Validated single-tube multiparameter flow cytometry approach for the assessment of minimal residual disease in multiple myeloma

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Supplements

Methods

Cell culture

Nine human multiple myeloma cell lines (MMCLs), IM-9, OPM-2, NCI-H929, L363, Karpas620, U266B1, MM1.S, RPMI8226 and MM1.R (mycoplasma free and assessed for genetic differences from the originals by the DSMZ, Heidelberg in 03/2016) were kindly provided by Dr. Julia Schüler (Oncotest, Freiburg, Germany) in 08/2016. All MMCLs were cultured in RPMI 1640 medium (Gibco life technologies, Darmstadt, Germany) containing 10% (V/V) fetal calf serum (Sigma-Aldrich Chemie GmbH, München, Germany) and 1% (V/V) Penicillin-Streptomycin (10,000U/ml) (Gibco life technologies, Darmstadt, Germany). Cells were incubated at 37°C and 5% CO_2 (HeraCell 240i CO_2 Incubator, Thermo Fisher Scientific GmbH, Darmstadt, Germany). Cells were harvested at a confluency of 70-80%.

Assay validation: the use of MM cell lines for the assessment of analytical specificity and stability (samples, sensitivity and antibody mix)

MMCLs or human peripheral blood mononuclear cells (PBMCs) were processed with the described bulk lysis protocol and stained with the 6- or 8-color panel. To assess the impact of light exposure on the staining quality, RPMI8226 were stained with the 6-color panel and directly measured and after 15, 30, 45 and 60 minutes (min) of light exposure, while kept on ice. To assess the time-dependent stability of the antibody-mix, the 6-color mix was prepared on 5 following days and L363 cells were stained with all 5 mixes at day 5 and directly measured after staining. To assess the stability of the sample staining, L363 cells were stained with the 6- and 8- color panel and measured directly after staining and every 20min for a timeframe of 3 hours. In-between measurements cells were kept on ice and light-protected. For these validation experiments 50,000 events were acquired at every time point. To check for stability and precision over time 30,000 MMCLs cells were spiked into PBMCs, stained with both panels and measured directly after staining and every 40min for an interval of 200min. For this validation experiment $3x10^6$ events were acquired for every time point with conditions as described above.

The analytical sensitivity, precision and linearity were assessed as follows: NCI-H929 cells were spiked into human PBMCs of HIs in 5 linear, defined concentrations (0.0007%; 0.0033%; 0.0066%; 0.033%; 0.0666%) for the dilution assay. Cells were stained as described above and 3,000,000 events per cell concentration were acquired.

Patient samples and characteristics

Two-hundred-and-five bone marrow (BM) samples from 163 consecutive multiple myeloma (MM) patients (some were measured sequentially) undergoing routine BM aspiration at the University Medical Center Freiburg between 04/17–04/19, and samples of healthy individuals (HIs) were included. Samples of patients diagnosed with monoclonal gammopathy of undetermined significance/smoldering multiple myeloma (MGUS/SMM) or MM were analyzed before treatment. MM patient samples were analyzed after 3 cycles of therapy or 1, 3, or 12 months post stem cell transplantation (SCT) for minimal residual disease (MRD) determination, depending on the routine time point of BM evaluation. Samples were analyzed directly after aspiration or whole BM was stored at 4°C and analyzed within 24 hours (h). Seventy-four samples at initial diagnosis (ID) (25 MGUS/SMM and 49 MM), 30 at progressive disease (PD) and 101 post treatment were measured. A total of 13 BM samples of HIs were included as controls.

Cytogenetic aberrations were detected in CD138 positive cells of the total BM using Fluorescence *in-situ* hybridization (FISH).

The analyses were carried out according to the Declaration of Helsinki and good clinical practice. This study was approved by the ethics committee of the University of Freiburg (No. 212/16). All patients gave written informed consent for the use of their samples for research purposes.

Sample processing, data acquisition and data analysis

BM samples were filtered and incubated with 1xNH_4Cl lysis solution for 15min on the shaker for erythrocyte lysis. After washing, cells were incubated with an anti-human FcR-Block (Thermo Fisher Scientific GmbH, Darmstadt, Germany) and directly stained with the cell-surface antibodies CD138APC, CD38PE-Cy7, CD45APC-H7, CD56PerCp-Cy5.5, CD27PE, CD19BV510 (Suppl. Table 1) included in both panels for 30min at 4°C in the dark. After washing, the 6-color samples were directly measured using a BD LSRFortessa (Becton Dickinson Biosciences, Heidelberg, Germany), which was daily validated with BD CS&T beads. Compensation was renewed every 4 months. The 8-color samples were additionally stained with the intracellular antibodies kappa-FITC and lambda-AF405 (Suppl. Table 1) using the Fix&Perm Kit (Thermo Fisher Scientific GmbH, Darmstadt, Germany) according to the manufacturer's instructions, washed once and measured directly. For all analyses, $3x10^6$ events were acquired. Unstained cells were used as controls. Samples were analyzed using Kaluza V2.1 (Beckman Coulter).

Statistical analyses

Data was analyzed using the paired t-test for pre-post comparisons and unpaired t-test for group comparisons, using t-tests with the assumption of normal distribution and the Mann-Whitney-U-test for patient samples with the assumption of no normal distribution. Progression-free and overall survival (PFS/OS) were calculated as time from BM aspiration until first progression (PFS) or death of any cause (PFS/OS). Patients without any event of interest were considered as censored observation at the time last seen alive without disease progression or death of any cause. PFS and OS were estimated using Kaplan-Meier method and compared using log-rank test. Statistical significance was defined as $P<0.05$. Statistical analyses were performed using GraphPadPrism V5.03 and SAS V9.2.

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Supplemental Table 1. Antibodies used for the 6- and 8-color panel.

Antigen/Fluorochrome	Isotype/Clone	Manufacturer
CD138 APC anti-human	IgG1 κ mouse/MI15	BioLegend GmbH, Fell
CD19 BV510 anti-human	IgG1 κ mouse/SJ25C1	Becton Dickinson Biosciences, Heidelberg
CD27 PE anti-human	IgG1 κ mouse/1A4CD27	Beckman Coulter GmbH, Krefeld
CD38 PE-Cy7 anti-human	IgG1 κ mouse/HB7	BioLegend GmbH, Fell
CD45 APC-H7 anti-human	IgG1 κ mouse/2D1	BioLegend GmbH, Fell
CD56 PerCP-Cy5.5 anti-human	IgG1 κ mouse/B159	Becton Dickinson Biosciences, Heidelberg
Kappa FITC anti-human	Rabbit/polyclonal F(ab‘)2	Agilent Technologies, Inc, Santa Clara, USA
Lambda AF405 anti-human	IgG1 κ mouse/1-155-22	Novus Biologicals, Littleton, Colorado, USA

Supplemental Table 2. Expression of antigens in myeloma cell lines using the 6-color panel compared to published literature results. For all 9 MMCLs triplicates were assessed.

	L363		NCI-H929		RPMI8226	
Antigen	**Mean % of positive cells**	**Literature** Ref.: 4–9	**Mean % of positive cells**	**Literature** Ref.: 5–11	**Mean % of positive cells**	**Literature** Ref.: 5–11
CD19	0.1	-	0.2	- (80%)	0.2	- (80%)
CD27	1.1	-	1.0	-	1.0	-
CD45	0.0	+/-	1.8	-	1.8	-
CD56	31.9	+	13.6	+ (25%)	13.6	+ (25%)
CD38	65.9	+	99.5	+ (100%)	99.5	+ (100%)
CD138	98.4	+	87.3	+	87.3	+

	IM-9		Karpas620		U266B1	
Antigen	**Mean % of positive cells**	**Literature** Ref.: 12	**Mean % of positive cells**	**Literature** Ref.:6,12,13	**Mean % of positive cells**	**Literature** Ref.: 4,5,7–11,14–16
CD19	84.4	+	2.4	-	0.0	- (100%)
CD27	0.1		4.6	-	0.0	-
CD45	98.8		1.7	-	0.0	-/+
CD56	0.0		8.0	+	14.8	+/-
CD38	10.3	-	97.6	+/-	96.1	(+)
CD138	89.1		95.7	+	42.5	+

	MM1.S		OPM-2		MM1.R	
Antigen	**Mean % of positive cells**	**Literature** Ref.: 9,11,17	**Mean % of positive cells**	**Literature** Ref.: 6,8,9	**Mean % of positive cells**	**Literature** Ref.: 9,11,17
CD19	0.1	- (99.8%)	0.4	-	0.4	- (99.8%)
CD27	0.0	-	1.5	-	1.5	-
CD45	0.2	- (99.7%)	13.1	-	0.4	- (99.7%)
CD56	0.7	+	97.9		1.5	(+/-)
CD38	100.0	+ (96.1%)	98.2	+	97.6	+ (96.1%)
CD138	75.6	+	88.6	+	88.9	+

Abbreviations: CD: cluster of differentiation, MMCLs: Multiple Myeloma cell lines.

Supplemental Table 3. Patient characteristics of MRD samples measured with 6- or 8-color panels: entire and 6/8-color patient description.

	Total cohort (n=91)[c]		6-color (n=54)		8-color (n=40)	
	No. (%)	**Median (range)**	**No. (%)**	**Median (range)**	**No. (%)**	**Median (range)**
Stage						
post CTx	10 (11)		3 (6)		7 (18)	
post ASCT/alloSCT	81 (89)		51 (94)		33 (82)	
Sex						
Female	41 (45)		22 (41)		20 (50)	
Male	50 (55)		32 (59)		20 (50)	
Age (years)						
ID		59 (28-84)		57 (28-74)		60 (32-84)
Sampling		62 (29-86)		59 (29-74)		65 (32-86)
Durie & Salmon (ID)						
I	10 (11)		6 (11)		4 (10)	
II	24 (26)		10 (19)		15 (38)	
III	57 (63)		38 (70)		21 (52)	
A/B	82 (90) / 9 (10)		50 (93) / 4 (7)		34 (85) / 6 (15)	
ISS (ID)						
I	35 (38)		23 (43)		12 (30)	
II	28 (31)		17 (31)		12 (30)	
III	28 (31)		14 (26)		16 (40)	
BM infiltration (%)						
Cytology		3 (0-28)		3 (0-28)		4 (0-20)
Pathology		5 (0-40)		5 (0-40)		0 (0-40)
Cytogenetics[a]						
Unfavorable	3 (3)		0 (0)		3 (8)	
Favorable	8 (9)		3 (6)		5 (12)	
Normal Karyotype	42 (46)		24 (44)		20 (50)	
Missing	38 (42)		27 (50)		12 (30)	
MM type						
IgG	70 (77)		43 (79)		29 (73)	
IgA	9 (10)		3 (6)		6 (15)	
Light chain only	12 (13)		8 (15)		5 (12)	
Light chain						
Kappa	67 (74)		43 (80)		27 (68)	
Lambda	24 (26)		11 (20)		13 (32)	
Last therapy before MRD determination						
PIs	70 (77)		42 (78)		29 (73)	
IMiDs	6 (7)		4 (8)		2 (4)	
Antibodies	14 (15)		6 (12)		9 (23)	
Others	1 (1)		1 (2)		0 (0)	
Remission[b]						
CR	18 (20)		6 (11)		14 (35)	
vgPR	52 (57)		42 (78)		12 (30)	
PR	18 (20)		5 (9)		12 (30)	
SD	3 (3)		1 (2)		2 (5)	
MM Progression						
Yes	15 (16)		12 (22)		3 (8)	
No	76 (84)		42 (78)		37 (92)	
Vital status						
Dead	1 (1)		1 (2)		0 (0)	
Alive	90 (99)		53 (98)		40 (100)	

Abbreviations: BM: bone marrow, CR: complete remission, del: deletion, ID: initial diagnosis, IMiDs: Immunomodulatory drugs, IMWG: International Myeloma Working Group, MM: multiple myeloma, no.: number, PIs: Proteasome Inhibitors, post alloSCT: post allogeneic stem cell transplantation, post ASCT: post autologous stem cell transplantation, post CTx: post chemotherapy, PR: partial remission, SD: stable disease, vgPR: very good partial remission.

[a] unfavorable defined as: +1q, t(4;14), t(14;16), del1p, cMYC, del17p; standard-risk defined as: Hyperdiploidy, t(11;14), del13q, Monosomy 13, del14q (IGH).

[b] according to IMWG criteria.

[c] some patients were measured more than ones during period of sampling. Every patient was only counted ones for each cohort. The number of both cohorts together was higher than of the entire cohort, because some patients were measured sequentially.

Supplemental Figure legends

Supplemental Figure 1. Comparison of our method with the EuroFlow standardized MRD assay and the MSKCC panel.

A. The two assays were compared side-by-side for the features regarding the assay (dark grey), the data (blue), the equipment needed (green), the costs (red) and the analyzed patient cohort (light grey).

B. The three assays were compared for the features regarding the markers, costs, sensitivity, number of needed cells and tube numbers.

h: hours.

Supplemental Figure 2. Gating strategy for the 6- and 8-color panel.

A. With the 6-color panel PCs were identified with the CD138/CD38 gate and further analyzed for their CD19/CD45 expression. In these 4 populations, expression of CD56/CD27 was analyzed and aPCs (red circle) could be distinguished from nPCs (green circle).

B. The gating strategy was based on the gating strategy of the 6-color panel. After analyzes of CD56/CD27 expression, populations were analyzed for their expression of intracellular kappa and lambda. Populations with an aberrant extracellular phenotype and monoclonal expression of either kappa or lambda were defined as aPCs. Populations with a normal extracellular phenotype were and polyclonal expression of kappa and lambda were defined as nPCs.

aPCs: aberrant plasma cells, CD: Cluster of differentiation, nPCs: normal plasma cells.

Supplemental Figure 3. Assay validation: stability of samples, assay precision and antibody mix

A-B. A MMCL was stained with the 6- (A) or 8- (B) color antibody panel to analyze the stability of the staining, lasers and machine over time. After staining, the cells were measured directly, and every 20min for a total time period of 3h. No significant differences in the expression levels of CD19, CD27, CD45, CD38 and CD138 could be observed. The expression level of CD56 significantly decreases after 40min (* p=0.0462) in the 6- and in the 8-color panel after 20min (** p=0.0092).

C-D. MMCL cells were spiked into human PBMCs of a healthy individual and stained with the 6- (C) or 8- (D) color panel to examine stability of sensitivity and precision of staining over time. After staining, the cells were directly measured and every 40min for a total time period of 200min. No significant differences in the number of cells and viability could be observed.

E. MMCL cells were stained with the antibodies included in both panels to check for the stability of fluorochromes during light exposure. After staining the cells were measured directly and after 15, 30, 45 and 60min of light exposure. No significant differences in the brightness of fluorochromes, expression levels or the viability of the cells could be observed.

F. An antibody mix of the 6-color panel was prepared every day on 5 following days to examine the stability of the antibody mix over time. MMCL cells were measured on day 5 with each mix prepared during the 5 days. No significant differences in the expression levels could be observed.

Graph depicted mean ± SD, paired t-test.

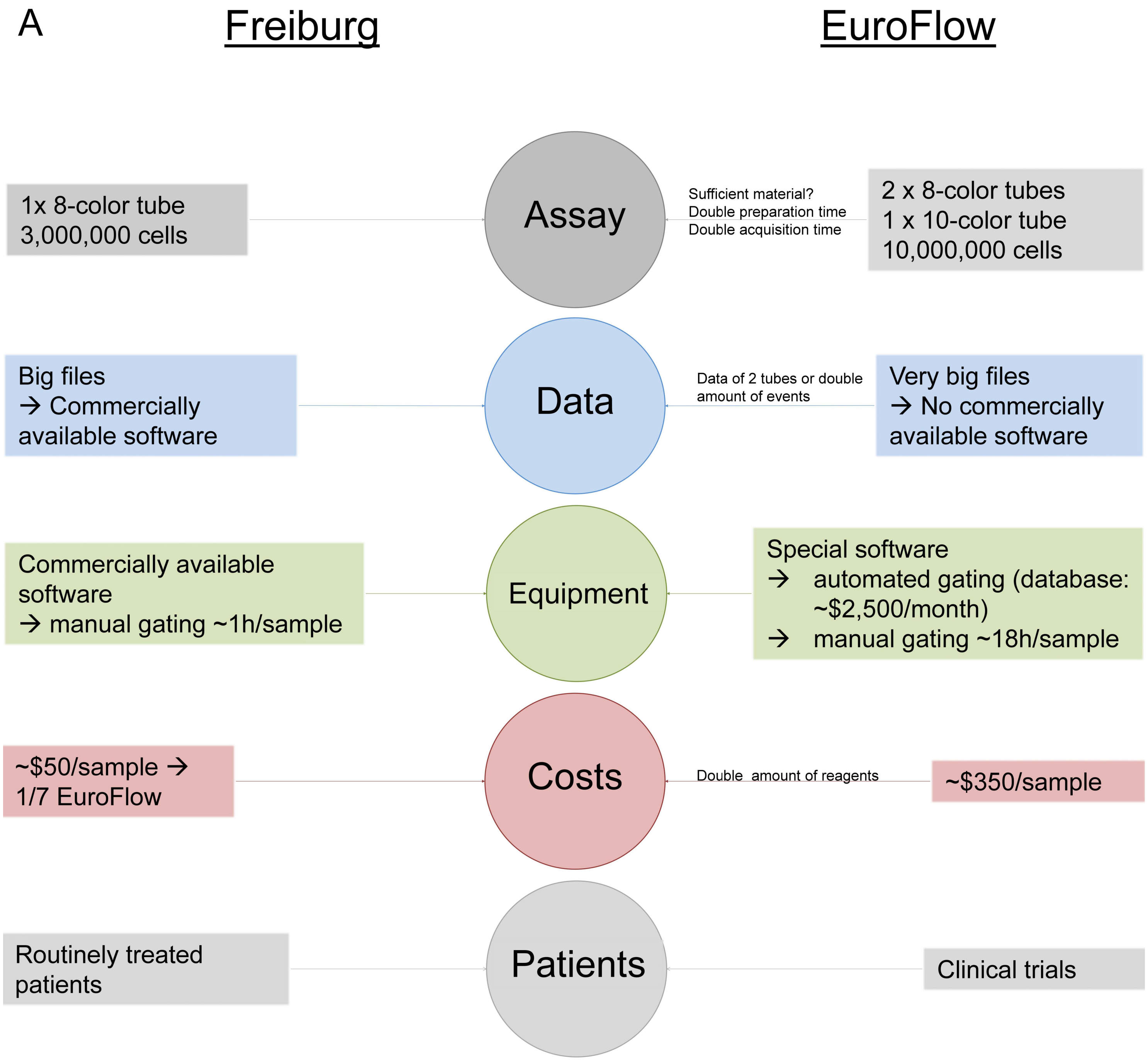
CD: cluster of differentiations, min: minutes, MMCL: Multiple Myeloma Cell Line, n: number, PBMCs: peripheral blood mononuclear cells, SD: standard deviation.

Supplemental Figure 4. Progression-free and overall survival of MRD- vs MRD+ samples and subgroup analyses.

A. MRD samples were divided into 4 groups according to MRD status and cytogenetic risk at ID. High-risk (HR) cytogenetics were defined as aberration +1q, t(4;14), t(14;16), del1p, cMYC, or del17p; standard-risk (SR) was defined as hyperdiploidy, t(11;14), del13q, Monosomy 13 or del14q (IGH).[1–3] The estimated median PFS for the MRD+/SR patients (n=40) was 17.9 months, the median PFS for the MRD+/HR (n=27) was 15.4 months, and for the MRD-/HR (n=6) and MRD-/SR (n=16) patients median PFS was not reached. There was a slight tendency towards a better PFS for the MRD-/SR compared to the MRD-/HR group. The MRD-/HR group had a better outcome than the MRD+/SR and MRD+/HR group. The hazard ratio (HR) (95% confidence interval (CI)) for the three groups compared to the MRD-/SR group was 2.528 (0.16-41.35) (MRD-/HR), 2.069 (0.25-17.06) (MRD+/SR) and 3.805 (0.45-32.00) (MRD+/HR).

B. The OS was also determined for the MRD level in combination with the cytogenetics. No big difference could be observed due to only one event censored.

BM: Bone Marrow, CI: confidence interval; del: deletion, HR: Hazard ratio, HR: High-risk, ID: initial diagnosis, MRD: Minimal residual disease, n: number, n.r.: not reached, OS: overall survival, PFS: progression-free survival, SR: standard-risk, t: translocation.



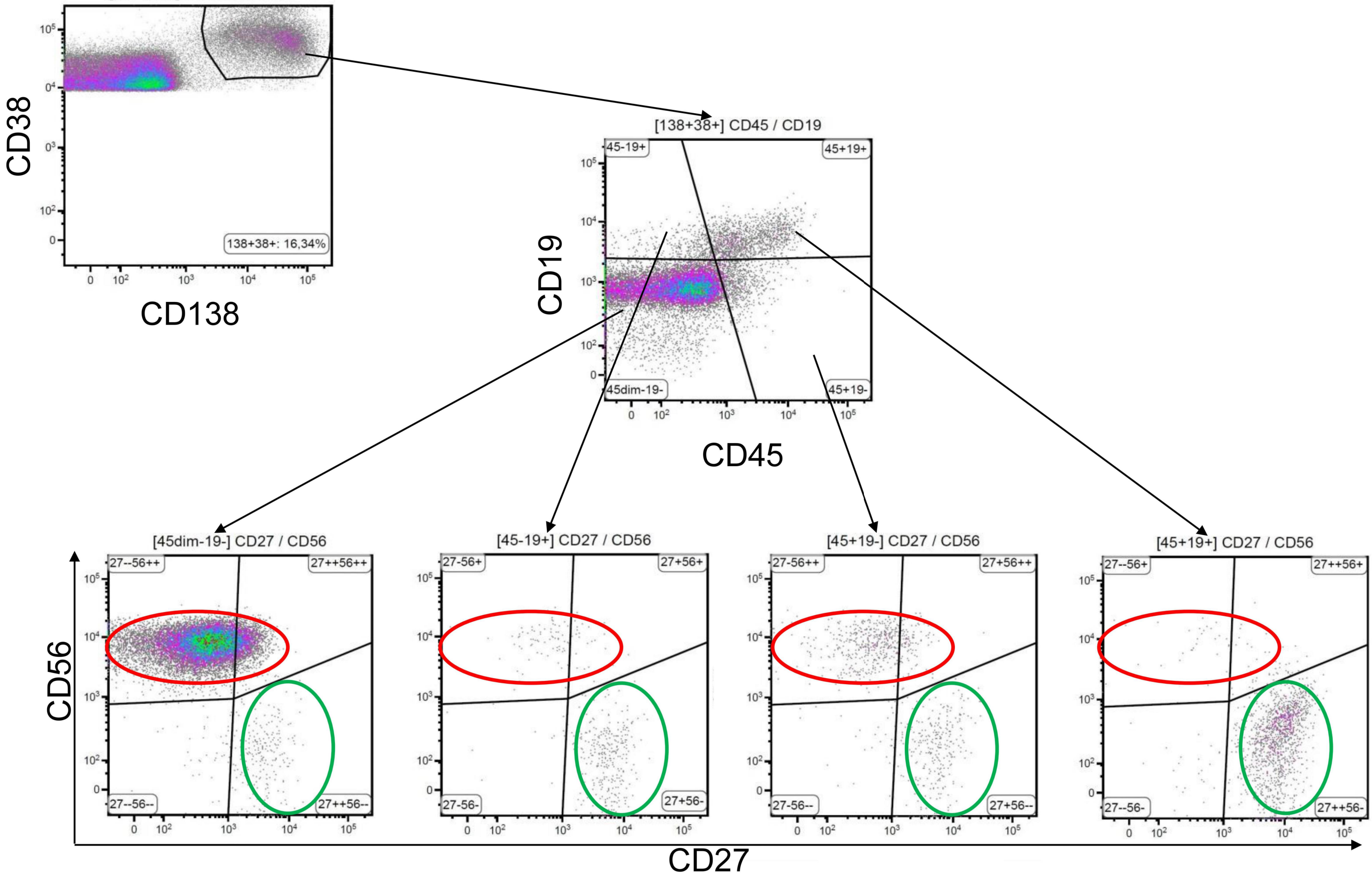
B

	Freiburg	**EuroFlow**	**MSKCC**
Markers	CD38, CD56, CD138, CD19, CD27, CD45, κ, λ	CD38, CD56, CD138, CD19, CD27, CD45, CD117, CD81, κ, λ	CD38, CD56, CD138, CD19, CD27, CD45, CD117, CD81, κ, λ
# of markers being used	8	10	10
Tubes	1	2	1
Number of cells being acquired	3,000,000	10,000,000	5,000,000
Costs	~$50/sample	~$350/sample	-
Sensitivity	10^{-5}	10^{-5}-10^{-6}	10^{-5}

Suppl. Figure 1

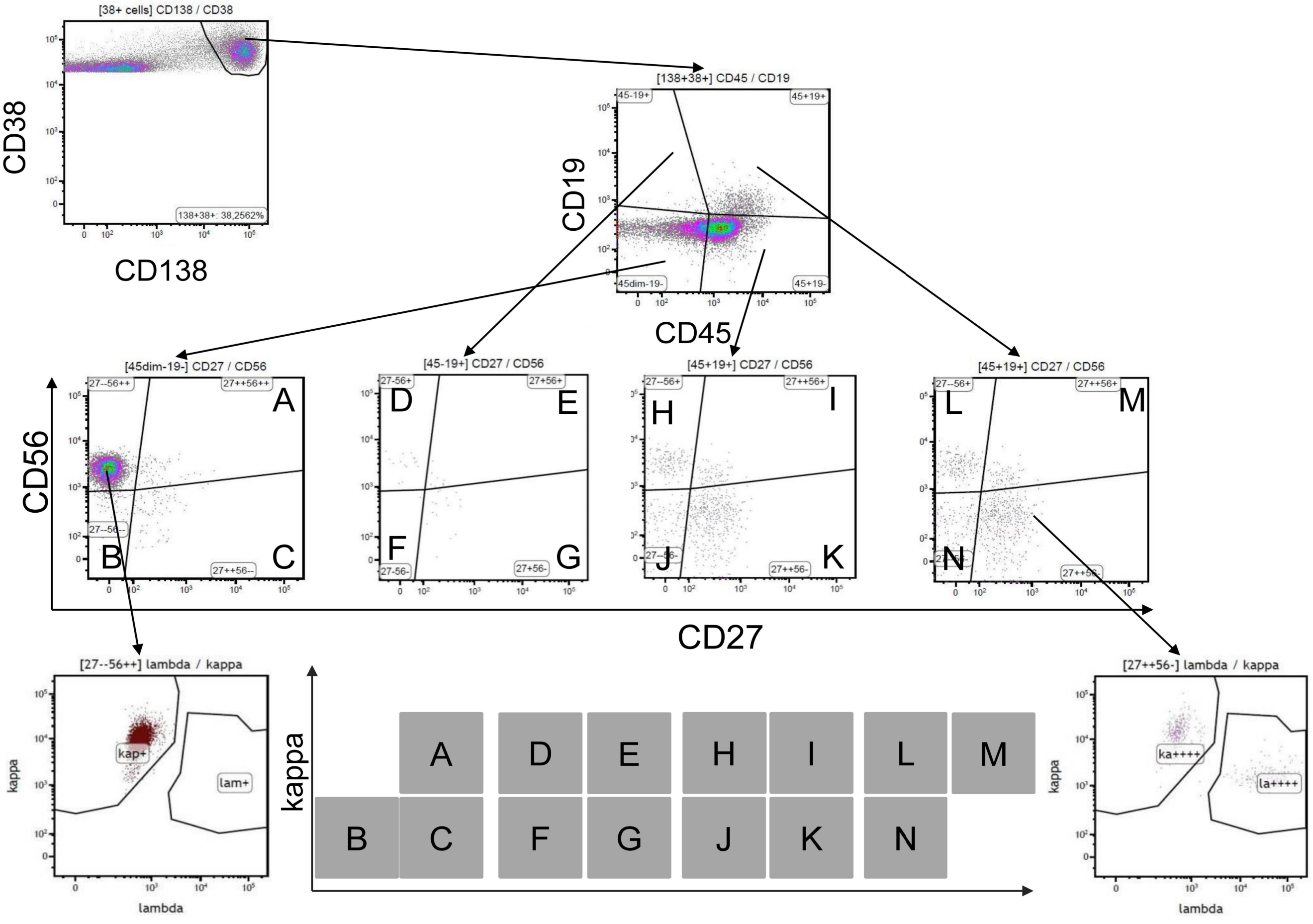
A

Gating 6-color panel

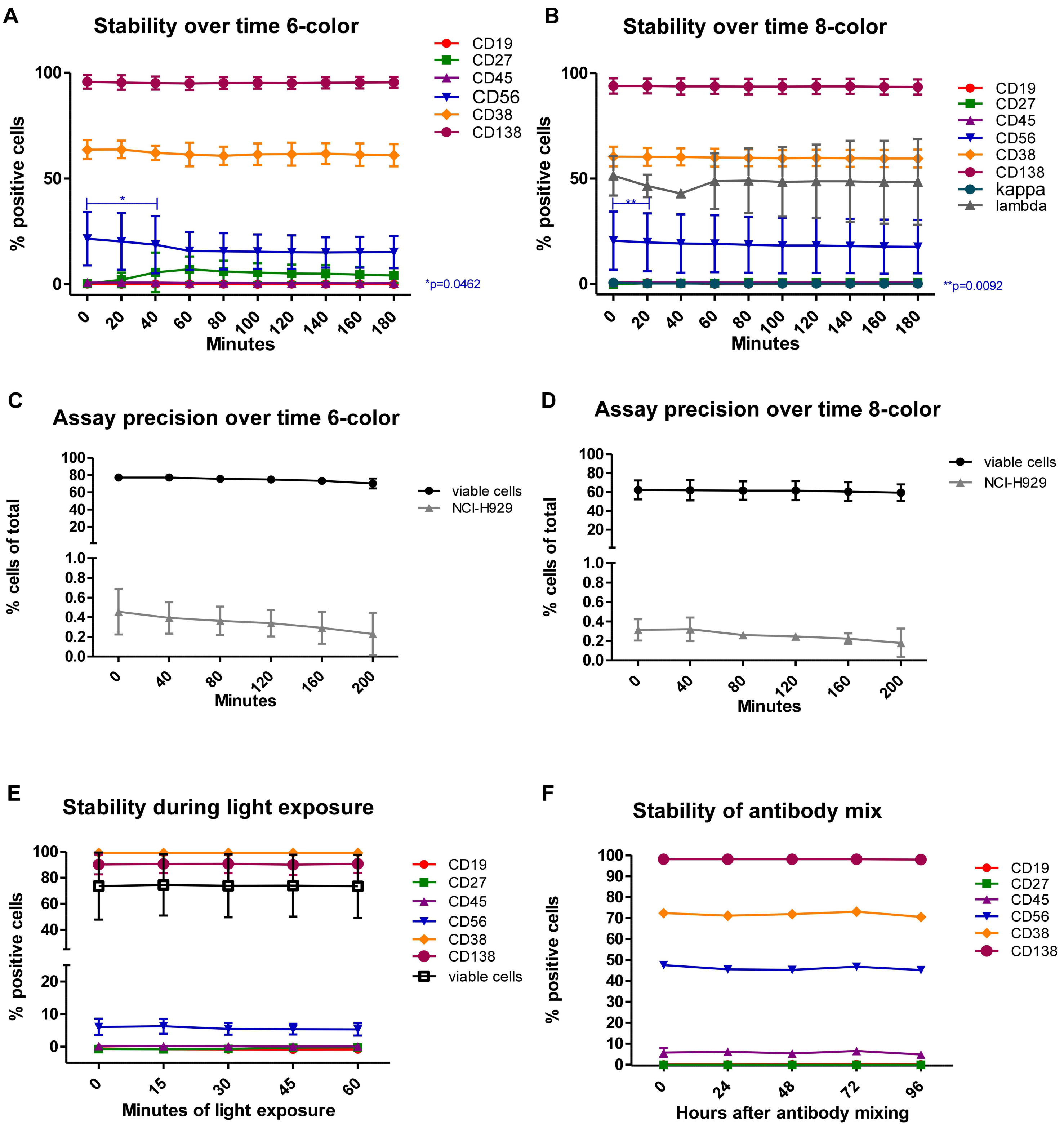


B

Gating 8-color panel

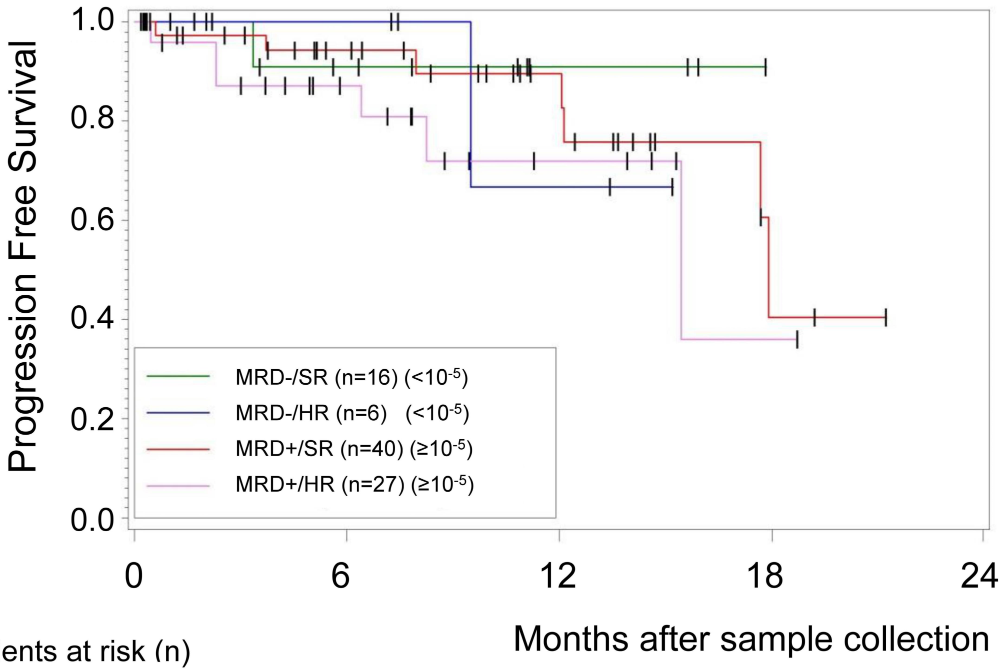


Suppl. Figure 2



Suppl. Figure 3

A



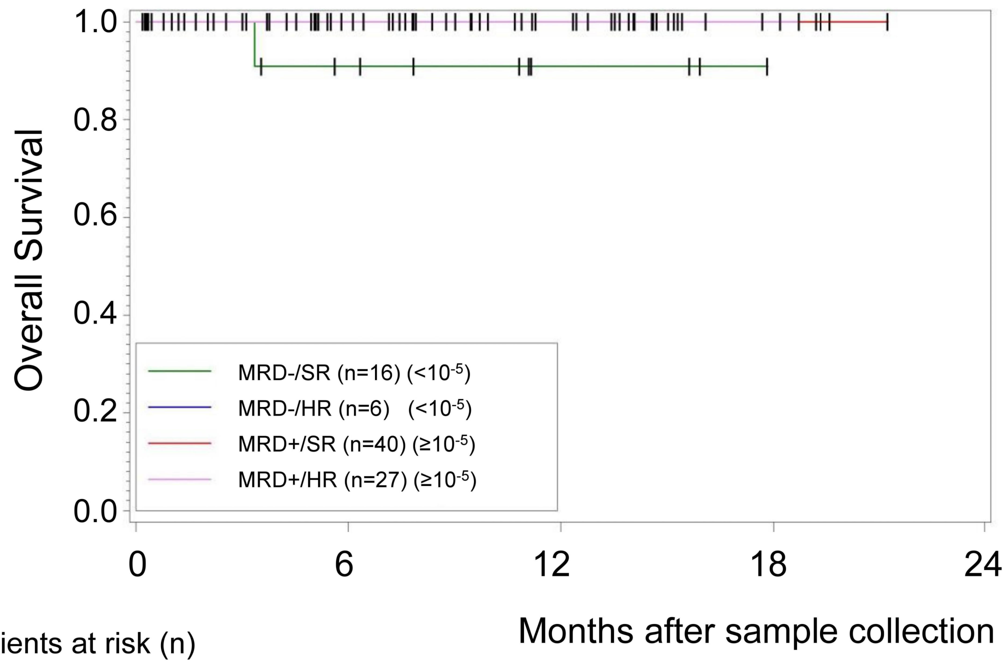
Median PFS
MRD-/SR: n.r.
MRD-/HR: n.r.
MRD+/SR: 17.9 months
MRD+/HR: 15.4 months

p= 0.5186

HR: 2.528 (MRD-/HR)
2.069 (MRD+/SR)
3.805 (MRD+/HR)

Patients at risk (n)					
MRD-/SR	16	8	3	0	0
MRD-/HR	6	5	2	0	0
MRD+/SR	40	24	13	2	0
MRD+/HR	27	14	5	1	0

B



Median OS
MRD-/SR: n.r.
MRD-/HR: n.r.
MRD+/SR: n.r.
MRD+/HR: n.r.

Patients at risk (n)					
MRD-/SR	16	8	3	0	0
MRD-/HR	6	5	3	0	0
MRD+/SR	40	25	15	5	0
MRD+/HR	27	17	7	1	0

Suppl. Figure 4