HIP-C Protocol whole blood

Version 3.3

- Added CD8a BV605 in the DC/mono/NK Panel

Equipment

12x75mm tubes
Pipettes 10-1000 µl
Sterile tips
Vortex
Pipetboy
25ml disposable pipettes
Flow cytometer with 405nm; 488nm; 640nm, 561nm

Reagents

Conjugated Antibodies
Phosphate buffered saline (PBS)
10x BD FACS Lysing Solution Cat#349202
FACS-buffer: 12,5x Stock: 10g NaN_3, 100ml 0,5M EDTA; 3,36g NaHCO3; 200ml H_2O; 100ml FCS; 500ml 20xPBS
BD Comp Beads Anti Mouse Ig Cat#51-90-9001229 / Cat#51-90-9001291
BD Multitest 6-Color TBNK Reagent incl. Trucount Tubes Cat#644611

Preparations

Dilute 10x BD FACS Lysing Solution 1:10 with H_2O
Dilute 12,5x FACS-buffer Stock 1:12,5 with H_2O
Prepare the antibody cocktails (see AK list FITMaN)

Staining Protocol

For the Compensation-control

1) Label 15 12x75mm tube with:
   a) unstained
   b) FITC (only needed in the B-cell Panel and DC/mono/NK Panel)
   c) AF488 (only needed in the T-Cell Panel)
   d) PE
   e) PerCP-Cy5.5
   f) PE-Cy7
   g) APC
   h) AF647 (only needed in the Treg Panel)
   i) APC-H7
   j) AF700
   k) V450
   l) V500
   m) BV605
   n) BV421 (only needed in the B-Cell Panel)
   o) BV605 (only needed in the T-cell Panel and DC/mono/NK Panel)

2) Place one drop BD CompBeads Negative Control in tube a)

3) Place one drop BD CompBeads Anti-Mouse Ig in each other tubes b) – o)

4) Add associated µl Antibody to each tube
   Example:

5µl SLAN FITC	in tube b) labeled FITC
1,25µl CD183 AF488	in tube c) labeled AF488
5 µl CD25 PE	in tube d) labeled PE

   and so on…

5) Mix gently

6) Incubate 20 minutes in the dark at RT

7) Centrifuge for 5 minutes at 400g

8) Remove the supernatant by pour off

9) Resuspend in 100-300µl FACS-buffer

10) Ready for analysing

For each panel

1) Place 100µl of gently mixed whole blood into appropriately labeled 12x75mm tube.

2) Add required amount PBS (see AK list FITMaN) to the appropriate tubes and mix gently

3) Add required amount of the antibody cocktail (see AK list FITMaN) to the appropriate tubes and mix gently.

4) Incubate 30 minutes in the dark at RT

5) Ery-lysing: Add 2ml 1x FACS BD Lysing Solution and mix immediately on a vortex device for 5secounds

6) Incubate 10 minutes in the dark at RT

7) Centrifuge for 5 minutes at 400g

8) Remove the supernatant by pour off

9) Wash the samples by resuspending in 2 ml FACS-buffer

10) Centrifuge for 5 minutes at 400g

11) Remove the supernatant by pour off

12) Resuspend in 100-300µl FACS-buffer

13) Ready for analysing

Instrument settings (to make sure that we all have the same)

We use a Fortessa with a 5 Laser configuration.
325nm, 405nm, 488nm, 640nm and 561nm FACS Diva Software 6.1.1 witch CST Module. For the FITMaN Panels we don´t need the 325nm UV Laser.

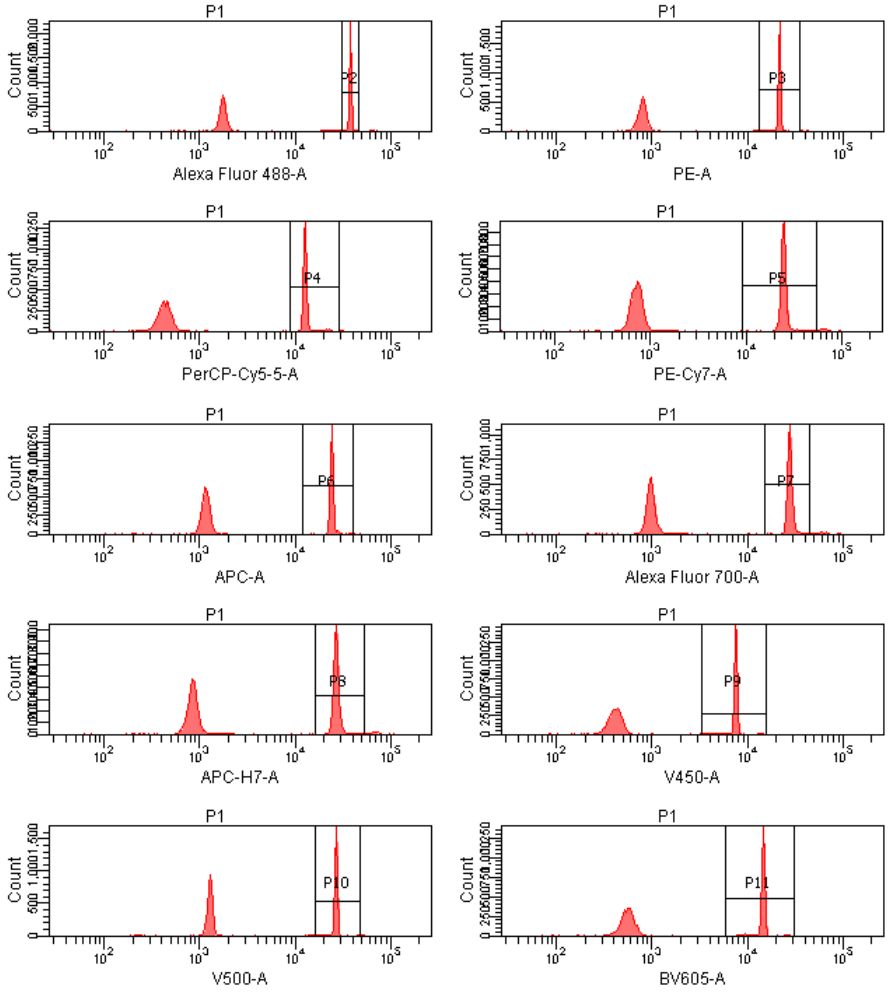
1. Start the Cytometer, and make the usual daily performance check with CST Beads.
2. Mix in one 12x75mm FACS Tube one drop CST-Beads, and 300 µl FACS Flow
3. Create a new Experiment and a new Specimen
4. Delete all Parameters in the Cytometer instrument settings you don´t need.
5. In the Cytometer instrument settings switch FSC-A, FSC-H, FSC-W, and SSC-A, SSC-H, SSC-W on. We will use these parameters for the doublet discrimination.

Status | Parameters | Threshold | Laser | Compensation | Ratio

Parameter	Voltage	Log	A	H	W
FSC	478	☐	☑	☑	☑
SSC	264	☐	☑	☑	☑
Alexa Fluor 488	544	☑	☑	☐	☐
PerCP-Cy5-5	539	☑	☑	☐	☐
PE	519	☑	☑	☐	☐
PE-Cy7	463	☑	☑	☐	☐
APC	529	☑	☑	☐	☐
Alexa Fluor 700	503	☑	☑	☐	☐
APC-H7	496	☑	☑	☐	☐
V450	399	☑	☑	☐	☐
V500	463	☑	☑	☐	☐
BV605	578	☑	☑	☐	☐

6. On a Global Worksheet create one Dot Plot with FSC-A vs. SSC-A and 10 Histogram-Plots with each color. AF488, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-H7, AF700, V450, V500 abd BV605
7. Generate a gate around the singlet beads, and gates around the brightest population each histogram.
8. Create statistic view to see the Means of all Parameters.

Population	Alexa Flu... Mean	PerCP-Cy... Mean	PE-A Mean	PE-Cy7-A Mean	APC-A Mean	Alexa Flu... Mean	APC-H7-A Mean	V450-A Mean	V500-A Mean	BV605-A Mean
P2	38,126	12,731	21,716	25,213	24,410	28,218	27,429	7,566	27,259	14,810
P3	37,782	12,943	21,546	26,173	24,523	29,048	28,405	7,491	27,020	14,695
P4	37,756	13,006	21,537	26,395	24,570	29,255	28,637	7,485	27,005	14,690
P5	38,149	12,770	21,735	25,357	24,457	28,349	27,577	7,571	27,279	14,822
P6	37,732	12,997	21,523	26,373	24,553	29,231	28,612	7,480	26,987	14,680
P7	38,208	12,723	21,762	25,135	24,421	28,160	27,355	7,583	27,320	14,842
P8	38,170	12,751	21,744	25,269	24,445	28,275	27,488	7,576	27,296	14,830
P9	37,766	13,007	21,544	26,399	24,574	29,259	28,642	7,488	27,013	14,694
P10	37,884	12,898	21,599	25,971	24,509	28,870	28,198	7,513	27,093	14,730
P11	37,757	13,013	21,539	26,422	24,576	29,279	28,666	7,485	27,006	14,691



9. Adjust the PMT-Voltages to get the following Geo Means + - 1000:
   Without any COMPENSATION!!!

AF488	38000
PE	22000
PerCP-Cy5.5	13000
Pe-Cy7	25000
APC	24000
APC-H7	26000
AF700	28000
V450	7000
V500	27000
BV605	15000

10. Create 4 new Experiments
    a. T-Cell Panel
    b. Treg Panel
    c. B-Cell Panel
    d. DC/Mono/NK Panel

11. Create in each Experiment a new Specimen

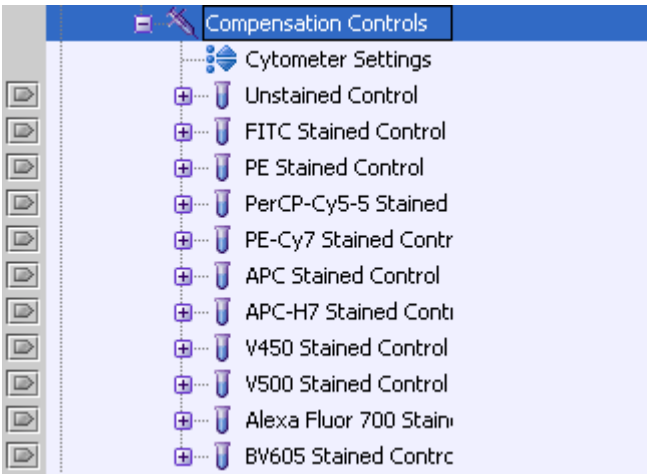
12. Delete in each Experiment all Parameters in the Cytometer instrument settings.

13. Add associated Parameters for each Experiment

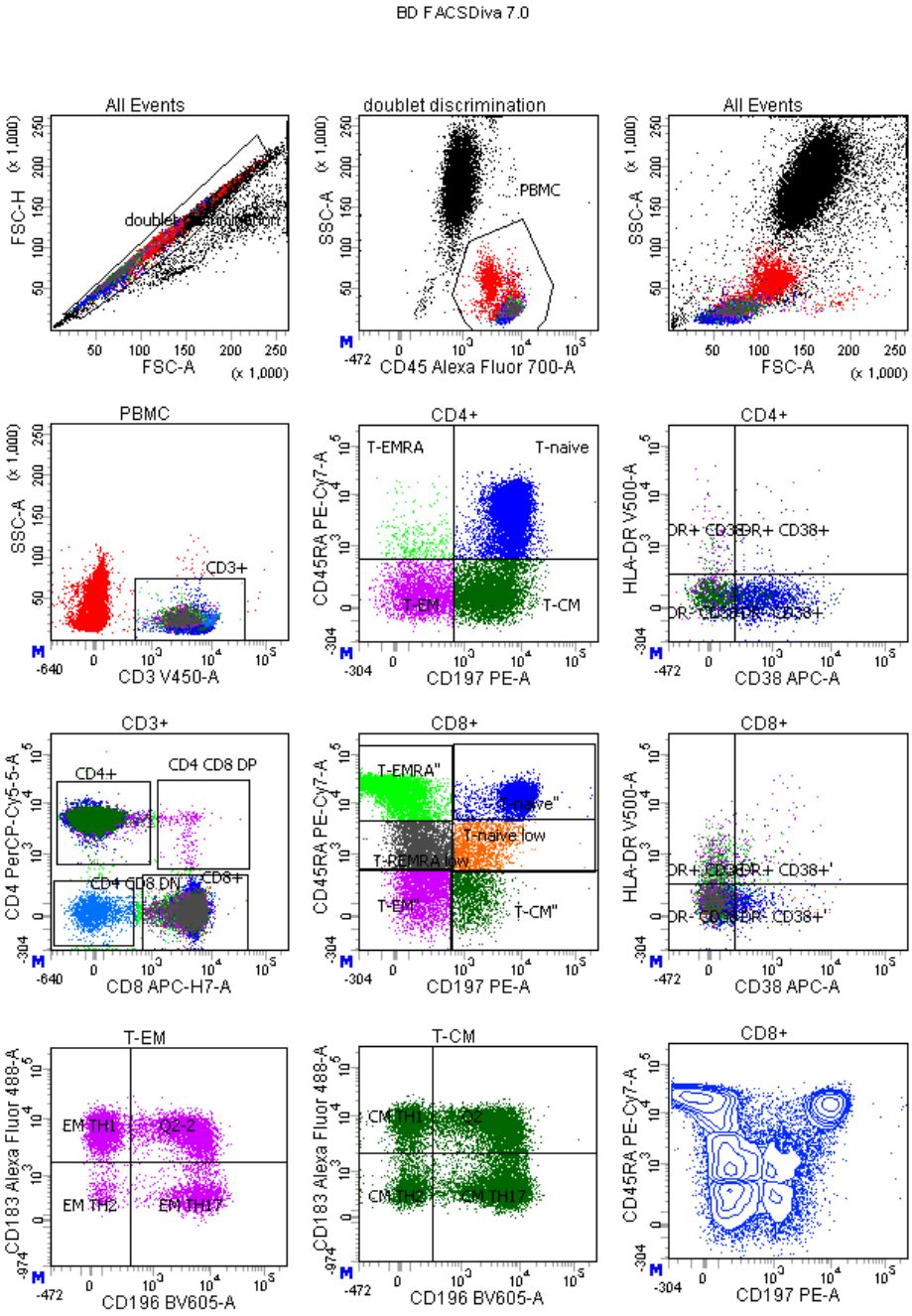
14. For the T-Cell Panel AF488, PerCP-Cy5.5, PE and so on
    for the Treg Panel PerCP-Cy5.5, PE, PE-Cy7 and so on
    for the B-Cell Panel FITC, PerCP-Cy5.5, PE, PE-Cy7 and so on
    for the DC/Mono/NK Panel FITC, PerCP-Cy5.5, PE and so on

15. Create in each Experiment a compensation Control
    Experiment =>Compensation Setup => Create compensation control

16. Measure the associated Compensation control Tubes in each
    Compensation control Specimen



17. Adjust the Gates in each histogram (normal worksheet) if it will not be automatically done
18. Calculate Compensation
    Experiment =>Compensation Setup =>Calculate compensation and “link and save” the settings to your Experiment
19. If compensation is fine, save the experiment “application settings” by right-clicking on cytometer settings. These application settings can be re-called in the next experiments, or duplicate the latest experiments/specimens
20. Create a new global worksheet in each experiment and a gating strategy like this



Population	#Events	%Parent	%Total
All Events	278,782	####	100.0
doublet discrimination	269,364	96.6	96.6
PBMC	139,113	51.6	49.9
CD3+	78,944	56.7	28.3
CD4+	56,153	71.1	20.1
T-EMRA	896	1.6	0.3
T-naive	32,577	58.0	11.7
T-EM	7,240	12.9	2.6
EM TH1	2,185	30.2	0.8
Q2-2	3,321	45.9	1.2
EM TH2	291	4.0	0.1
EM TH17	1,443	19.9	0.5
T-CM	15,440	27.5	5.5
CM TH1	2,600	16.8	0.9
Q2	4,862	31.5	1.7
CM TH2	1,860	12.0	0.7
CM TH17	6,118	39.6	2.2
DR+ CD38-	2,202	3.9	0.8
DR+ CD38+	978	1.7	0.4
DR- CD38-	23,906	42.6	8.6
DR- CD38+	29,067	51.8	10.4
CD8+	20,564	26.0	7.4
DR+ CD38-'	3,872	18.8	1.4
DR+ CD38+'	842	4.1	0.3
DR- CD38-'	13,523	65.8	4.9
DR- CD38+'	2,327	11.3	0.8
T-naive low	1,780	8.7	0.6
T-EMRA"	5,424	26.4	1.9
T-naive"	5,911	28.7	2.1
T-EM"	2,804	13.6	1.0
T-CM"	1,475	7.2	0.5
T-REMRA low	3,399	16.5	1.2
CD4 CD8 DP	218	0.3	0.1
CD4 CD8 DN	1,809	2.3	0.6

Population	#Events
All Events	278,782
doublet discrimination	269,364
PBMC	139,113
CD3+	78,944
CD4+	56,153
T-naive	32,577
T-EMRA	896
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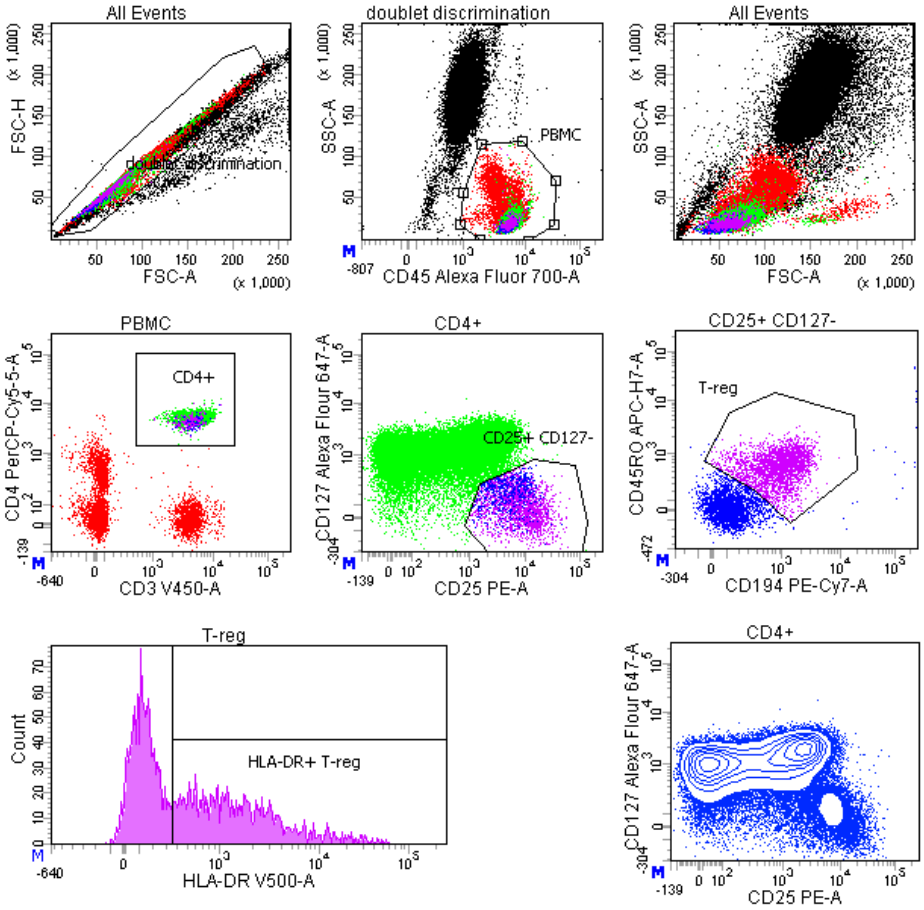
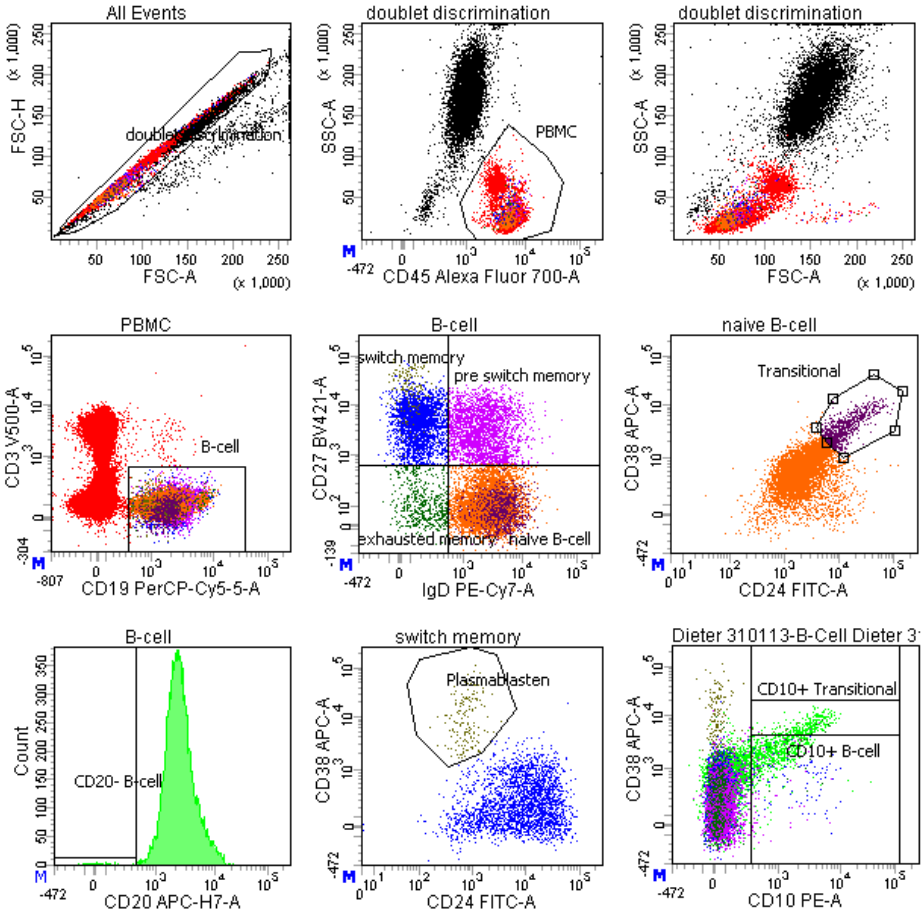
EM TH1	2,185
EM TH2	291
EM TH17	1,443
T-CM	15,440
CM TH1	2,600
CM TH2	1,860
CM TH17	6,118
DR+ CD38-	2,202
DR+ CD38+	978
DR- CD38-	23,906
DR- CD38+	29,067
CD8+	20,564
T-naive"	5,911
T-naive low	1,780
T-EMRA"	5,424
T-REMRA low	3,399
T-EM"	2,804
T-CM"	1,475
DR+ CD38-'	3,872
DR+ CD38+'	842
DR- CD38-'	13,523
DR- CD38+'	2,327
CD4 CD8 DP	218
CD4 CD8 DN	1,809

BD FACSDiva 7.0

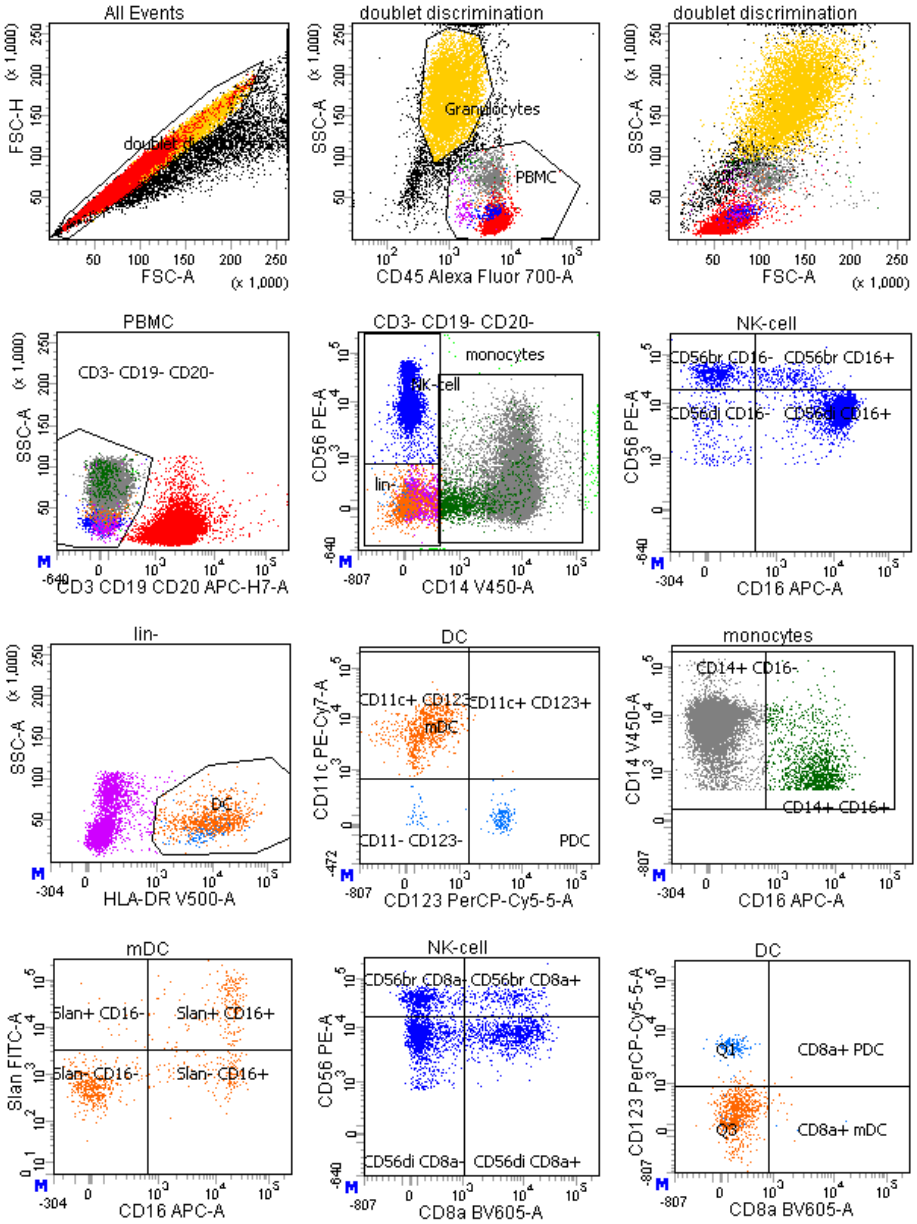


Population	#Events	%Parent	%Total
All Events	296,358	####	100.0
doublet discrimination	288,069	97.2	97.2
PBMC	147,936	51.4	49.9
CD4+	58,516	39.6	19.7
CD25+ CD127-	5,159	8.8	1.7
T-reg	2,561	49.6	0.9
HLA-DR+ T-reg	1,331	52.0	0.4

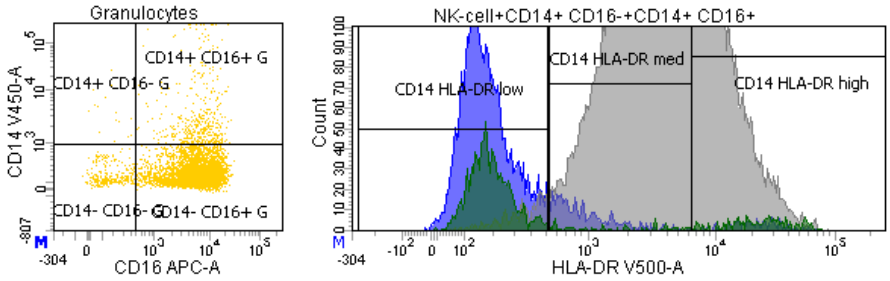
BD FACSDiva 7.0



Population	#Events	%Parent	%Total
All Events	275,587	####	100.0
doublet discrimination	268,496	97.4	97.4
PBMC	135,496	50.5	49.2
B-cell	12,655	9.3	4.6
CD20- B-cell	173	1.4	0.1
switch memory	2,747	21.7	1.0
Plasmablasten	162	5.9	0.1
pre switch memory	2,416	19.1	0.9
exhausted memory	553	4.4	0.2
naive B-cell	6,939	54.8	2.5
Transitional	714	10.3	0.3
CD10+ Trans	614	86.0	0.2


All Events
doublet
FSC-H (x 1,000)
FSC-A (x 1,000)
doublet discrimination
Granulocytes
PBMC
SSC-A (x 1,000)
CD45 Alexa Fluor 700-A
doublet discrimination
SSC-A (x 1,000)
FSC-A (x 1,000)
PBMC
CD3- CD19- CD20-
SSC-A (x 1,000)
CD3 CD19 CD20 APC-H7-A
CD3- CD19- CD20-
monocytes
NK-cell
lin-
CD56 PE-A
CD14 V450-A
NK-cell
CD56br CD16-
CD56br CD16+
CD56di CD16-
CD56di CD16+
CD56 PE-A
CD16 APC-A
lin-
SSC-A (x 1,000)
DC
HLA-DR V500-A
DC
CD11c+ CD123-
mDC
CD11c+ CD123+
CD11- CD123-
PDC
CD11c PE-Cy7-A
CD123 PerCP-Cy5-5-A
monocytes
CD14+ CD16-
CD14+ CD16+
CD14 V450-A
CD16 APC-A
mDC
Slan+ CD16-
Slan+ CD16+
Slan- CD16-
Slan- CD16+
Slan FITC-A
CD16 APC-A
NK-cell
CD56br CD8a-
CD56br CD8a+
CD56di CD8a-
CD56di CD8a+
CD56 PE-A
CD8a BV605-A
DC
Q1
CD8a+ PDC
Q3
CD8a+ mDC
CD123 PerCP-Cy5-5-A
CD8a BV605-A

BD FACSDiva 7.0



Population	#Events	%Parent	%Total
All Events	300,000	####	100.0
doublet discrimination	275,560	91.9	91.9
PBMC	114,194	41.4	38.1
CD3- CD19- CD20-	23,268	20.4	7.8
NK-cell	3,139	13.5	1.0
CD56br CD16-	560	17.8	0.2
CD56br CD16+	291	9.3	0.1
CD56di CD16-	217	6.9	0.1
CD56di CD16+	2,071	66.0	0.7
CD56br CD8a-	628	20.0	0.2
CD56br CD8a+	285	9.1	0.1
CD56di CD8a-	1,314	41.9	0.4
CD56di CD8a+	912	29.1	0.3
lin-	4,019	17.3	1.3
DC	1,064	26.5	0.4
CD11c+ CD123-	858	80.6	0.3
CD11c+ CD123+	4	0.4	0.0
CD11- CD123-	40	3.8	0.0
PDC	162	15.2	0.1
mDC	863	81.1	0.3
Slan+ CD16-	17	2.0	0.0
Slan+ CD16+	216	25.0	0.1
Slan- CD16-	471	54.6	0.2
Slan- CD16+	159	18.4	0.1
Q1	190	17.9	0.1
CD8a+ PDC	3	0.3	0.0
Q3	856	80.5	0.3
CD8a+ mDC	15	1.4	0.0
monocytes	16,130	69.3	5.4
CD14+ CD16-	14,907	92.4	5.0
CD14+ CD16+	1,230	7.6	0.4
CD14 HLA-DR low	1,246	7.7	0.4
CD14 HLA-DR med	10,659	66.1	3.6
CD14 HLA-DR high	4,237	26.3	1.4
Granulocytes	134,038	48.6	44.7
CD14+ CD16- G	278	0.2	0.1
CD14+ CD16+ G	8,958	6.7	3.0

Population	#Events
NK-cell	3,139
CD56br CD16-	560
CD56br CD16+	291
CD56di CD16-	217
CD56di CD16+	2,071
CD56br CD8a-	628
CD56br CD8a+	285
CD56di CD8a-	1,314
CD56di CD8a+	912
monocytes	16,130
CD14+ CD16-	14,907
CD14+ CD16+	1,230
DC	1,064
CD11c+ CD123-	858
CD11c+ CD123+	4
CD11- CD123-	40
mDC	863
Slan+ CD16-	17
Slan+ CD16+	216
Slan- CD16-	471
Slan- CD16+	159
CD8a+ mDC	15
PDC	162
CD8a+ PDC	3
Granulocytes	134,038
CD14+ CD16- G	278
CD14+ CD16+ G	8,958
CD14- CD16- G	5,910
CD14- CD16+ G	118,892
CD14 HLA-DR low	1,246
CD14 HLA-DR med	10,659
CD14 HLA-DR high	4,237

21. Adjust Threshold in FSC (maybe delete it) and set an new Threshold in AF700 at 500
22. Measure 300.000 counts if possible.
    Alternative: a specific population stopping gate should be determined for each panel. E.g. 40’000 CD4 T-cells, or 30,000 B-cells etc. These numbers should be calculated in a way that the downstream hierarchy and less represented populations are in a sufficient number. In this way, in case of different cell frequencies, the cell populations are still numerically comparable among samples

	Tcell-Panel	Preis	µl	reicht für	Clone	Treg-Panel	Preis	µl	reicht für	Clone	Bcell-Panel	Preis	µl	reicht für	Clone	DC/mono/NK	Preis	µl	reicht für	Clone
Fitc											CD24					Slan				
AF488	CD183 (CXCR3)				G025H7										ML5					
Filter 530/30	1,25µl = 1:120	206,82	500	400	BL#353710						10µl =1:15				BD#555427	5µl = 1:30	230,00	1000	200	Miltenyi#130-093-027
PerCP-Cy5.5	CD4				RPA-T4	CD4				RPA-T4	CD19				HIB19	CD123				7G3
Filter 695/40	1,25µl = 1:120	237,20	250	200	BD#560650	1,25µl = 1:120	237,20	250	200	BD#560650	2,5µl = 1:60	237,20	250	100	BD#561295	10µl = 1:15	174,41	500	50	BD#560904
PE-TexasRED																				
Filter 610/20																				
PE	CD197 (CCR7)				150503	CD25				M-A251	CD10				HI 10a	CD56				MY31
Filter 586/15	10µl = 1:15	279,06	2000	200	BD#560765	5µl = 1:30	286,03	2000	400	BD#555432	10µl = 1:15				BD#332776	5µl = 1:30	442,50	2000	400	BD#345810
PE-Cy5																				
Filter 660/20																				
PE-Cy7	CD45RA				HI100	CD194 (CCR4)				1G1	IgD				IA6-2	CD11c				B-LY6
Filter 780/60	2,5µl = 1:60	199,33	250	100	BD#560675	2,5µl = 1:60	344,83	500	200	BD#557864	2,5µl = 1:60	101,53	500	200	BL#348210	2,5µl = 1:60	209,29	250	100	BD#561356
APC	CD38				HIT2						CD38				HIT2	CD16				B73.1
AF647						CD127				hIL-7R-M21										
Filter 670/30	10µl = 1:15	357,79	2000	200	BD#555462	10µl = 1:15	164,44	500	50	BD#560905	10µl = 1:30	357,79	2000	200	BD#555462	5µl = 1:30	204,31	250	50	BD#561304
AF700	CD45				HI30	CD45				HI30	CD45				HI30	CD45				HI30
Filter 730/45	0,5µl = 1:300	169,22			BL#304024	0,5µl = 1:300	169,22			BL#304024	0,5µl = 1:300	169,22			BL#304024	0,5µl = 1:300	169,22			BL#304024
APC-H7	CD8				SK1	CD45RO				UCHL1	CD20				L27	CD3+CD19+CD20	471,40	500	200	CD3 BD#641397 Clone SK7
Filter 780/60	2,5µl = 1:60	471,40	500	200	BD#641400	2,5µl = 1:60	199,33	250	100	BD#561137	2,5µl = 1:60	471,40	500	200	BD#641396	2,5µl = 1:60	204,31	250	100	CD19 BD#560727 Clone HIB19
V450	CD3				UCHT1	CD3				UCHT1						CD14				MoP9
Brilliant Violet 421											CD27				O323					
Filter 450/50	2,5µl = 1:60	305,96	600	240	BD#560365	2,5µl = 1:60	305,96	600	240	BD#560365	2,5µl = 1:60	131,61	500	200	BL#302824	2,5µl = 1:60	305,96	600	240	BD#560349
V500	HLA-DR				L243(G46-6)	HLA-DR				L243(G46-6)	CD3				UCHT1	HLA-DR				L243(G46-6)
Filter 525/50	2,5µl = 1:60	393,67	500	200	BD#561224	2,5µl = 1:60	393,67	500	200	BD#561224	2,5µl = 1:60	393,67	500	200	BD#561416	2,5µl = 1:60	393,67	500	200	BD#561224
Brilliant Violet 605	CD196 (CCR6)				G034E3											CD8a				RPA-T8
Filter 605/40	1,25µl = 1:120	308,35	500	400	BL#353420											1,25µl = 1:120		125	100	BL#301039
100µl Vollblut+																				
Mastermix:	34,25 µl					26,75µl					43µl					41,75µl				
	+15,75 µl PBS					+23,25µl PBS					+7 µl PBS					+ 8,25 µl PBS				

Trucount to determine the absolute cell number

1) Place 50µl of gently mixed whole blood into appropriately labeled 12x75mm Trucount tube.

2) Add 20µl antibody cocktail (6-Color TBNK) to the appropriate tubes and mix gently.

3) Incubate 30 minutes in the dark at RT

4) Ery-lysing: Add 400µl 1x FACS BD Lysing Solution and mix immediately on a vortex device for 5secounds

5) Incubate at least 15 minutes in the dark at RT

6) Ready for analyzing. Measure within 60 minutes

Instrument settings

1. Use the same Instrument settings like in FITMaN.

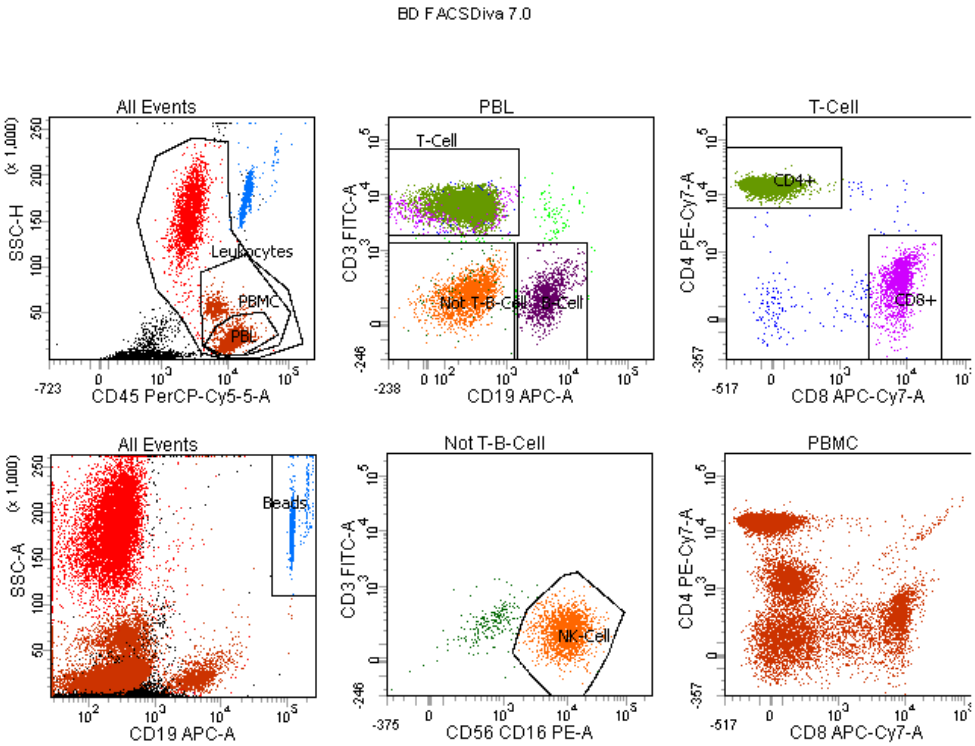
2. Set the FSC SSC to Log-Scale

3. delete the FSC and the AF700 Threshold and set an new Threshold to PerCP-Cy5.5 at 500

4. Create a Global Worksheet and the gating strategy like in the example

5. Adjust voltage of FSC SSC like in the example

6. Measure 50.000 counts
   Alternative: a specific population stopping gate should be determined.
   E.g. 20’000 Lymphocytes.



Population	#Events	%Parent	%Total
All Events	30,949	####	100.0
Leukocytes	20,673	66.8	66.8
PBL	10,001	32.3	32.3
T-Cell	6,519	65.2	21.1
CD8+	1,580	24.2	5.1
CD4+	4,736	72.6	15.3
B-Cell	1,134	11.3	3.7
Not T-B-Cell	2,229	22.3	7.2
NK-Cell	2,008	90.1	6.5
PBMC	11,652	37.6	37.6
Beads	4,799	15.5	15.5

Calculate the absolute cell number:

events in cell population beads/test
---------- X ---------- = cell population absolute count
events in bead region test volume (50µl)