The impact of bilirubin ditaurate on platelet quality during storage.

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Supplementary Material 1

HPLC Method

Briefly, HPLC-PDA analysis was performed using a Shimadzu Prominence HPLC system consisting of an online degassing unit (DGU20A5R), solvent delivery unit (LC-20AT), autosampler (SIL-20 AC), column oven (CTO-20) and photo diode-array (SPD-M20A), connected in series. Separation was achieved via reverse phase C18 column (GraceSmart™ RP-C18, 150mm x 4.6mm, 3µm; Grace Davidson, Australia), which was preceded by a guard column (GraceSmart™ RP-C18, 3µm; Grace Davidson, Australia) and an UltraLine HPLC in-line filter (Restek, 0.5µm; Shimadzu, Sydney, Australia), respectively. The column oven and autosampler were set to 45˚C and 4˚C, respectively, with an initial flow rate of 1.6mL·min^{-1}. Starting mobile phase consisted of 20% organic B (100% HPLC grade MeOH) and 80% aqueous A (10mM NH_4OAc in 25% HPLC grade MeOH and 75% Milli-Q purified H_2O). A linear gradient was applied, reaching 90% B at 4.5 minutes, remaining until 7 minutes. From 7 to 11.5 minutes, the mobile phase was set at 20% B. To assist equilibration, flow rate remained at 1.6mL·min^{-1} until 6 minutes where it increased to 2.3mL·min^{-1} at 6.35 minutes, remaining until 8.5 minutes. From 8.5 to 9.5 minutes, the flow rate decreased to 1.6mL·min^{-1} and remained until 11.5 minutes. The total run time including adequate column re-equilibration was 11.5 minutes. For UCB and BRT extraction, 160µL of extractant (1:4 DMSO:MeOH) was added to 40µL of PPM sample, mixed via vortex for 10 seconds before centrifugation (21 500RCF; 10 minutes) to pellet protein. The supernatant was diluted (2:1) with Milli-Q H_2O upon addition to a HPLC vial, prior to being placed in the autosampler where 40µL was injected for analysis. Commercially prepared BRT and UCB (Frontier Scientific Inc. Logan, UT, USA) served as external standards with retention times of 4.1 and 5.1 minutes respectively. Extraction efficiencies of these compounds from PPM was 95% and 91% for BRT and UCB, with standard curves indicating great linearity over concentration ranges of 1.5625µM – 100µM (r^2 =0.9993) and 0.15625µM – 10µM (r^2 =0.9995)

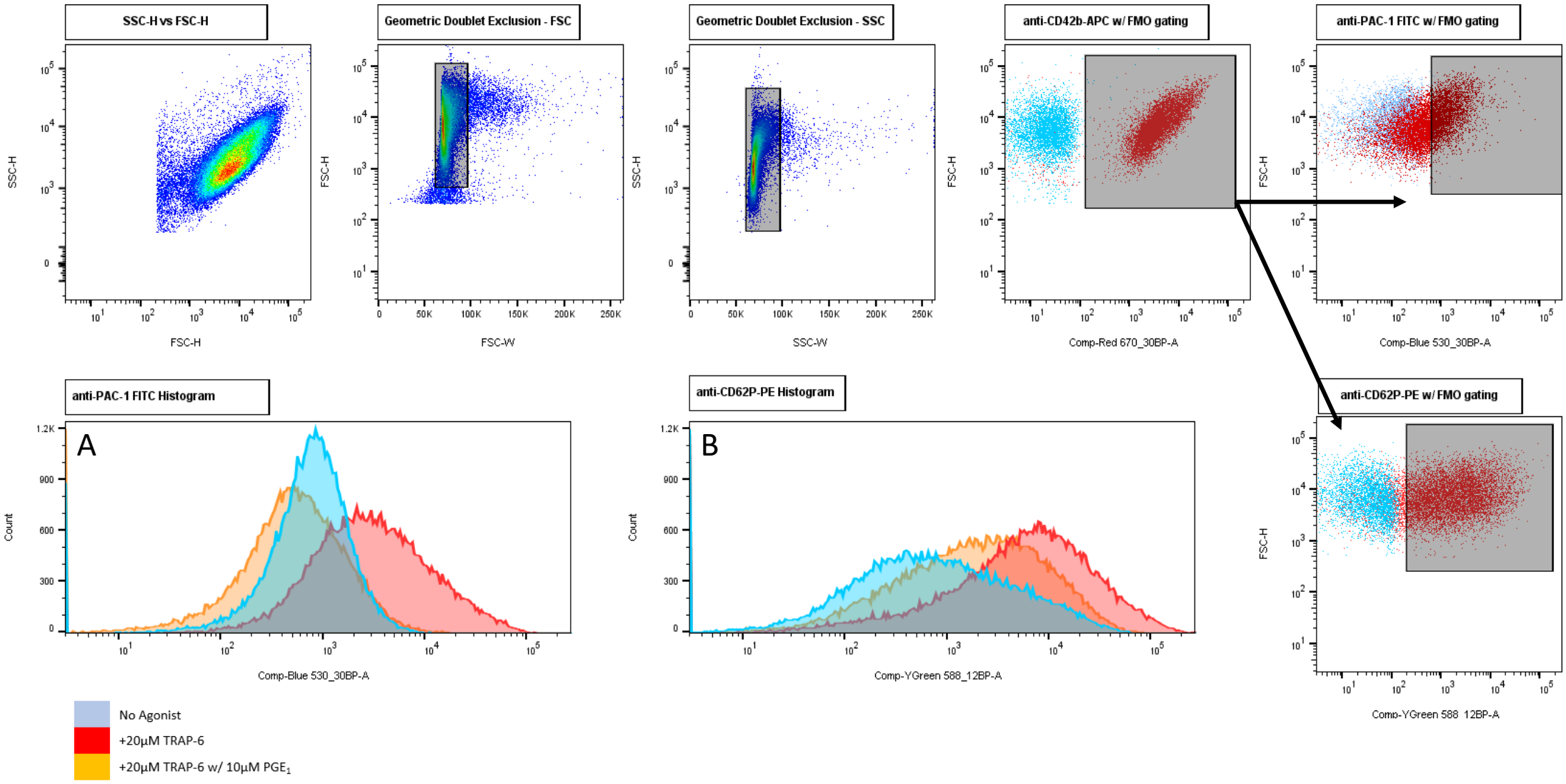
respectively. An approximate LOQ of 70nM and 800nM was reported for UCB and BRT respectively (Supplementary Material 9).

Supplementary Material 2

Cytometer Setup

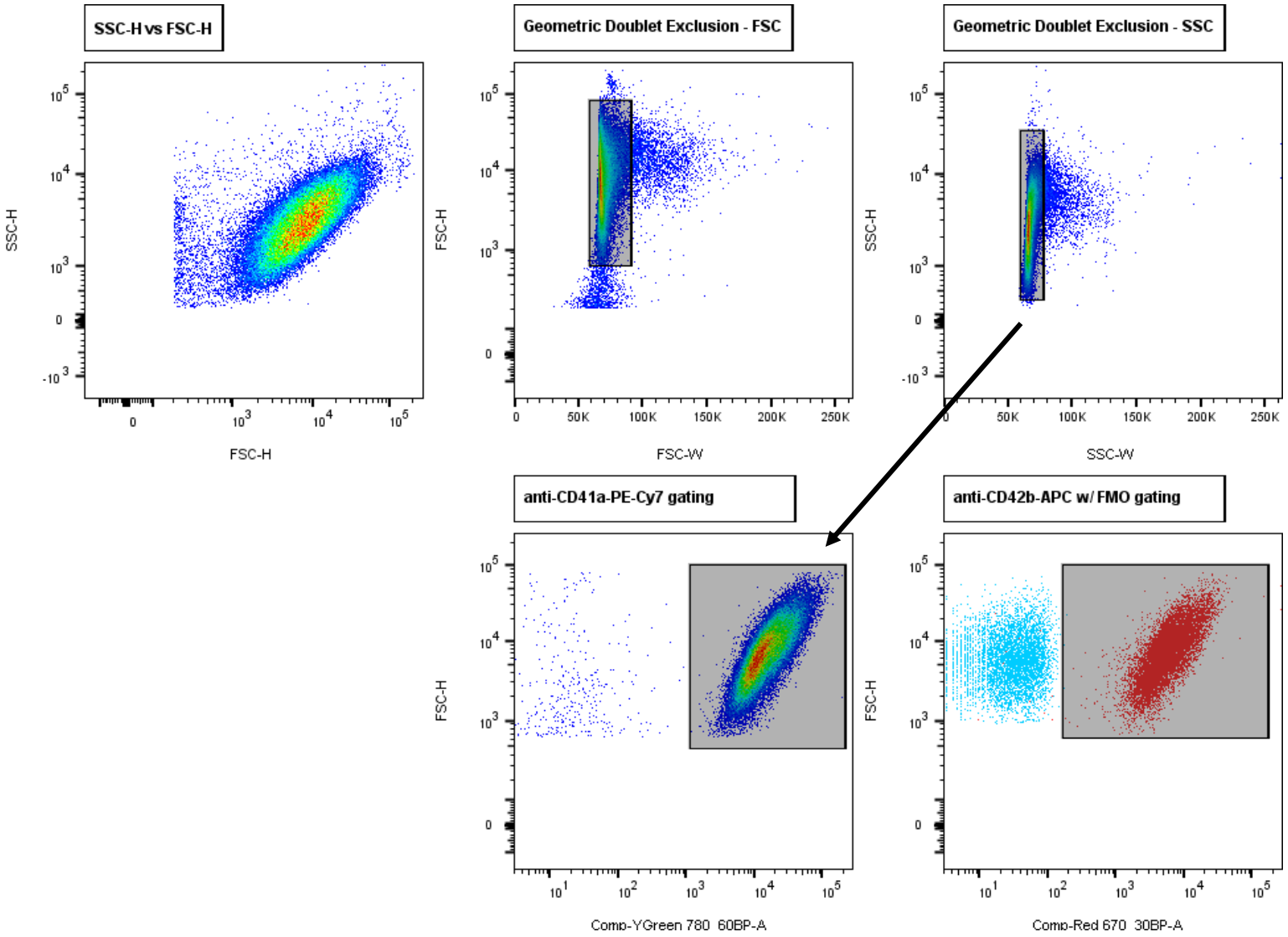
Flow Cytometric Analysis *parts previously published* [1] The BD SORP LSR II Fortessa flow cytometer was equipped with 355nm, 405nm, 488nm, 561nm and 640nm laser lines with detector filter sets for FITC (530/30BP), PE (588/12BP), PE-Cy7 (780/60BP), APC (670/30BP), BV421 (450/50BP), MitoSOX Red (585/42BP), MitoSPY Green (530/30BP) and Zombie Violet (450/50BP). The Guava EasyCyte 5HT flow cytometer was equipped with a single 488nm laser line and detector sets for Green (525/30), Yellow (583/26) and Red (690/50). Setup and tracking of cytometer performance was undertaken daily with OEM reference beads (CS&T FACSDiva 8 or Guava easyCheck Kit) with additional QC by way of the 6th peak method (RCP-30-5a-6, Spherotech USA)[2] All fluorescence values reported as median fluorescence intensity (MFI). Prior to analysis titration of fluorescent dye or antibody concentration was by way of staining index (not shown).[3] Methods utilizing antibody conjugated fluorophores were compensated with BD CompBeads (Anti-Mouse Ig, κ/Negative Control 552843) where 1µL of antibody was incubated with 1 drop of both positive and negative beads in the same manner as analytical samples. Fluorogenic dyes were compensated by way of a single stained sample either incubated at double the relevant analytical concentration (MitoSPY Green) or pharmacological treatment to induce the relevant marker upregulation. These samples were then mixed with unstained samples immediately prior to acquisition.[4] MitoSOX Red was analysed by 355nm laser line excitation due to the greater specificity of the specific superoxide product (2-OH-MitoE+) using this wavelength.[5,6] Relevant Fluorescence Minus One (FMO) controls were undertaken where necessary to establish percentage positive gating. All flow cytometric data analysis undertaken in FlowJo V10 (FlowJo LLC USA). The gating strategy adopted for both pulse geometry doublet exclusion and target population identification is presented in the following figures.

Supplementary Material 3



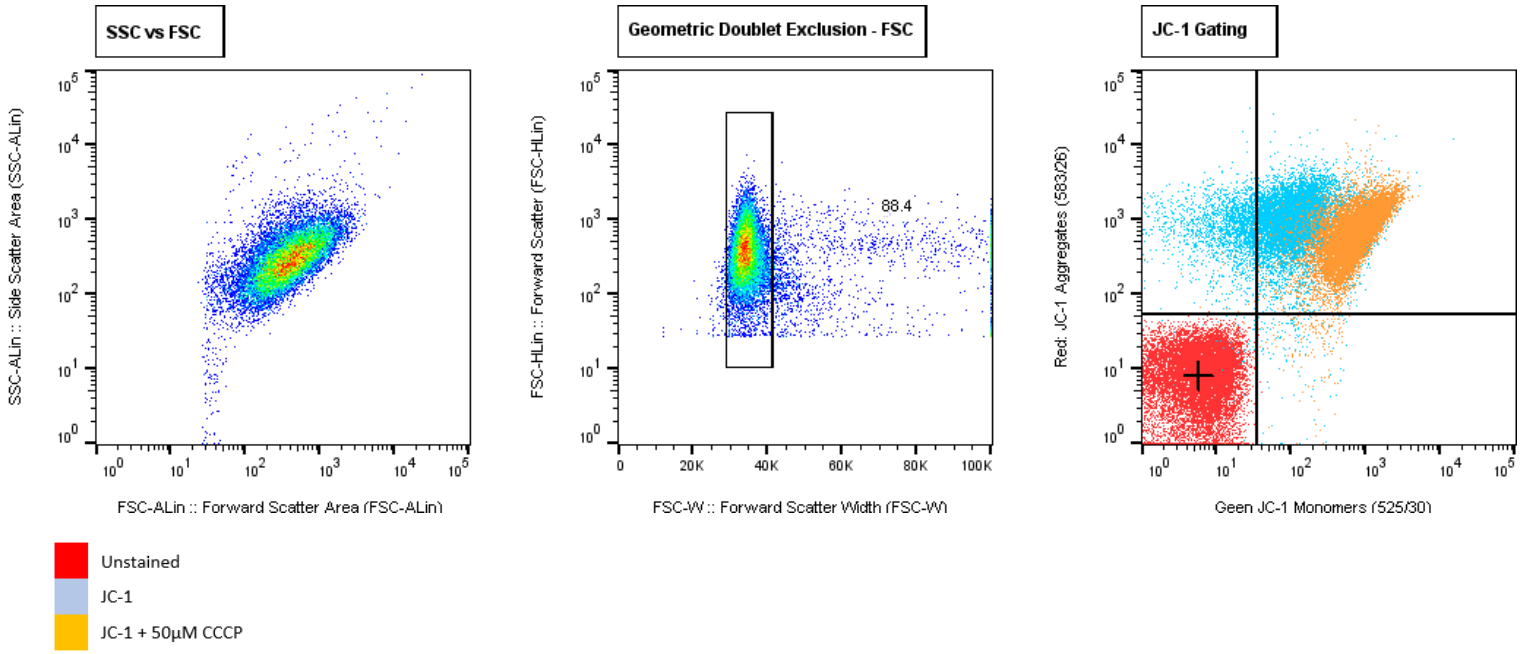
Representative flow cytometric gating strategy for exclusion of doublets by geometric pulse gating and analysis of platelet activation. **A)** Representative histogram of basal PAC-1 upregulation and following 20µM TRAP-6 stimulation with and without 10µM PGE_1 pre-treatment. **B)** Representative histogram of basal P-selectin expression and following 20µM TRAP-6 stimulation with and without 10µM PGE_1 pre-treatment.

Supplementary Material 4



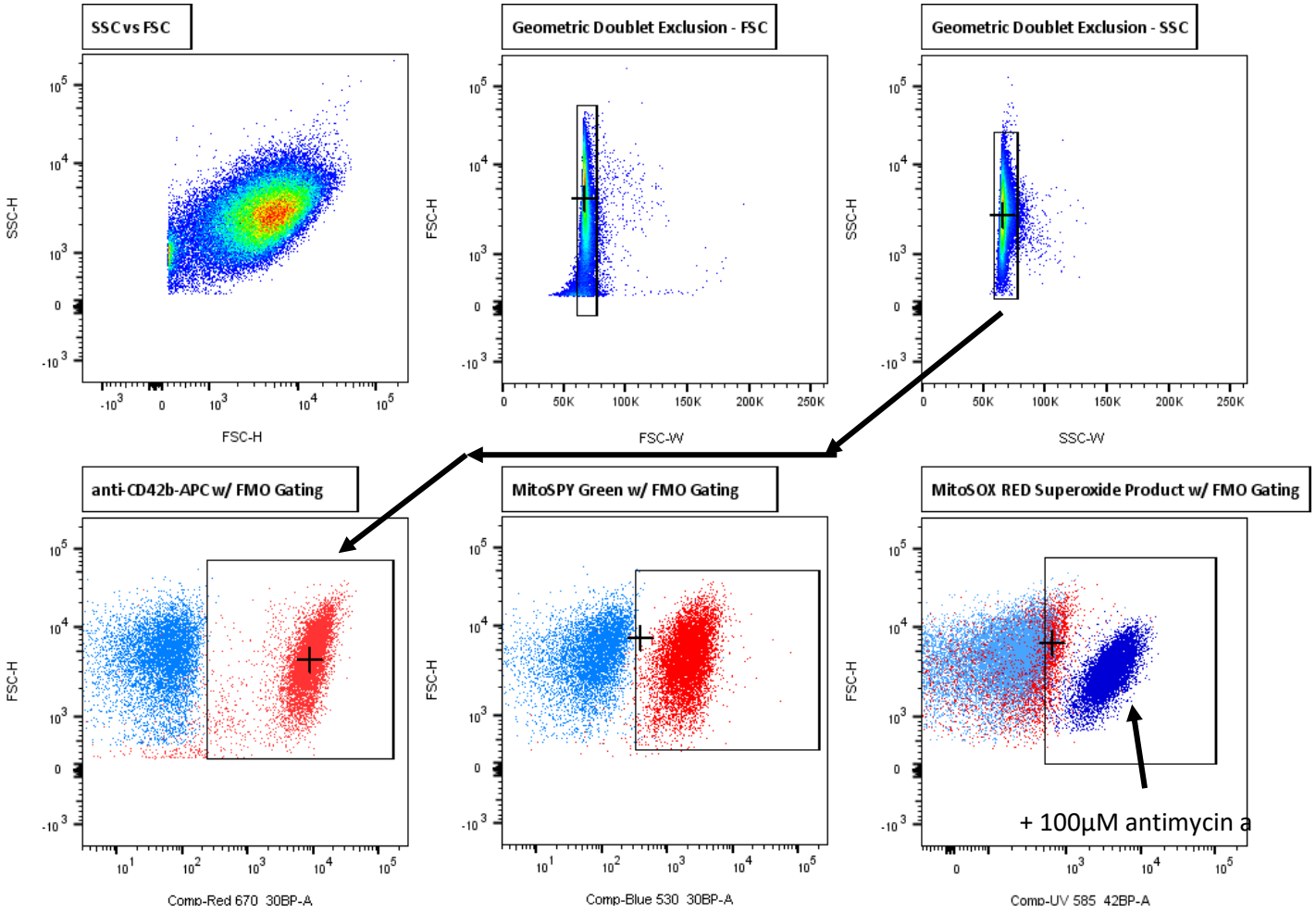
Representative flow cytometric gating strategy for exclusion of doublets by geometric pulse exclusion and analysis of platelet GPIbα (CD42b).

Supplementary Material 5



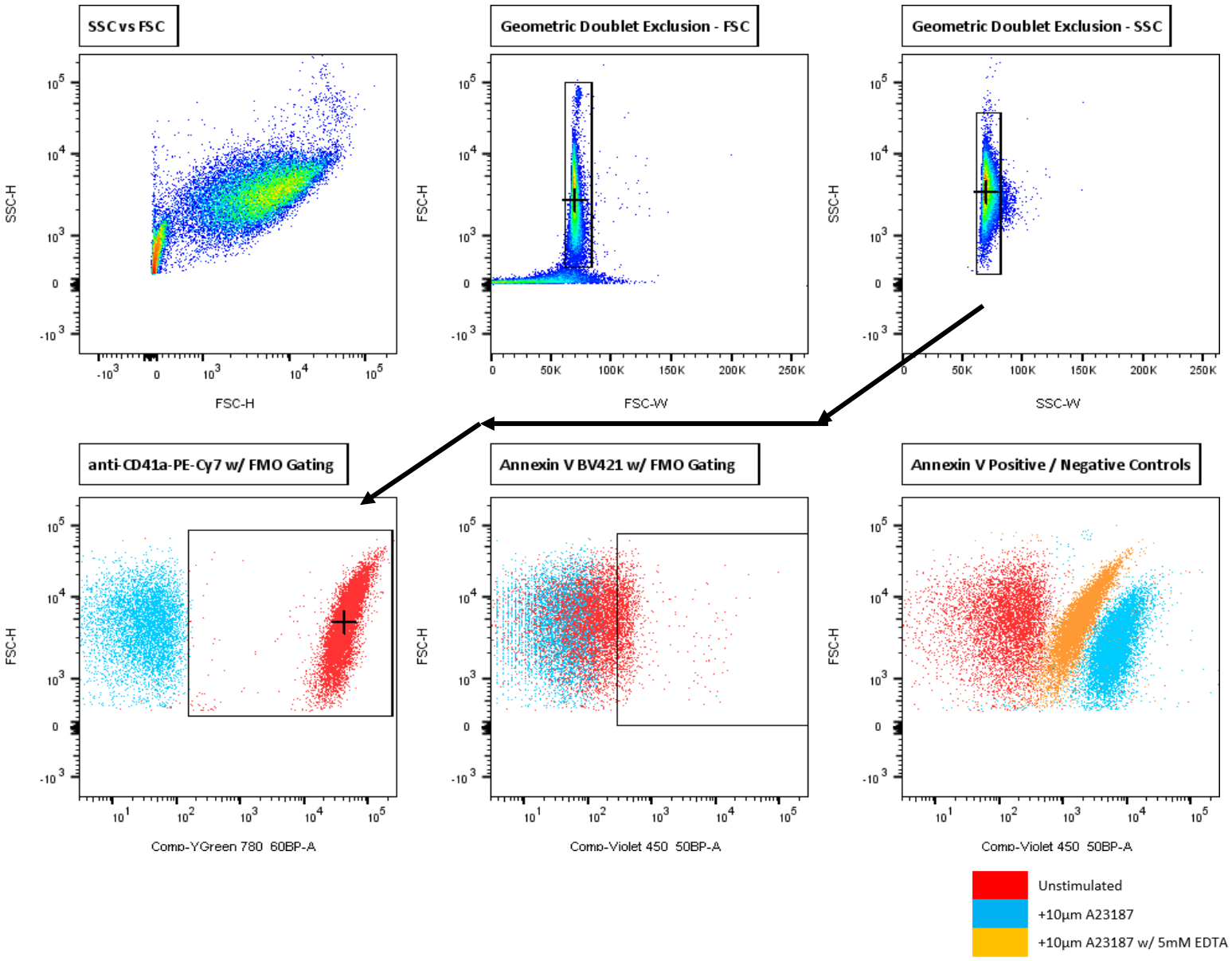
Representative flow cytometric gating strategy for exclusion of doublets by geometric pulse exclusion and analysis of platelet ΔΨm by JC-1. JC-1 red to green shift by way of 50µM CCCP addition prior to incubation (orange).

Supplementary Material 6



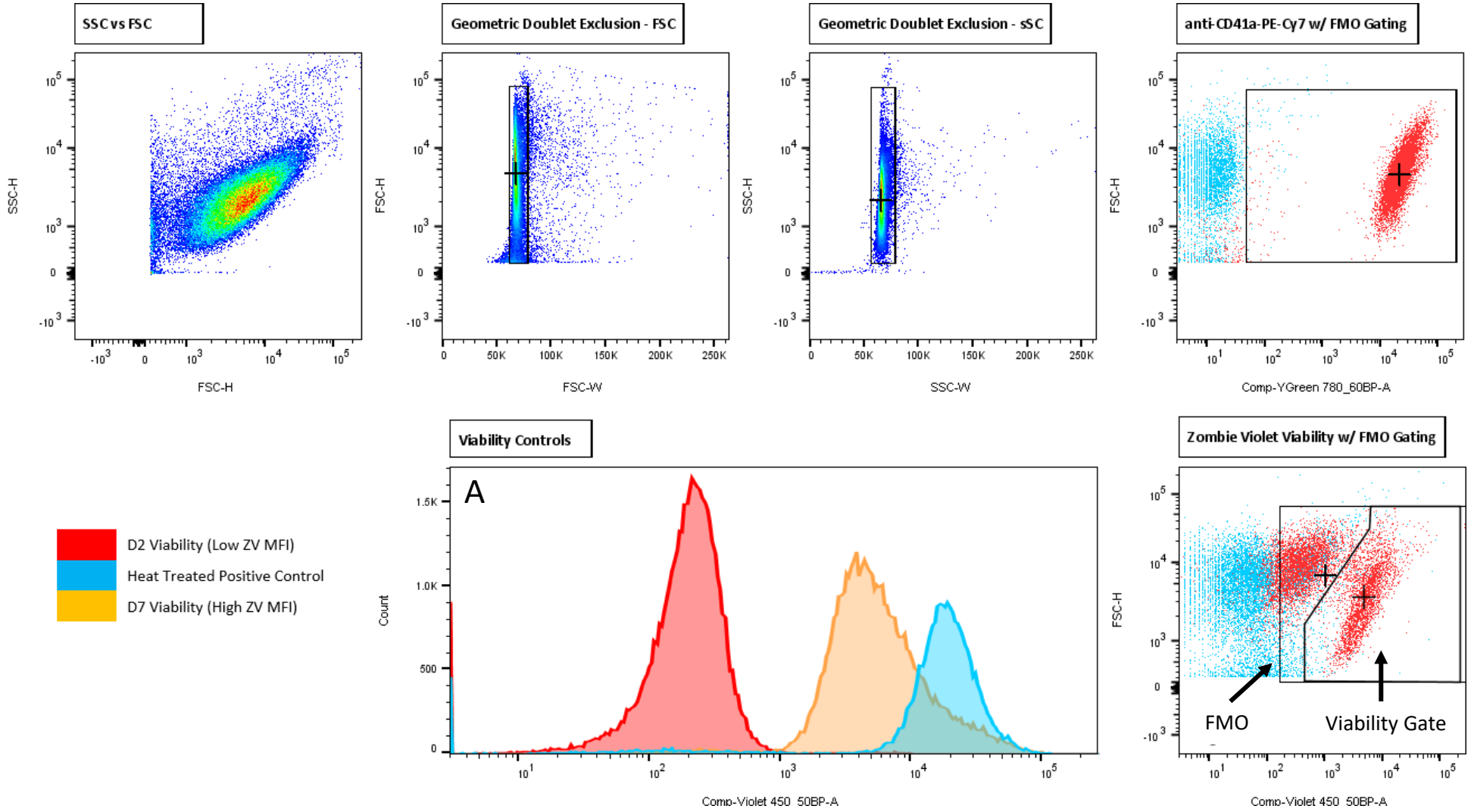
Representative flow cytometric gating strategy for exclusion of doublets by geometric pulse exclusion and analysis of mitochondrial superoxide generation.

Supplementary Material 7



Representative flow cytometric gating strategy for exclusion of doublets by geometric pulse exclusion and analysis of platelet phosphatidylserine expression by way of Annexin V staining.

Supplementary Material 8

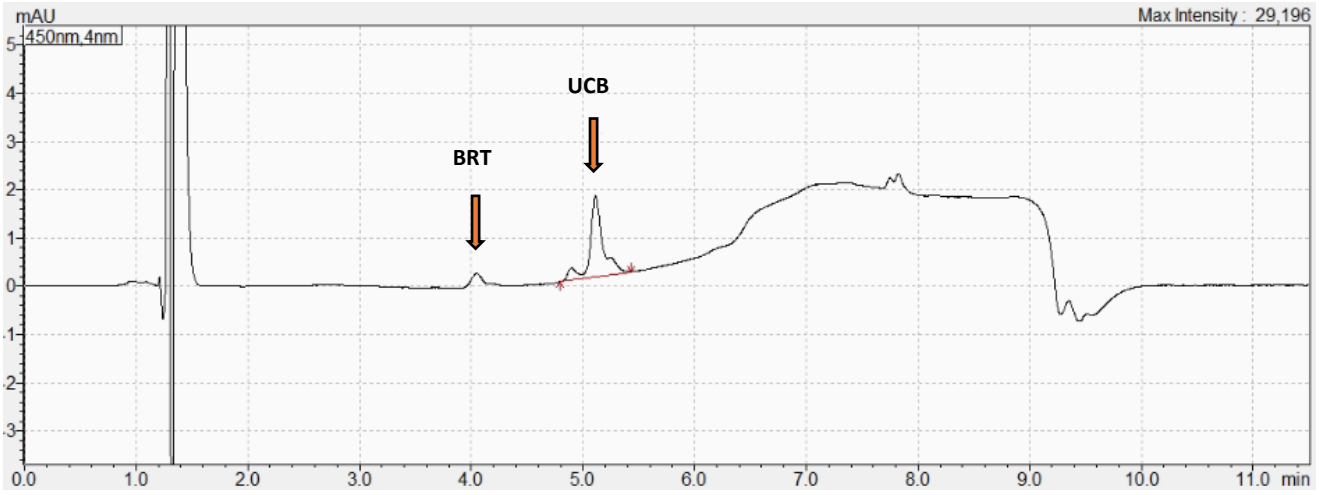


Representative flow cytometric gating strategy for exclusion of doublets and analysis of platelet viability by way of Zombie Violet Viability Dye. **A)** Representative histogram of Zombie Violet (ZV) MFI of platelets exhibiting high viability (D2) and low relative ZV MFI, platelets exhibiting low viability (D7) and high relative ZV MFI and heat-treated platelets for positive control of dead platelets. Data reported within MS is % viability, representative histograms for illustrative purposes only. Generation of % viability by way of gating exhibited above.

Supplementary Material 9

A) Representative chromatographic trace of an injected sample with the characteristic UCB peak (three total peaks) and BRT peak highlighted. **B)** UCB and BRT standard curve between 0.015625µM and 10µM and 1.5626µM and 100µM respectively. Reporting $R^2 = 0.9995$ and $R^2 = 0.9993$ for UCB and BRT respectively. **C)** Retention time, [ ], LOQ, noise and accuracy % of UCB standard curve presented in B. **D)** Retention time, [ ], LOQ, noise and accuracy % of BRT standard curve presented in B. **E)** Representative chromatographic traces of QC material, generated during method development. Briefly, appropriate volumes of a mixed UCB and BRT standard were spiked into 1mL of matrix material (300µL platelet poor plasma & 700µL SSP+) to generate a tri level of QC material. Level 0 was blank matrix material, Level 1 was +1µM UCB and + 10µM BRT, Level 2 was + 5µM UCB and + 30µM BRT and Level 3 was + 10µM UCB and + 50µM BRT. Aliquots of 40µL of each QC level were prepared in 0.625mL centrifuge tubes and snap frozen in liquid nitrogen and stored at -80°C, mimicking sample handling of analytical samples. When appropriate, one aliquot of each level was thawed to room temperature prepared for HPLC quantification was described in the Methods section. Following approximately 5 or 6 sample injections, a single QC level was injected for analysis. Level 0 was immediately run before analytical acquisition with the Level 4 QC injected immediately proceeding the final analytical sample, prior to the shutdown and cleaning method file. QC stability was tracked during and between analytical runs, with no significant degradation or difference reported between each of the batched runs.

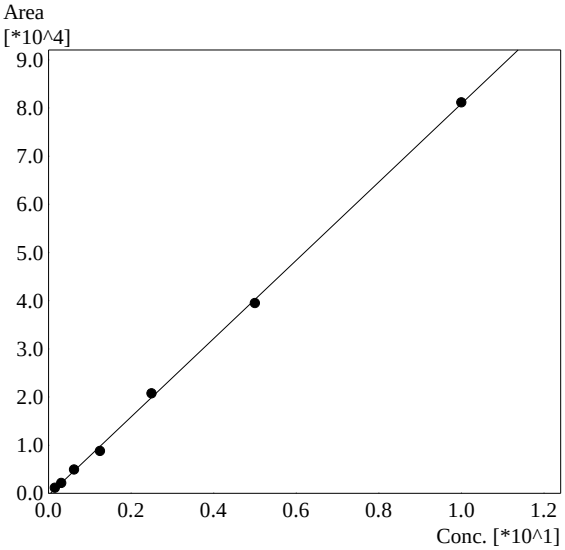
A)



B

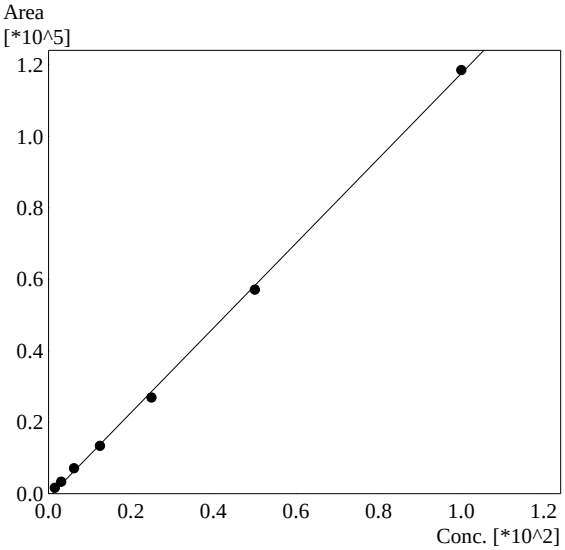
Calibration Curve

Name : UCB
Quantitative Method : External Standard
Function : f(x)=8124.89*x-398.330 (Linear)
R2 = 0.9995286



#	Conc.(uM)	MeanArea	Area	%CV
1	0.15625	1141	1187	3.73
			1103	
			1133	
2	0.3125	2185	2254	2.76
			2162	
			2140	
3	0.625	4981	4953	0.48
			4994	
			4995	
4	1.25	8798	8921	1.22
			8735	
			8736	
5	2.5	20744	20820	1.02
			20505	
			20905	
6	5	39481	39601	0.64
			39189	
			39652	
7	10	81112	80758	1.95
			79734	
			82842	

Name : BRDT
Quantitative Method : External Standard
Function : f(x)=1185.78*x-1036.02 (Linear)
R2 = 0.9993121



#	Conc.(uM)	MeanArea	Area	%CV
1	1.5625	1676	1719	3.83
			1706	
			1602	
2	3.125	3401	3439	1.74
			3332	
			3431	
3	6.25	7136	7162	0.83
			7068	
			7179	
4	12.5	13366	13390	1.18
			13198	
			13512	
5	25	26860	26970	1.19
			26501	
			27109	
6	50	57076	56980	0.62
			57469	
			56780	
7	100	118536	116629	1.56
			118653	
			120326	

C

<< PDA >>

ID#1 Compound Name: UCB

Title	Ret. Time	Conc.	Quantitative Limit	Noise	Accuracy[%]
std_ucb 0.15625_003.lcd	5.167	0.195	0.23	18.82	124.9
std_ucb 0.15625_026.lcd	5.158	0.185	0.26	19.44	118.3
std_ucb 0.15625_044.lcd	5.178	0.188	0.28	17.78	120.6
std_ucb 0.3125_004.lcd	5.167	0.326	0.20	18.25	104.4
std_ucb 0.3125_027.lcd	5.165	0.315	0.24	18.65	100.8
std_ucb 0.3125_045.lcd	5.171	0.312	0.21	15.36	100.0
std_ucb 0.625_005.lcd	5.166	0.659	0.12	11.62	105.4
std_ucb 0.625_028.lcd	5.155	0.664	0.20	16.35	106.2
std_ucb 0.625_046.lcd	5.168	0.664	0.17	12.91	106.2
std_ucb 1.25_006.lcd	5.163	1.147	0.15	14.43	91.8
std_ucb 1.25_029.lcd	5.159	1.124	0.15	12.15	89.9
std_ucb 1.25_047.lcd	5.164	1.124	0.14	10.98	89.9
std_ucb 2.5_007.lcd	5.157	2.612	0.17	15.98	104.5
std_ucb 2.5_030.lcd	5.151	2.573	0.19	15.17	102.9
std_ucb 2.5_048.lcd	5.152	2.622	0.17	13.40	104.9
std_ucb 5_008.lcd	5.164	4.923	0.13	12.33	98.5
std_ucb 5_031.lcd	5.150	4.872	0.14	11.08	97.4
std_ucb 5_049.lcd	5.159	4.929	0.16	12.46	98.6
std_ucb 10_009.lcd	5.159	9.989	0.12	10.45	99.9
std_ucb 10_032.lcd	5.167	9.863	0.12	9.87	98.6
std_ucb 10_050.lcd	5.155	10.245	0.10	7.69	102.5
Average	5.162	2.835	0.17	14.06	103.2
%RSD	0.139	119.990	28.248	23.726	8.775

ID#2 Compound Name: BRDT

Title	Ret. Time	Conc.	Quantitative Limit	Noise	Accuracy[%]
std_ucb 0.15625_003.lcd	-	-	-	-	0.0
std_ucb 0.15625_026.lcd	-	-	-	-	0.0
std_ucb 0.15625_044.lcd	-	-	-	-	0.0
std_ucb 0.3125_004.lcd	-	-	-	-	0.0
std_ucb 0.3125_027.lcd	-	-	-	-	0.0
std_ucb 0.3125_045.lcd	-	-	-	-	0.0
std_ucb 0.625_005.lcd	-	-	-	-	0.0
std_ucb 0.625_028.lcd	-	-	-	-	0.0
std_ucb 0.625_046.lcd	-	-	-	-	0.0
std_ucb 1.25_006.lcd	-	-	-	-	0.0
std_ucb 1.25_029.lcd	-	-	-	-	0.0
std_ucb 1.25_047.lcd	-	-	-	-	0.0
std_ucb 2.5_007.lcd	-	-	-	-	0.0
std_ucb 2.5_030.lcd	-	-	-	-	0.0
std_ucb 2.5_048.lcd	-	-	-	-	0.0
std_ucb 5_008.lcd	-	-	-	-	0.0
std_ucb 5_031.lcd	-	-	-	-	0.0
std_ucb 5_049.lcd	-	-	-	-	0.0
std_ucb 10_009.lcd	-	-	-	-	0.0
std_ucb 10_032.lcd	-	-	-	-	0.0
std_ucb 10_050.lcd	-	-	-	-	0.0
Average	-	-	-	-	0.0
%RSD	0.000	0.000	0.000	0.000	0.000

D

Summary(Compound)

<< PDA >>

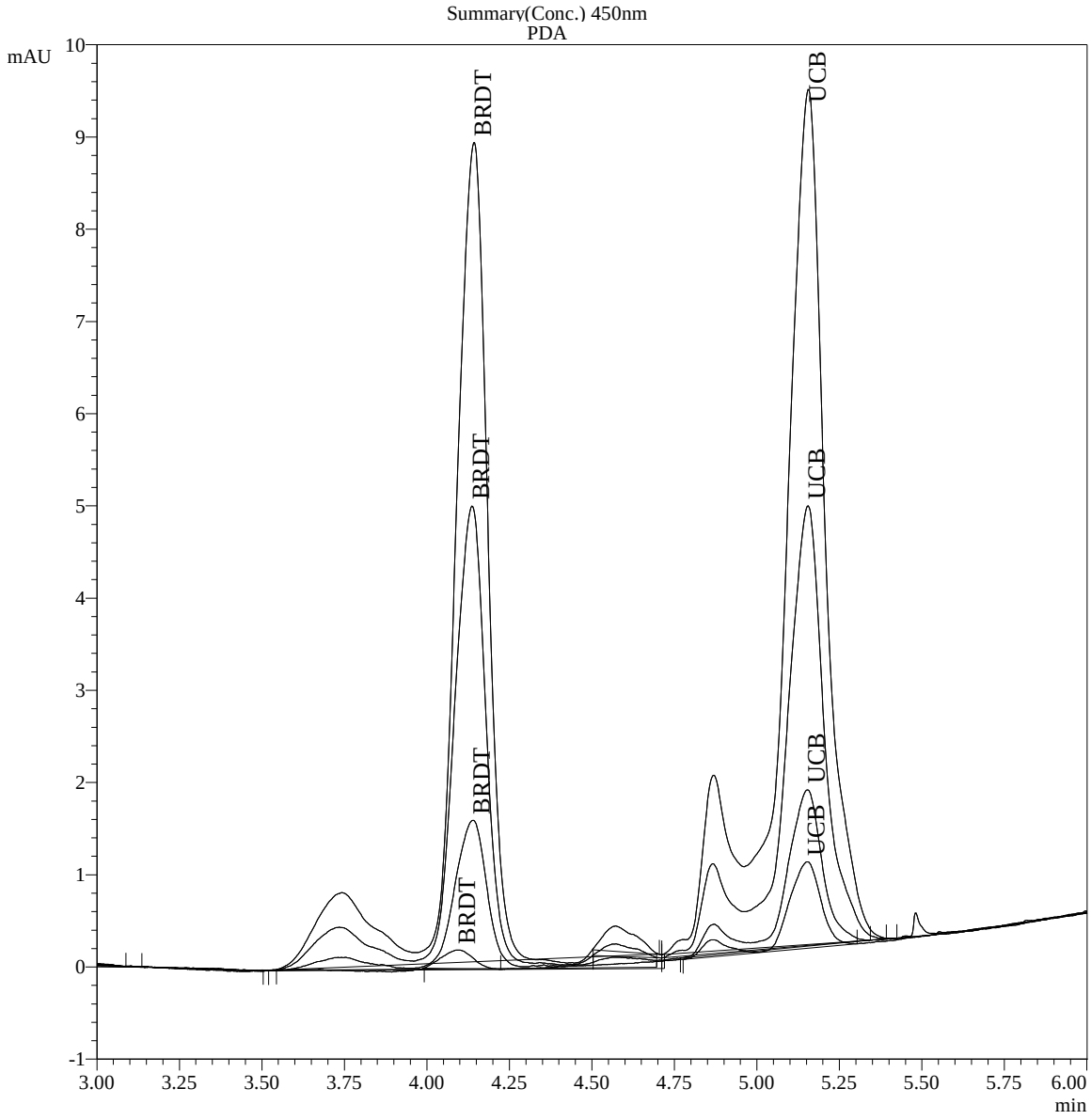
ID#1 Compound Name: UCB

Title	Ret. Time	Conc.(uM)	Quantitative Limit	Noise	Accuracy[%]
std_brt 1.5625_012.lcd	-	-	-	-	0.0
std_brt 1.5625_035.lcd	-	-	-	-	0.0
std_brt 1.5625_053.lcd	-	-	-	-	0.0
std_brt 3.125_013.lcd	-	-	-	-	0.0
std_brt 3.125_036.lcd	-	-	-	-	0.0
std_brt 3.125_054.lcd	-	-	-	-	0.0
std_brt 6.25_014.lcd	-	-	-	-	0.0
std_brt 6.25_037.lcd	-	-	-	-	0.0
std_brt 6.25_055.lcd	-	-	-	-	0.0
std_brt 12.5_015.lcd	-	-	-	-	0.0
std_brt 12.5_038.lcd	-	-	-	-	0.0
std_brt 12.5_056.lcd	-	-	-	-	0.0
std_brt 25_016.lcd	-	-	-	-	0.0
std_brt 25_039.lcd	-	-	-	-	0.0
std_brt 25_057.lcd	-	-	-	-	0.0
std_brt 50_017.lcd	-	-	-	-	0.0
std_brt 50_040.lcd	-	-	-	-	0.0
std_brt 50_058.lcd	-	-	-	-	0.0
std_brt 100_018.lcd	-	-	-	-	0.0
std_brt 100_041.lcd	-	-	-	-	0.0
std_brt 100_059.lcd	-	-	-	-	0.0
Average	-	-	-	-	0.0
%RSD	0.000	0.000	0.000	0.000	0.000

ID#2 Compound Name: BRDT

Title	Ret. Time	Conc.(uM)	Quantitative Limit	Noise	Accuracy[%]
std_brt 1.5625_012.lcd	4.128	3.01	1.78	14.72	192.9
std_brt 1.5625_035.lcd	4.123	3.01	1.41	9.92	192.3
std_brt 1.5625_053.lcd	4.112	2.91	1.95	12.82	186.0
std_brt 3.125_013.lcd	4.127	3.39	0.74	9.58	108.4
std_brt 3.125_036.lcd	4.117	3.28	1.03	11.70	104.9
std_brt 3.125_054.lcd	4.121	3.51	0.99	10.46	112.4
std_brt 6.25_014.lcd	4.126	6.63	0.65	9.23	106.1
std_brt 6.25_037.lcd	4.115	6.34	0.76	10.02	101.4
std_brt 6.25_055.lcd	4.119	6.67	0.91	11.21	106.7
std_brt 12.5_015.lcd	4.127	12.21	0.61	8.76	97.7
std_brt 12.5_038.lcd	4.111	12.13	0.81	10.35	97.1
std_brt 12.5_056.lcd	4.115	12.24	0.74	9.03	97.9
std_brt 25_016.lcd	4.135	22.63	0.72	11.79	90.5
std_brt 25_039.lcd	4.116	22.48	0.54	7.39	89.9
std_brt 25_057.lcd	4.108	24.88	0.76	9.77	99.5
std_brt 50_017.lcd	4.119	49.34	0.14	2.00	98.7
std_brt 50_040.lcd	4.112	48.89	0.39	5.34	97.8
std_brt 50_058.lcd	4.113	49.33	0.69	9.15	98.7
std_brt 100_018.lcd	4.119	100.36	0.46	6.76	100.4
std_brt 100_041.lcd	4.118	100.76	0.33	4.39	100.8
std_brt 100_059.lcd	4.110	101.33	0.81	10.60	101.3
Average	4.119	28.35	0.82	9.29	113.4
%RSD	0.172	120.002	53.387	31.059	28.809

E



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Title	Sample Name	UCB	BRDT
std_QC Level 0_021.lcd	QC Level 0	0.93 uM	2.74 uM
std_QC Level 1_022.lcd	QC Level 1	1.78 uM	11.72 uM
std_QC Level 2_023.lcd	QC Level 2	5.44 uM	32.40 uM
std_QC Level 3_024.lcd	QC Level 3	10.76 uM	51.49 uM

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