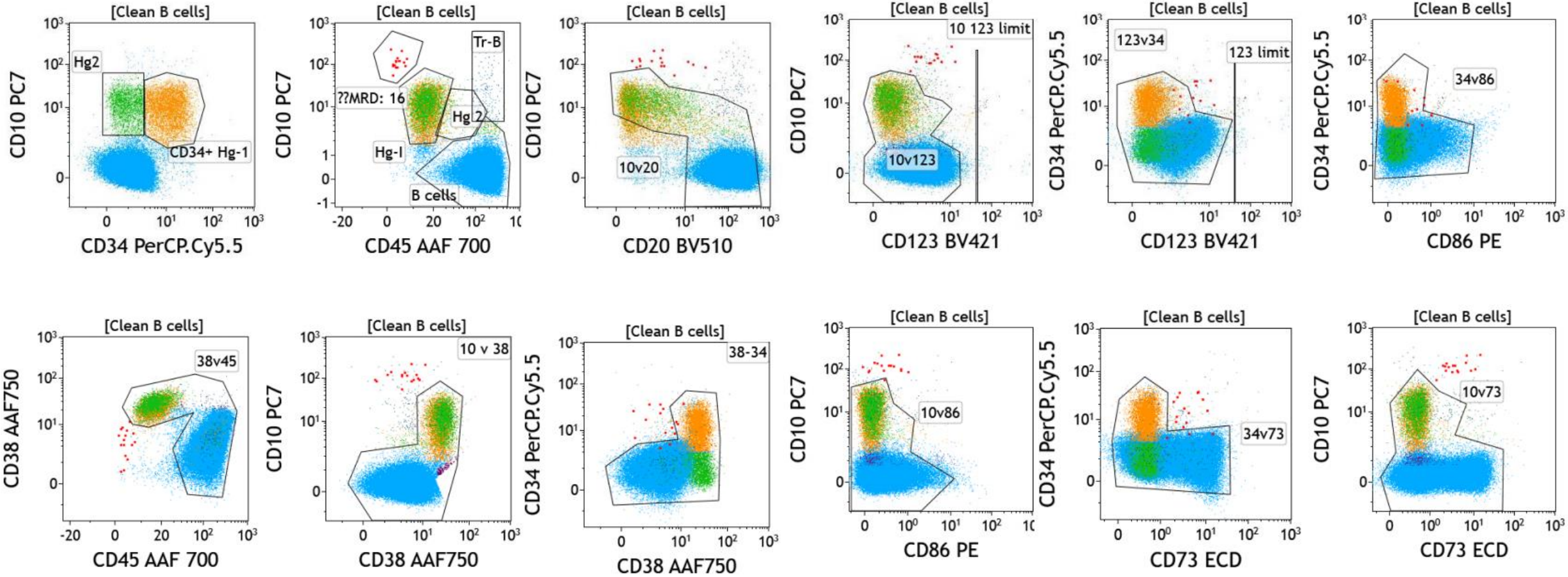
10 v 73
CD10 PC7-A
CD73 ECD-A

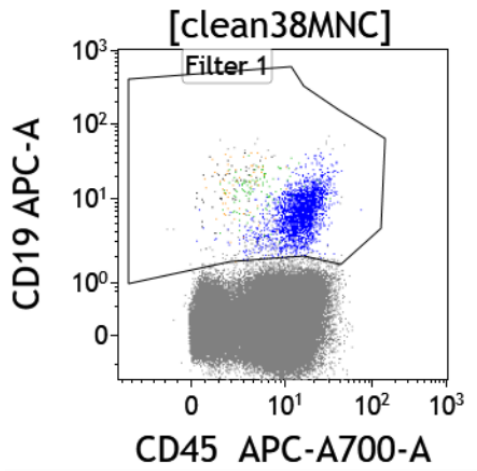

[19+ B]
10v38
CD10 PC7-A
CD38 APC-A750-A

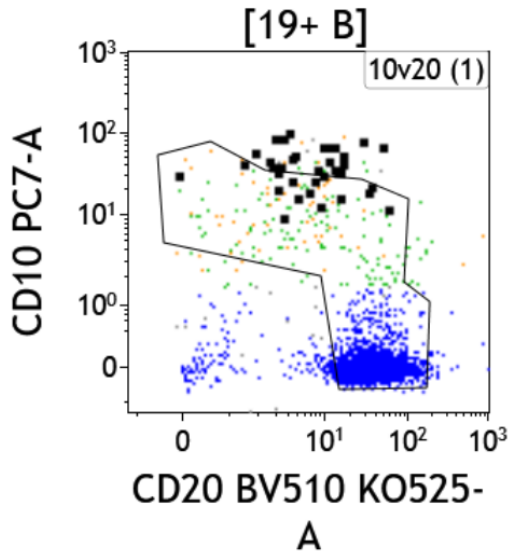

[19+ B]
19limit
CD34 PerCP 5.5 PC5.5-A
CD19 APC-A

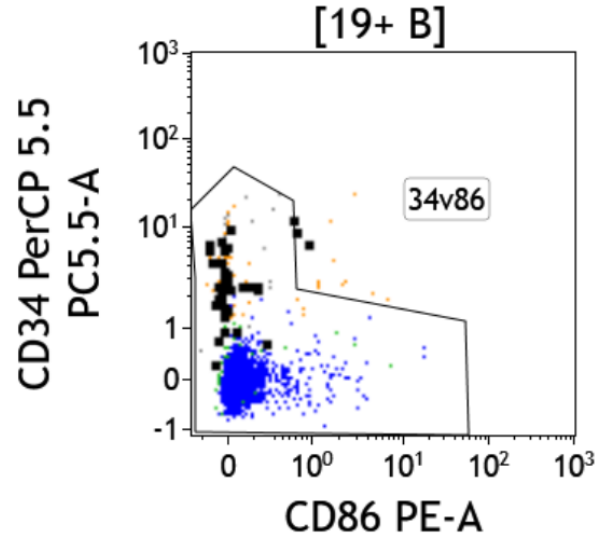
Are these cells normal?

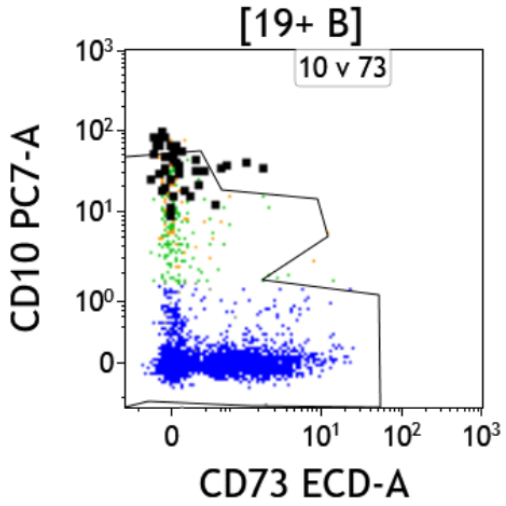


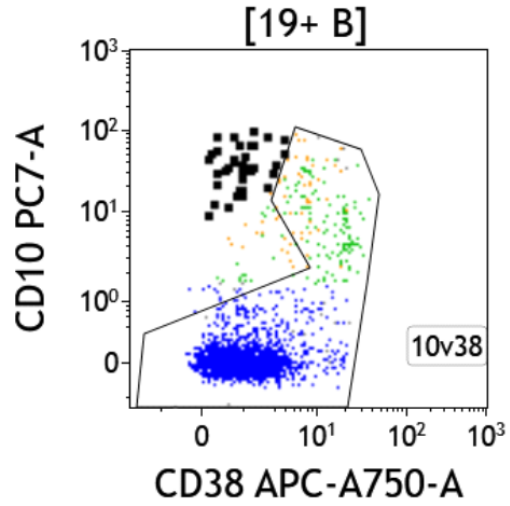
Tembhare et al. Cytometry Part B 2019;00B: 1-11

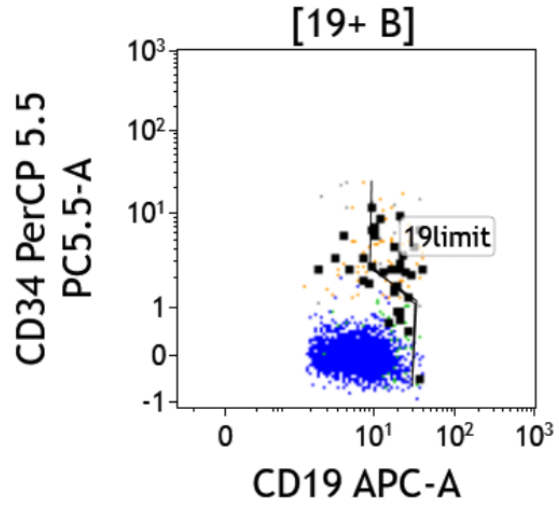

[clean38MNC]
Filter 1
CD19 APC-A
CD45 APC-A700-A


[19+ B]
10v20 (1)
CD10 PC7-A
CD20 BV510 KO525-A

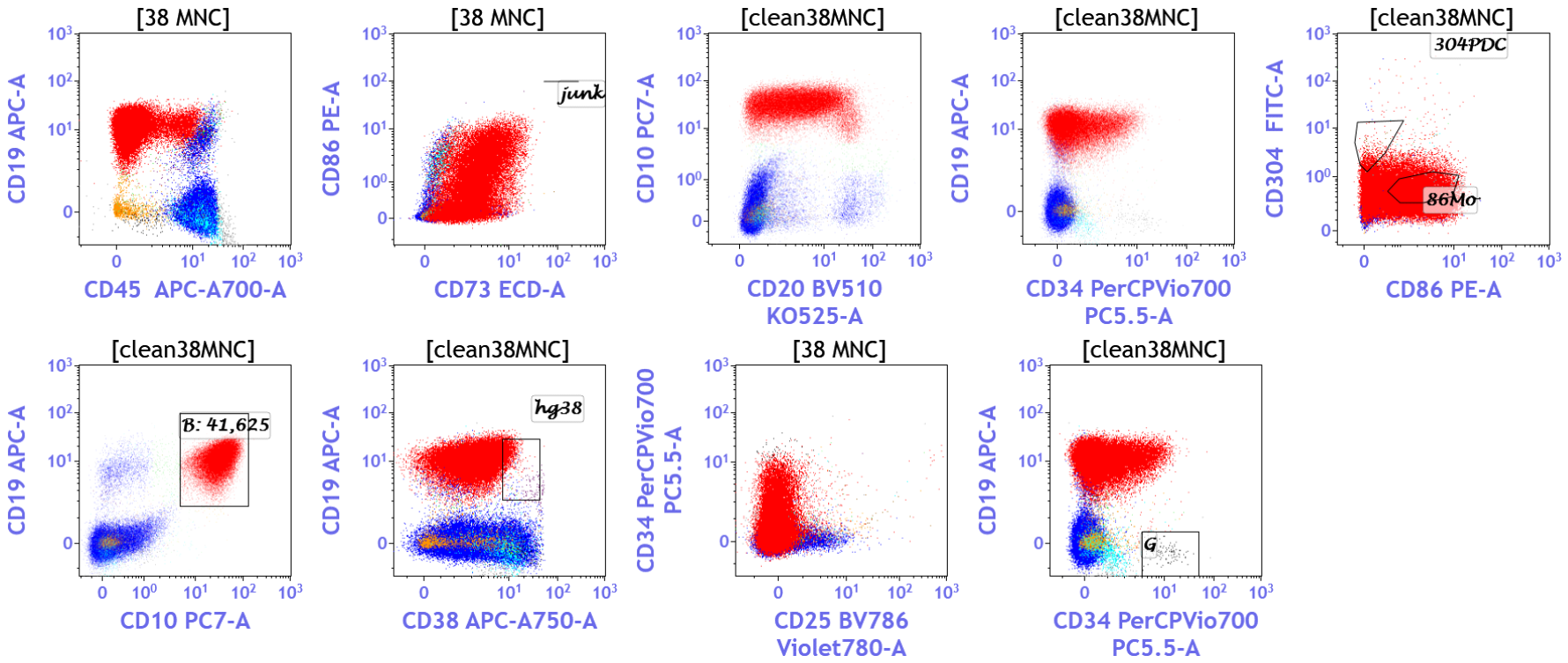

[19+ B]
34v86
CD34 PerCP 5.5 PC5.5-A
CD86 PE-A


[19+ B]
10 v 73
CD10 PC7-A
CD73 ECD-A


[19+ B]
10v38
CD10 PC7-A
CD38 APC-A750-A


[19+ B]
19limit
CD34 PerCP 5.5 PC5.5-A
CD19 APC-A

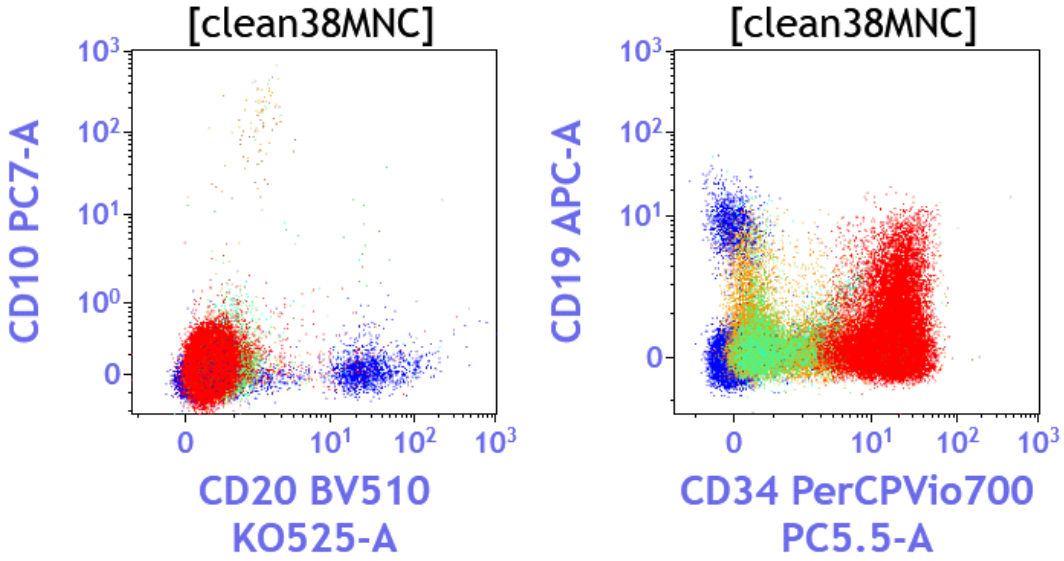
3 months later, presented with B lymphoid blast crisis

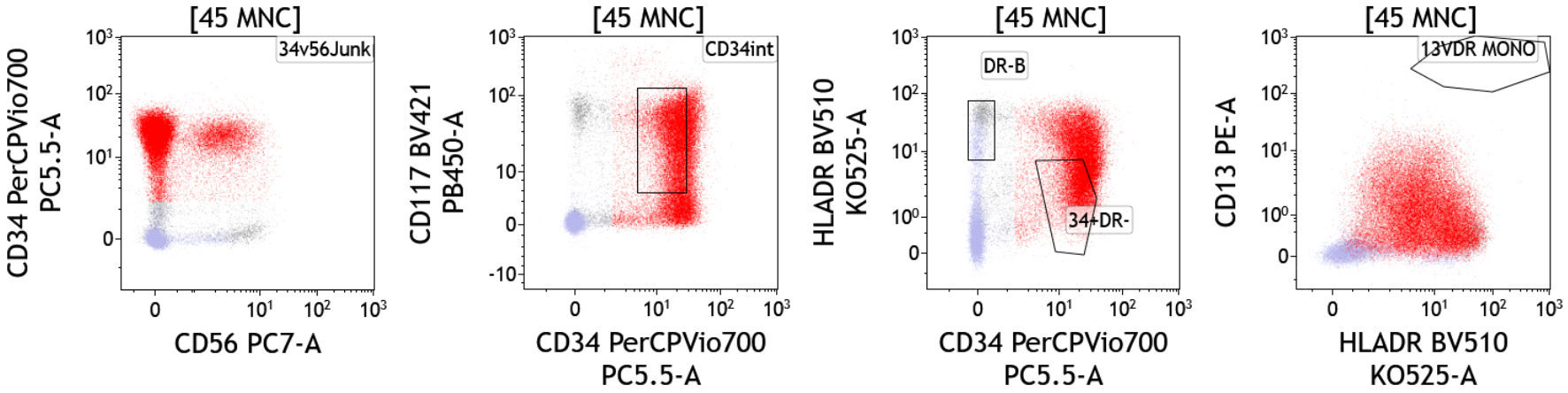


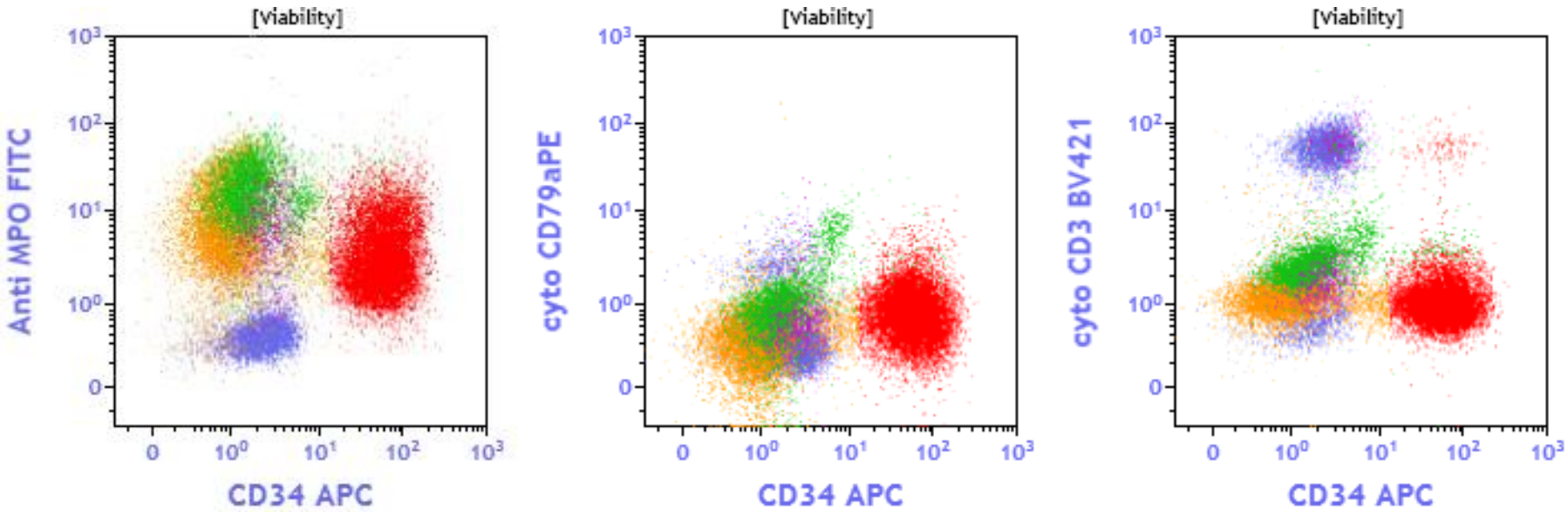
Vijayasekharan et al. Cytometry Part B DOI: 10.1002/cyto.b.21958

Case 10

- 12 yrs Male
- Presented with fever and high TLC
- Hb 8.5 TLC 35 Plt 80
- BMA

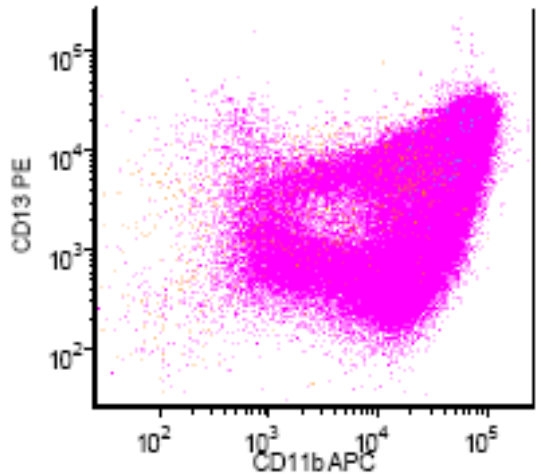


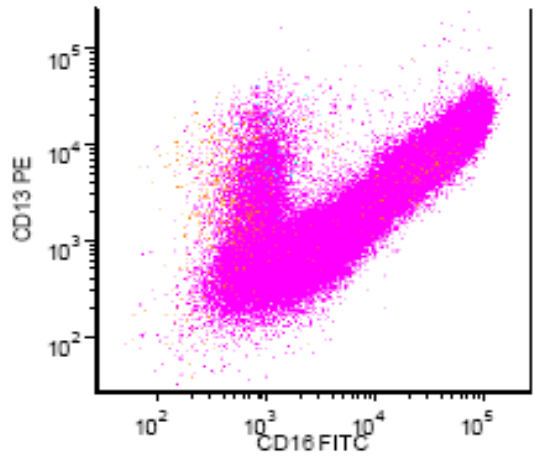




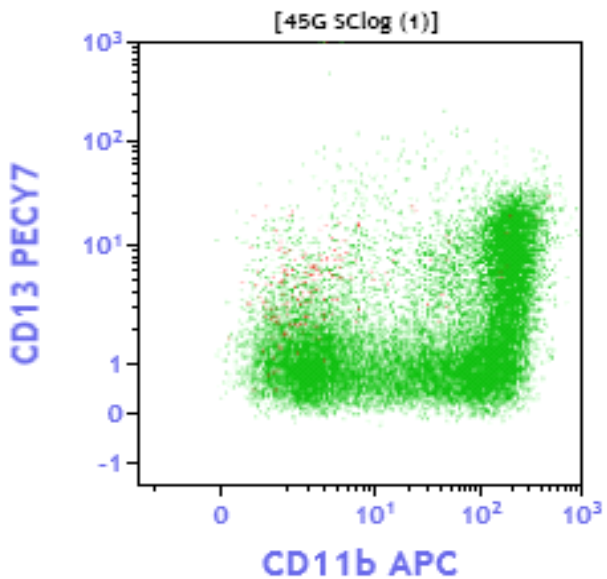
- AML, RUNX1-RUNX1T1 rearranged

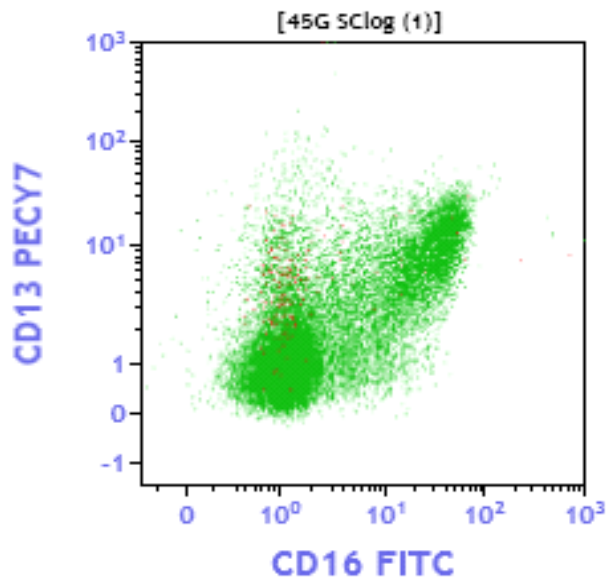
Normal Maturation Pattern

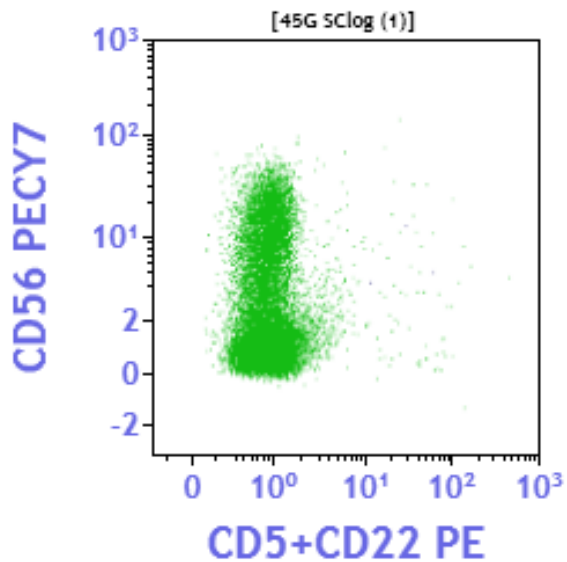


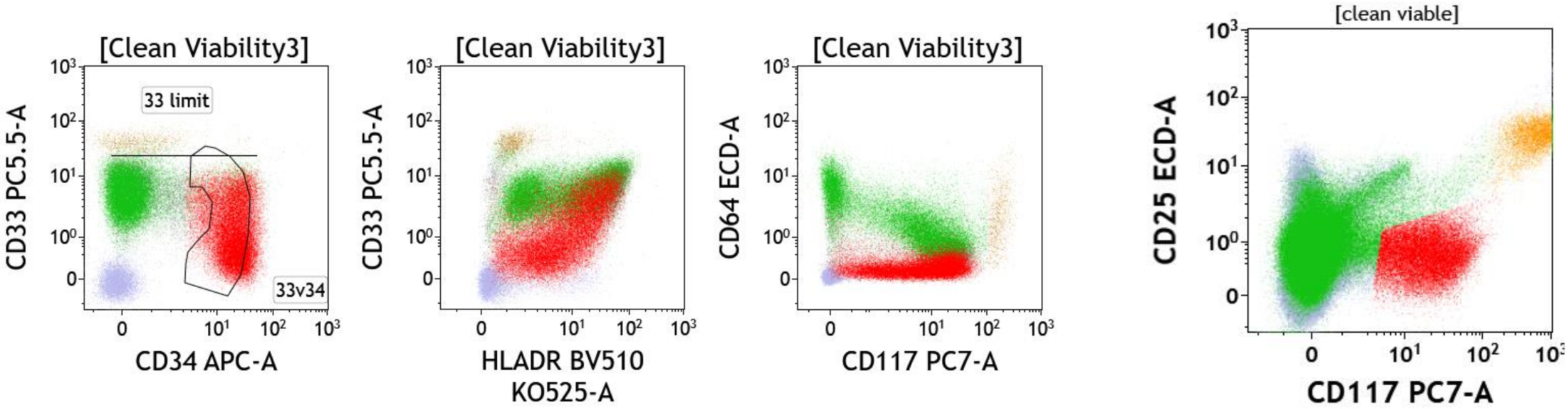


Our Case







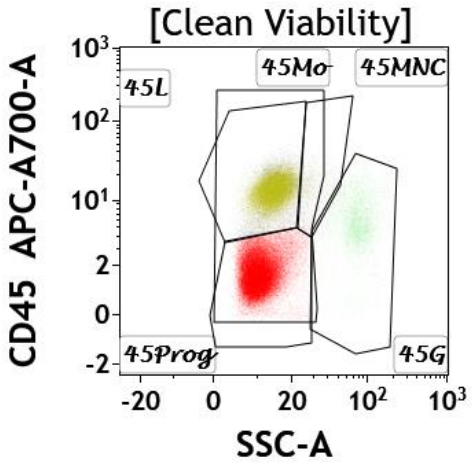


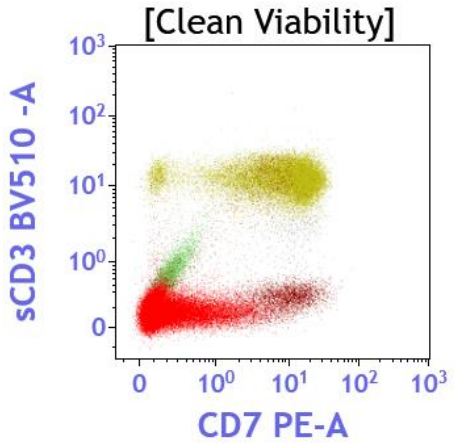
- **AML, abnormal myeloid blasts and granulocyte maturation, CD25+ ?clonal mast cells**
- **RUNX1-RUNX1T1 rearranged, KITD816V mutated**

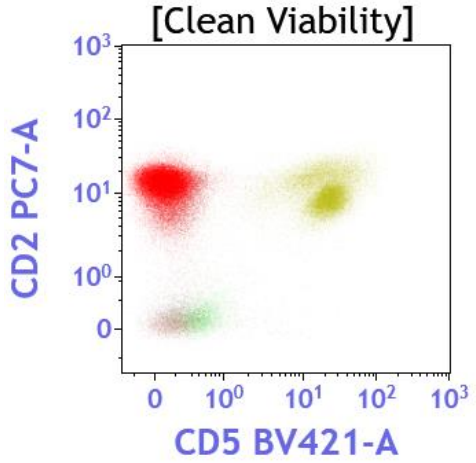
- **Learning points:**
- **Look for aberrancies in otherwise well-conserved maturation pattern**
- **Single cell nature of analysis – evaluate all the cells**

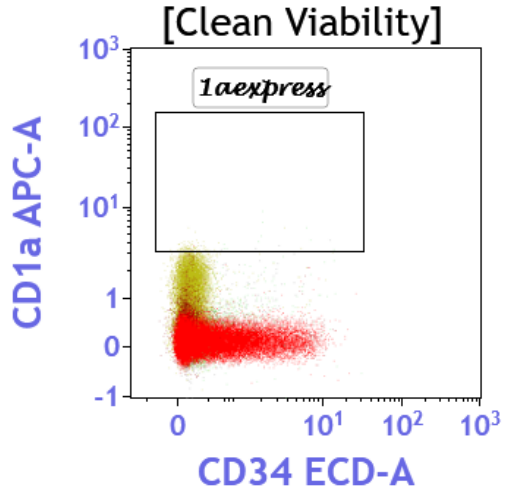
Case 11

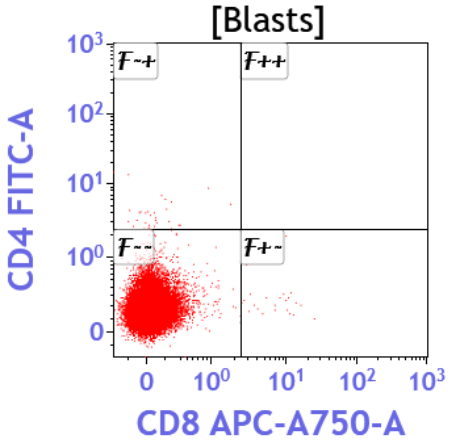
32Y/F

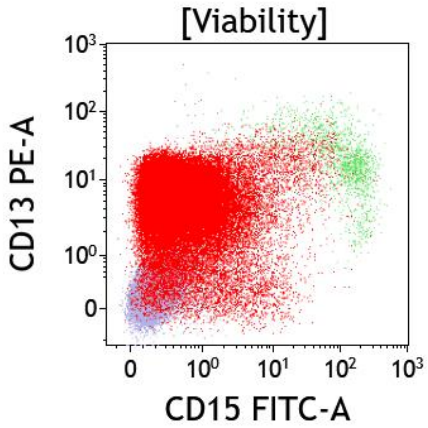


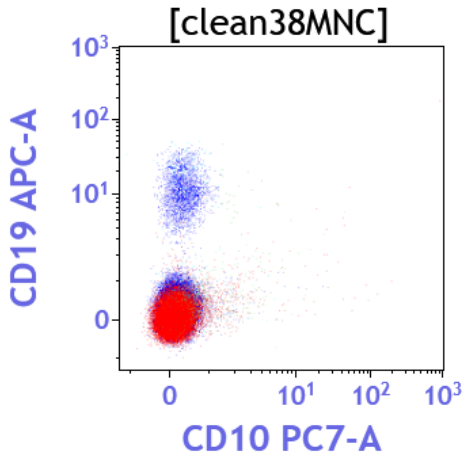


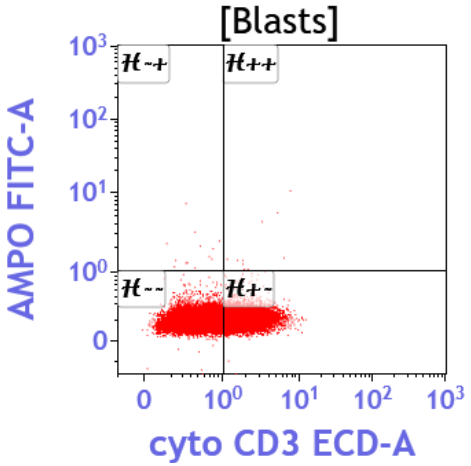












ETPALL

Received ALL-like induction - MRD negative

Matched-sibling ASCT in Jan 2014

Relapse after 21 months

Salvage induction-no morphological remission

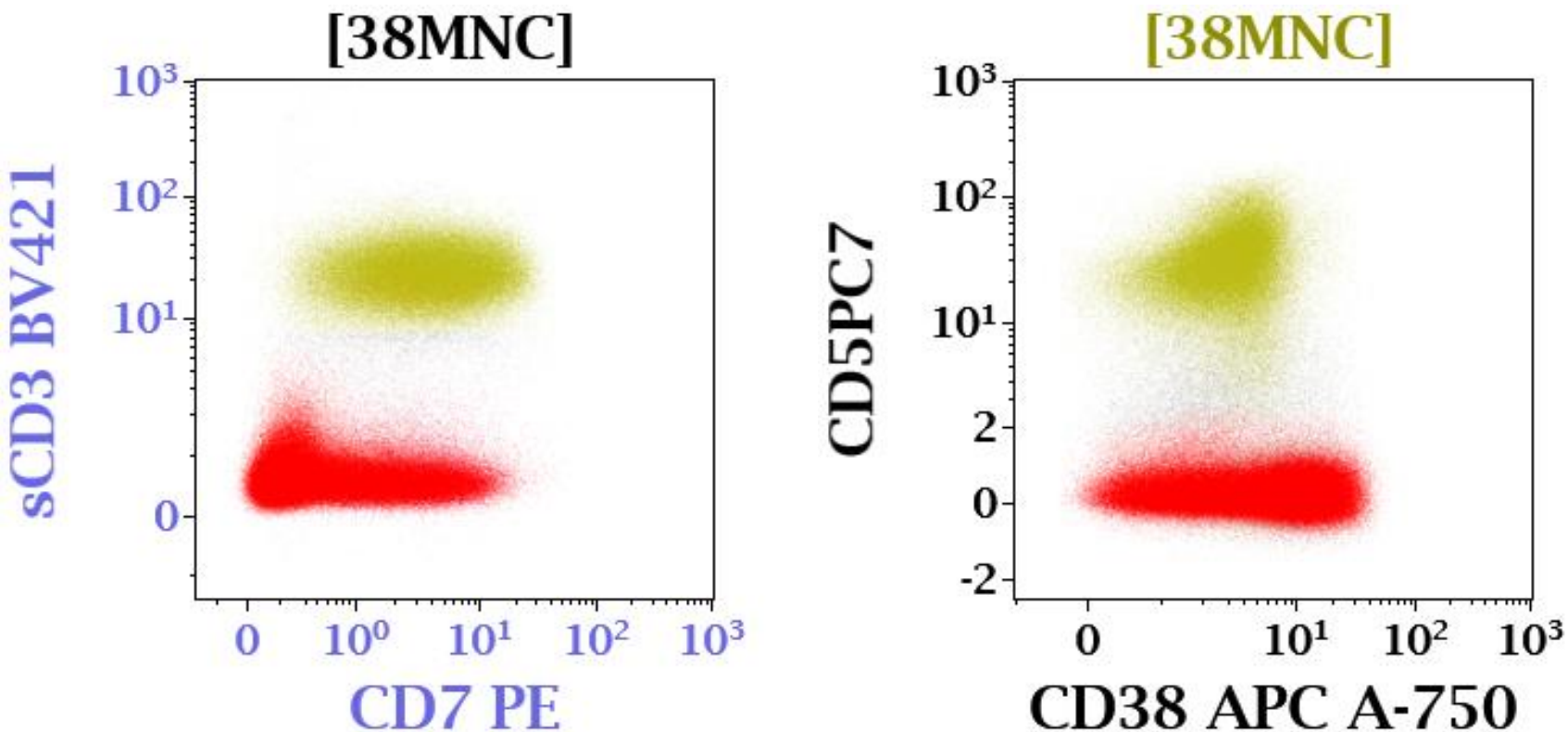
FLAG IDA – morphological remission but 0.17% MRD

Second ASCT in Feb 2016

Received prophylactic Donor-lymphocyte infusion – developed GVHD

Relapse again after 18 months

Salvage chemotherapy – 16.5% MRD


[38MNC]
sCD3 BV421
CD7 PE
10^3
10^2
10^1
0
10^0
10^1
10^2
10^3
[38MNC]
CD5PC7
CD38 APC A-750
10^3
10^2
10^1
2
0
-2
0
10^1
10^2
10^3

Received ALL-like induction - MRD negative

Matched-sibling ASCT in Jan 2014

Relapse after 21 months

Salvage induction-no morphological remission

FLAG IDA – morphological remission but 0.17% MRD

Second ASCT in Feb 2016

Received prophylactic DLI – developed GVHD

Relapse again after 18 months

Salvage chemotherapy – 16.5% MRD

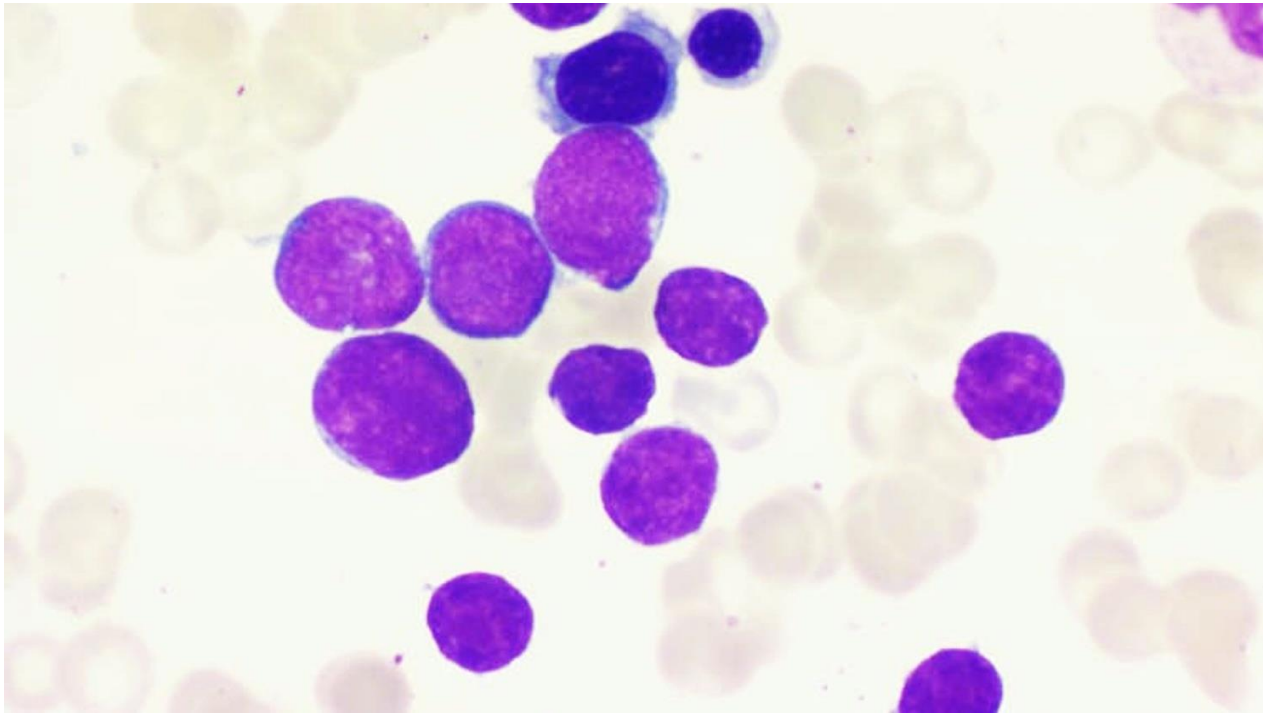
MRD negative after 3rd and 8th dose

Is in continuing remission at 2021

CASE 12

72 yr male C/o fatigue and loss of appetite for 2-3 months,

fever x 2 months

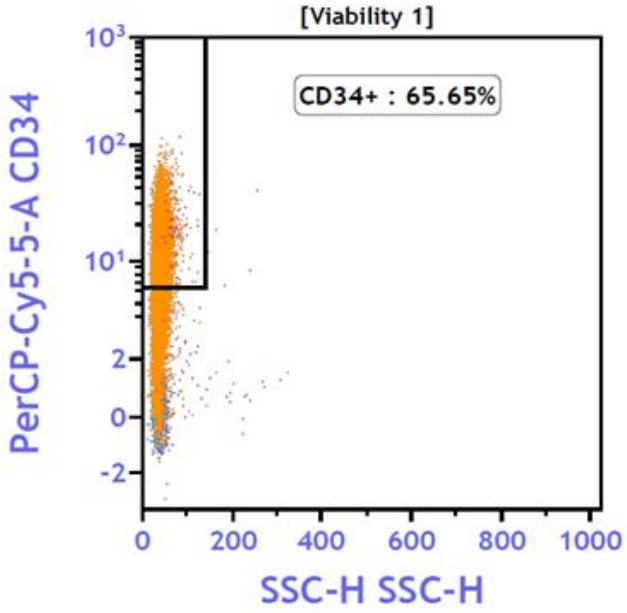


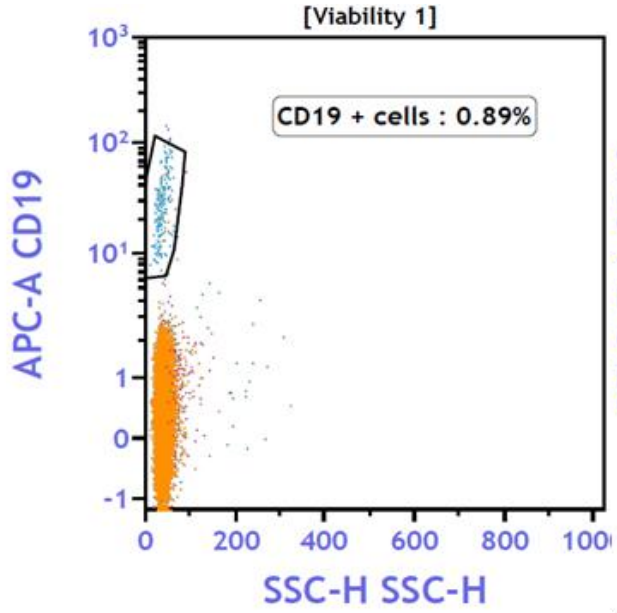
CBC: Hb- 9.9 g/dL

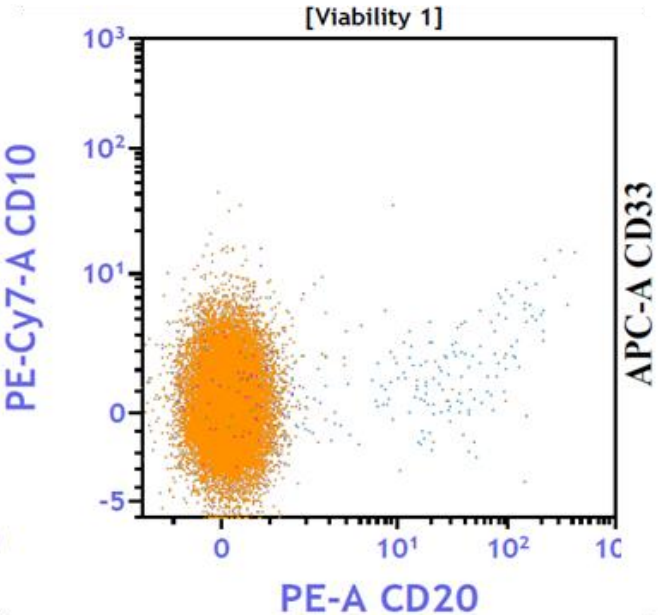
TLC - 13 x 10^9/L

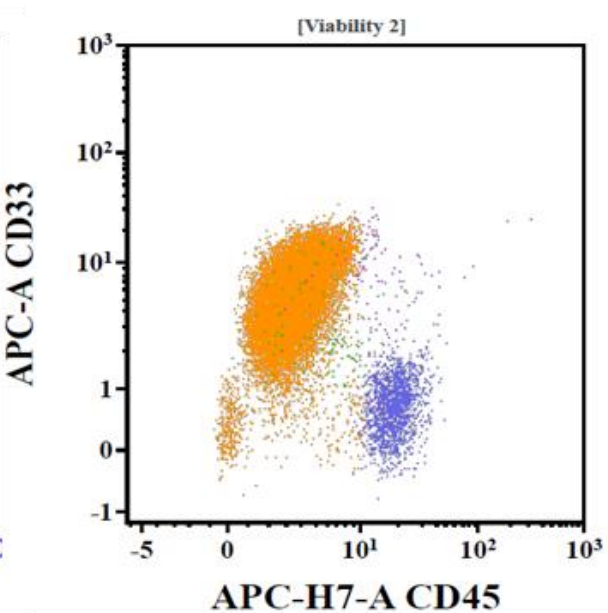
Plt - 33 x 10^9/L

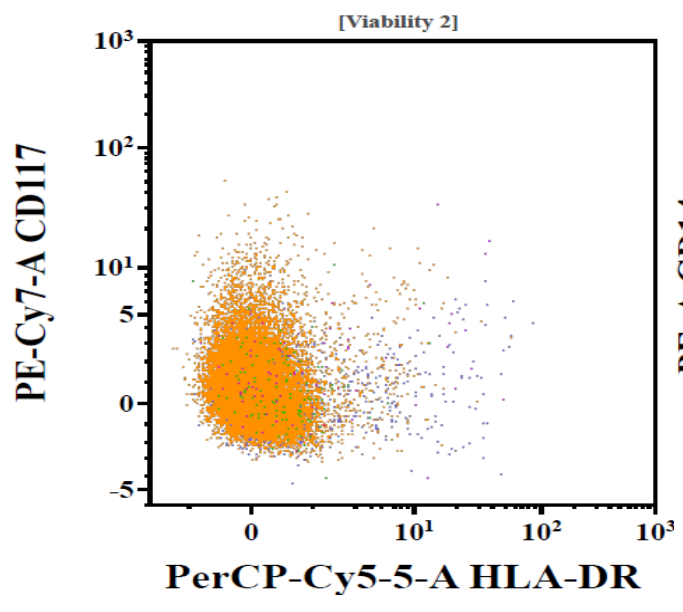
D/C: Blasts- 90%, PMN- 2% and Lym- 8%

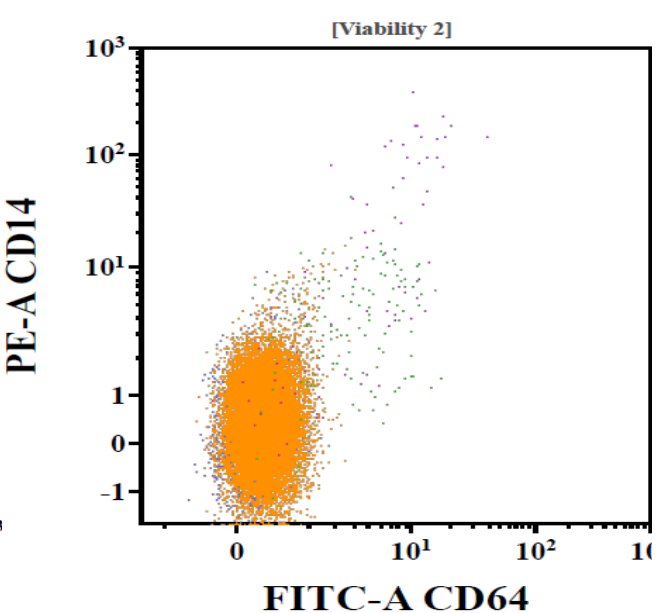

[Viability 1]
CD34+ : 65.65%
PerCP-Cy5-5-A CD34
SSC-H SSC-H

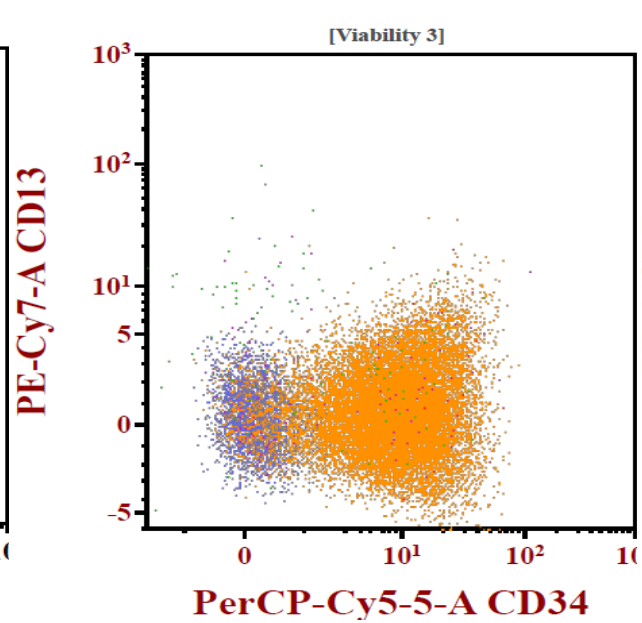

[Viability 1]
CD19 + cells : 0.89%
APC-A CD19
SSC-H SSC-H

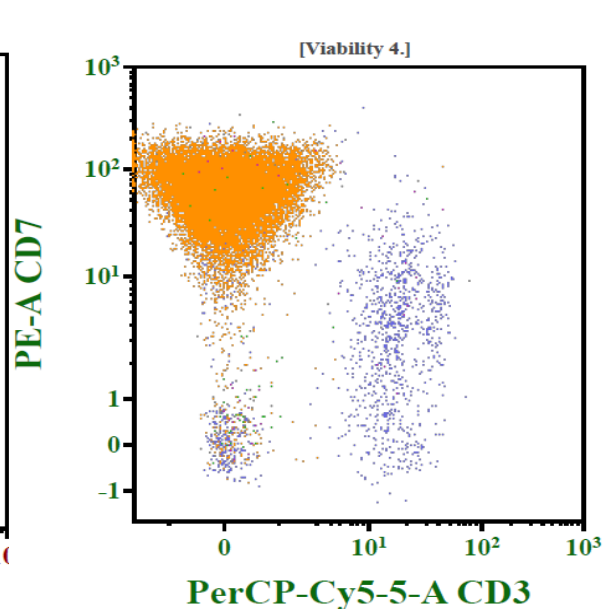

[Viability 1]
PE-Cy7-A CD10
PE-A CD20

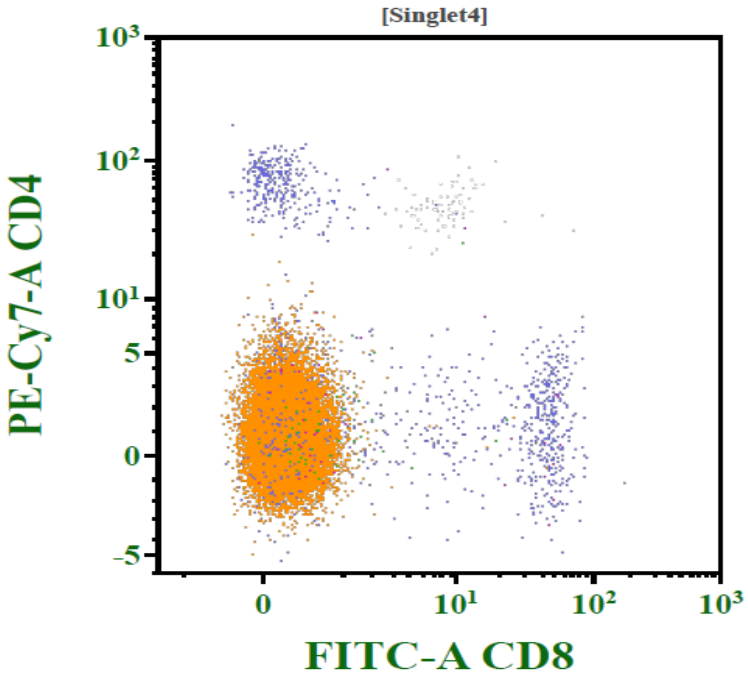

[Viability 2]
APC-A CD33
APC-H7-A CD45

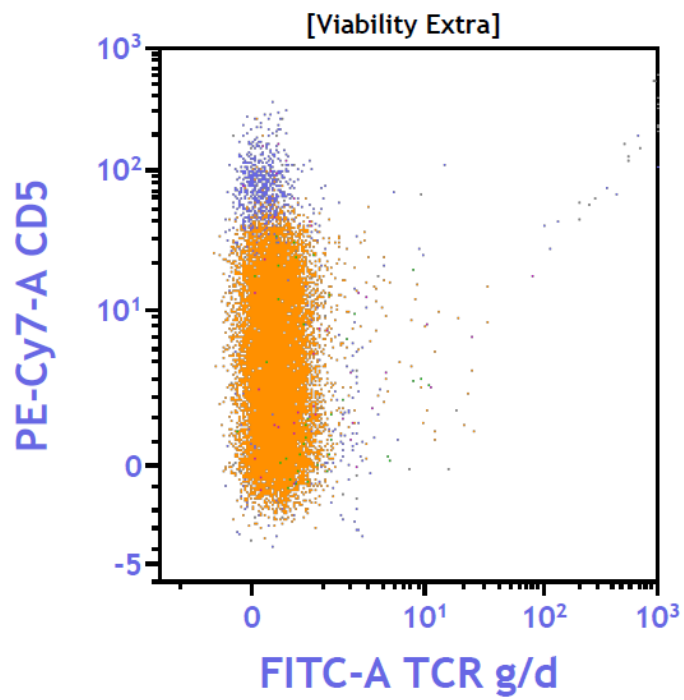

[Viability 2]
PE-Cy7-A CD117
PerCP-Cy5-5-A HLA-DR

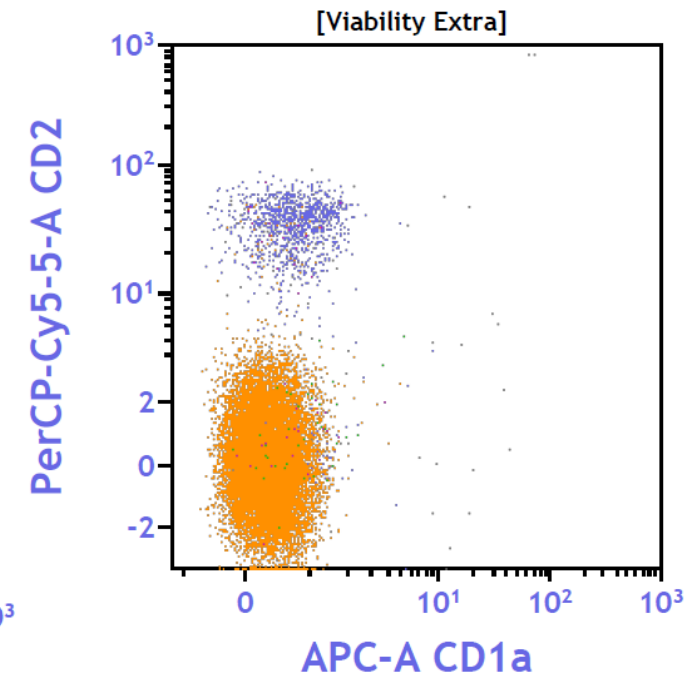

[Viability 2]
PE-A CD14
FITC-A CD64

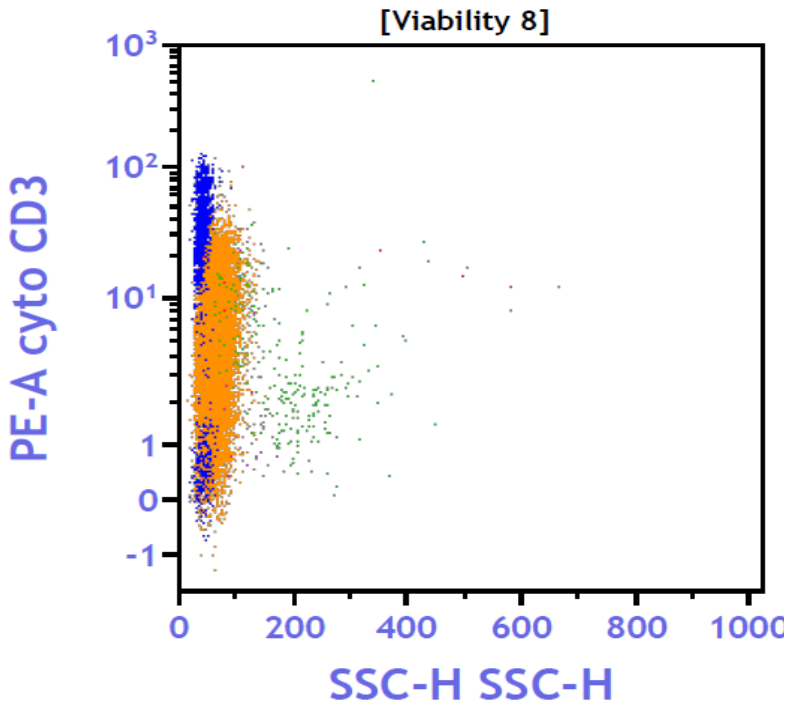

[Viability 3]
PE-Cy7-A CD13
PerCP-Cy5-5-A CD34

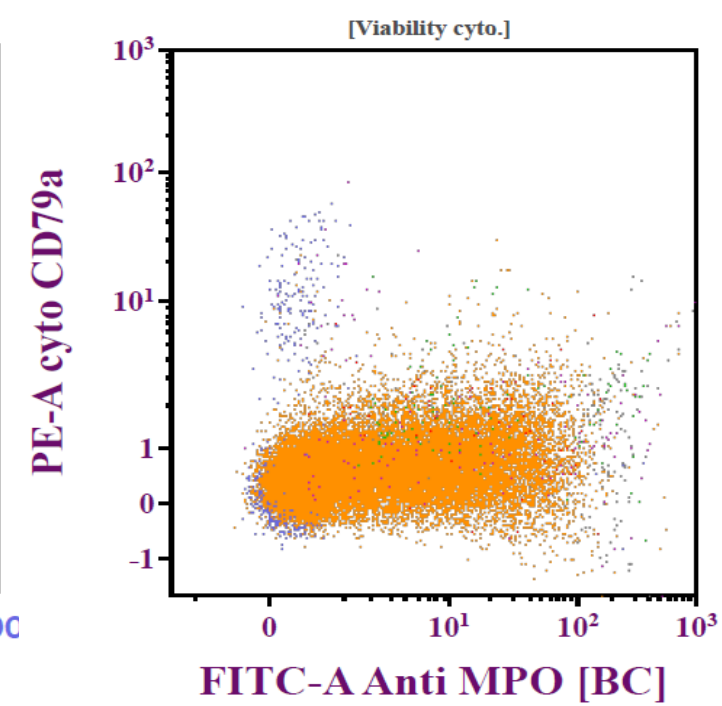

[Viability 4.]
PE-A CD7
PerCP-Cy5-5-A CD3

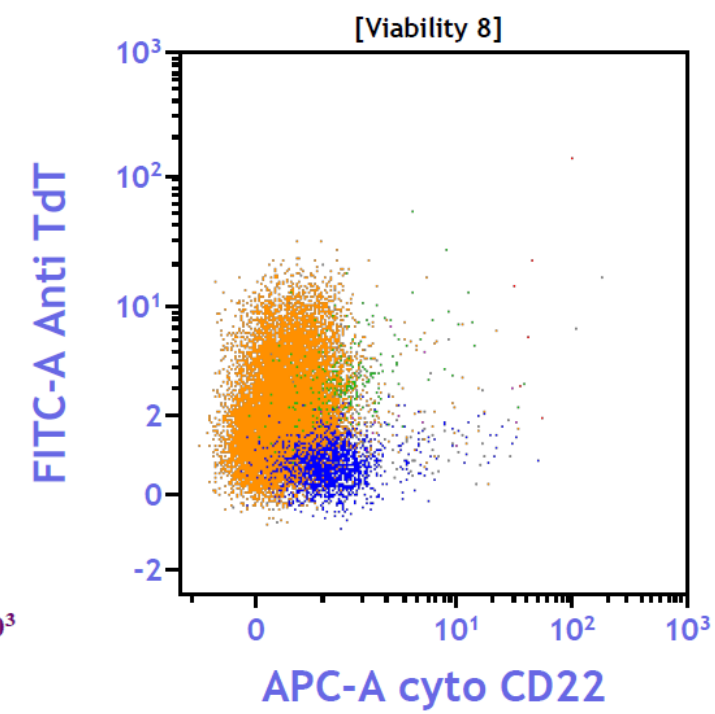

[Singlet4]
PE-Cy7-A CD4
FITC-A CD8


[Viability Extra]
PE-Cy7-A CD5
FITC-A TCR g/d

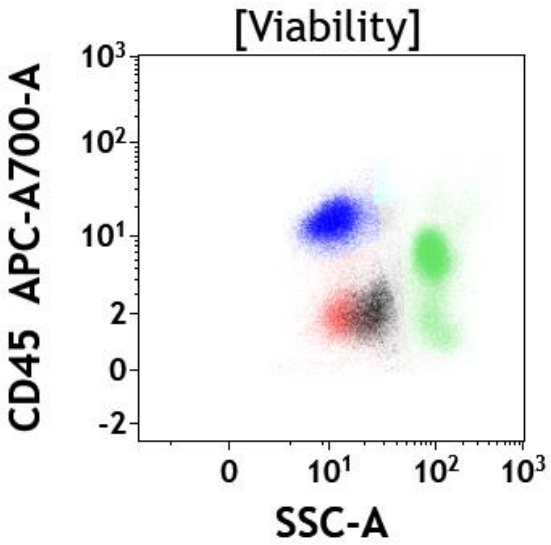

[Viability Extra]
PerCP-Cy5-5-A CD2
APC-A CD1a

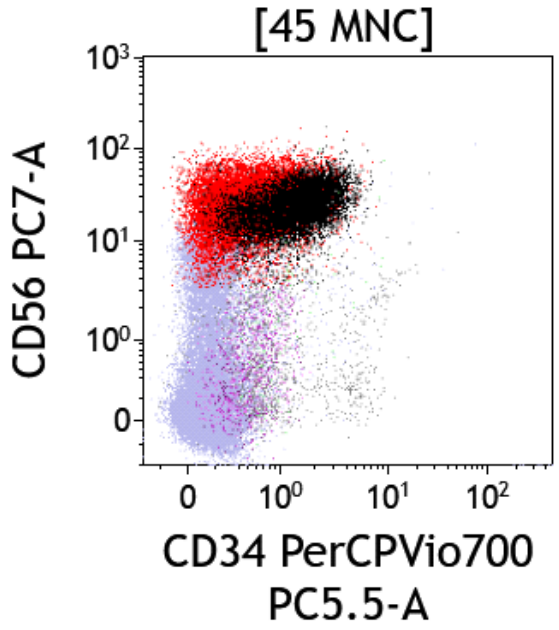

[Viability 8]
PE-A cyto CD3
SSC-H SSC-H

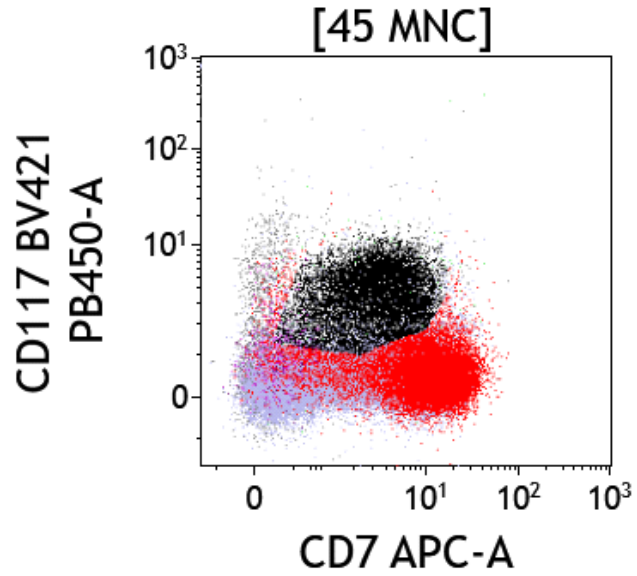

[Viability cyto.]
PE-A cyto CD79a
FITC-A Anti MPO [BC]

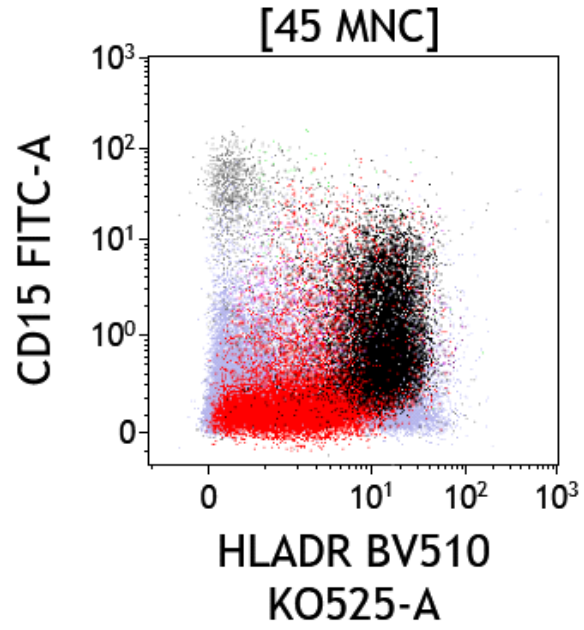

[Viability 8]
FITC-A Anti TdT
APC-A cyto CD22

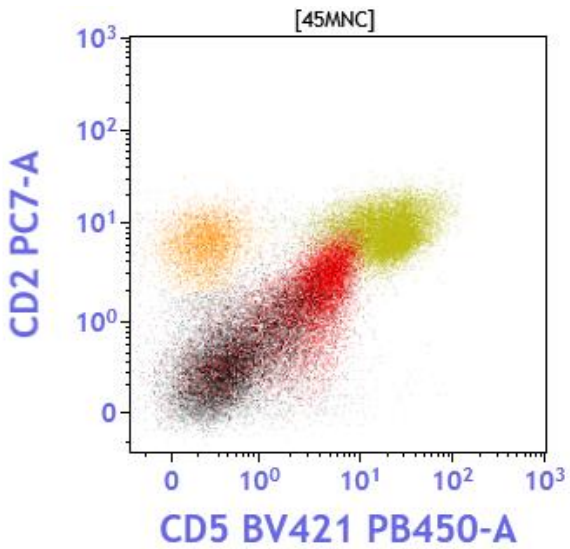
Mixed Phenotypic Acute Leukemia (Biphenotypic-T/Myeloid)

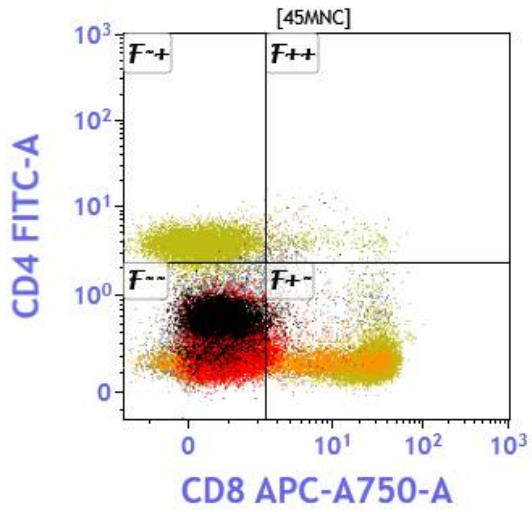

[Viability]
CD45 APC-A700-A
SSC-A

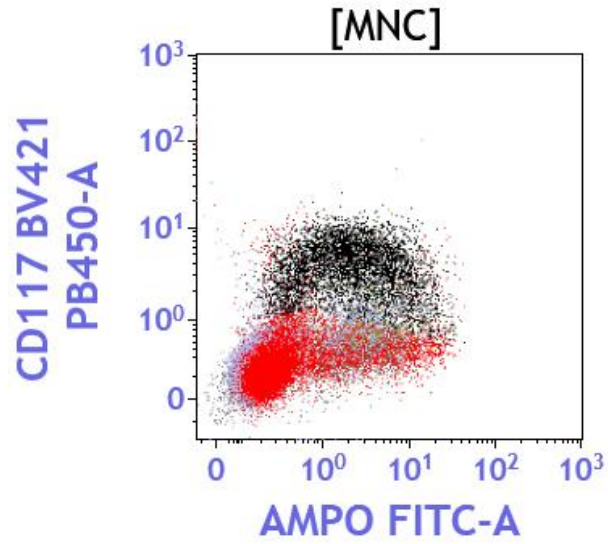

[45 MNC]
CD56 PC7-A
CD34 PerCPVio700
PC5.5-A

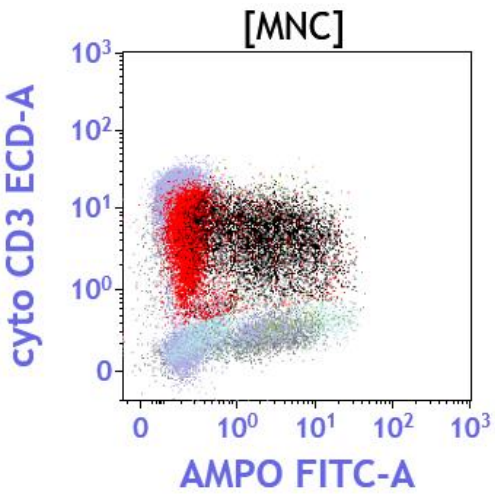

[45 MNC]
CD117 BV421
PB450-A
CD7 APC-A


[45 MNC]
CD15 FITC-A
HLADR BV510
KO525-A


[45MNC]
CD2 PC7-A
CD5 BV421 PB450-A


[45MNC]
F-+
F++
F--
F+-
CD4 FITC-A
CD8 APC-A750-A


[MNC]
CD117 BV421
PB450-A
AMPO FITC-A


[MNC]
cyto CD3 ECD-A
AMPO FITC-A

Bilineal MPAL (T/Myeloid)

- **Learning points:**
- **Acute leukemia of ambiguous lineage and MPAL are heterogenous neoplasms.**
- **May show combination of complex phenotypes.**
- **Detailed description of all the immunophenotypically discernible clones**

Sample TMH report

FINAL BONE MARROW REPORT

MORPHOLOGY

Cellularity Normocellular

Erythroid Series

Cellularity Reduced

Erythroid Cells 04

Megakaryocytic Cells

Cellularity Reduced

Myeloid Series

Promyelocytes	00
Myelocytes	12
Metamyelocytes	03
Polymorphs	06
Basophils	00
Eosinophils	12
Monocytes	04
Promonocytes	00

Lymphoid Series

Mature Lymphocytes 05

Plasma Cells

Mature 01

Abnormal Cells 53 % Blasts.

Descriptive Morphology Auer rods seen. Dysgranulopoiesis is noted.
Myeloid series cells show salmon pink cytoplasm.

CYTOCHEMISTRY

MPO Positive

IMMUNOPHENOTYPIC PROFILE

Immunophenotyping analysis results are as follows : Kindly note that the below results were determined on 73.4 of the gated event on Side scatter/CD45 plots

CD34	Bright	**CD45**	Dim-Neg	**CD38**	Moderate
HLA-DR	Moderate	**CD25**	Subset	**CD10**	Negative
CD20	Negative	**CD19**	Dim-Neg	**CD22**	Negative
CD73	Negative	**CD86**	Dim-Neg	**CD123**	Variable
CD1a	Negative	**CD2**	Negative	**CD4**	Negative
CD7	Negative	**CD8**	Negative	**CD16**	Negative
CD56	Moderate	**sCD3**	Negative	**CD11b**	Negative
CD13	Variable	**CD15**	Variable	**CD33**	Moderate
CD117	Variable	**CD36**	Negative	**CD64**	Dim-Neg
CD14	Negative	**CD11c**	Negative	**CD42b**	Negative
cyCD3	Negative	**AMPO**	Positive	**cyCD79a**	Negative
CD5	Dim-Neg	**TCR-GD**	Negative		

Instrument	BC Cytoflex	**Cell Preparation Method**	Bulk-Lyse-Stain-Wash
Software	Kaluza(BC)	**Acquisition Technologist**	MR. SITARAM GHOGALE

Sample TMH report

FINAL BONE MARROW REPORT

Impression Morphologic & immunophenotypic findings are consistent with acute myeloid leukemia.
Advise: Cytogenetic and molecular genetic studies including RUNX1-RUNX1T1 and KIT mutation.

Comments Independent immunophenotypic analysis revealed 73.4% abnormal myeloid blasts expressing above mentioned immunophenotype.
Granulocytes gated at 2.9% of all viable cells have low light side scatter characteristics, show asynchronous maturation pattern with respect to CD13 vs CD11b vs CD16, and overexpression of CD56.

Req Dt/Time	15-04-2021	/	1:13:04PM
Coll. Dt/Time :	15-04-2021	/	4:15:47PM
Recd. Dt/Time :	15-04-2021	/	7:51:59PM
Prelim Dt/Tm :	22-04-2021	/	12:11:19PM
Commit Dt/Time	22-04-2021	/	12:31:41PM

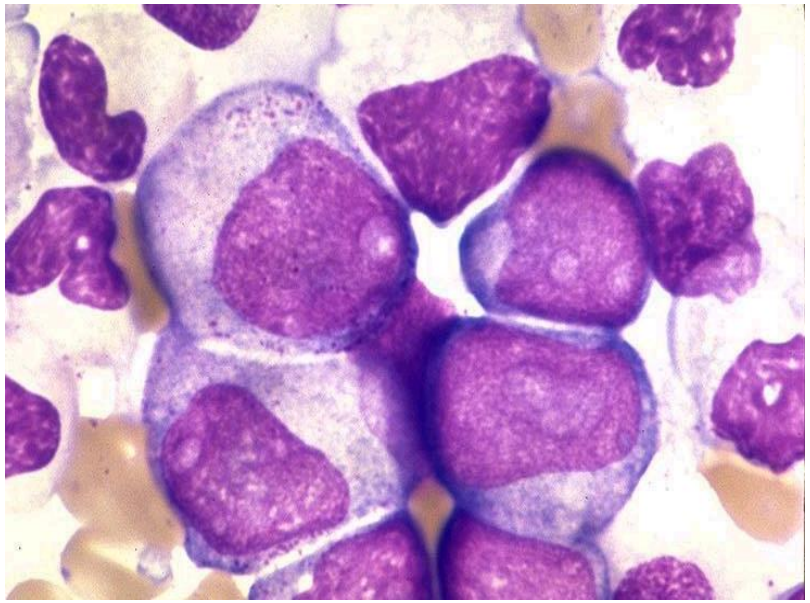
Entered By
BHAGYASHRI PRASAD VEDPATHAK

Prepared By
DR. ANU SINGH

Finalized By
DR. GAURAV CHATTERJEE
ASSISTANT PROFESSOR,
HAEMATOPATHOLOGY

Role of FCM in AL

- **Confirmation** of presence of **abnormal** blasts
- Accurate **quantification** of blasts
- **Lineage determination** and **characterization** of abnormal blasts.



- FCM can provide reliable **prognostic information**
- Typical FCM marker expression can reliably **predict disease genotype**.
- FCM evaluation of biomarkers can predict **response to targeted therapy**
- FCM based **pattern-analysis** identifies aberrant maturation

- FCM is a practically useful, **highly sensitive** technique to **monitor treatment response** and quantitate measurable residual disease (MRD).

Thank you!

- Prof Sumeet Gujral
- Prof P. G. Subramanian
- Prof Nikhil Patkar
- Prof Prashant Tembhare
- Dr Sweta Rajpal

- And team!!