March 24th, 2023

2023_ILT_06

1250 c/l/l 80/l · 950 c/l/l 60/l · 1010 c/l/l 60/l · 1630 c/l/l 60/l · 1450 c/l/l 60/l · 540 c/l/l 40/l · 550 c/l/l 40/l

Specimen	Status	Location	Conc	Date	Tasks	Volume	Ly (1:100)	Ly+Mon	Total	3E+6	.3E+6
Inf171-9 a-3	HU ↗ SQ45	(4)	<9/17			2	7.76	12.1			
Inf248-4 a-3	HEU-lo ♀ SFH8	(5)				0.75	4.16	8.08	3.12		
248 a-4	SFH9	(6)	<157			0.75	3.67	8.51	2.75 (+2.93)		
Inf023-5 a-3	HEU-hi ♀ RQ42	(3)	<1687			0.75	5.39	9.07	4.04		
023 a-4	RQ43	(7)	<187			0.75	5.02	8.72	3.76 (+3.9)		
ND050	Adult Norm	(1)	2x10^7 <147	1/13/23		1.5	6.82	9.14			
ND006	Adult SC	(2)	1.5x10^6 <127	1/20/23		1.5	8.33	10.5			

11:19 thaw start ...
27 cond
12:23 stain
(12:56) Not enough ~~for 248 or 23~~
:00 aliquots
:24 Rox cells ✓

+Inf248-4 SFH4 a-5 Box 1B
+Inf023-5 RQ44 a-5

5M · SC 3M · ~1.5 · 2.5 · ~150 · 3M · 3M · 3M · 1.3M

Inf171	15.52	÷ 2
Inf248	~8.82	÷ 2.5
Inf023	11.07	÷ 2.5
ND050	10.23	÷ 1.5
ND006	12.495	÷ 1.5

Incubation @ 2:55pm (3½ hours) → 8:55pm
Ab prep @ 8:11pm
Prepped @ 8:35pm
9:04pm spin down
L/D @ 9:20 → 35
Sc's aliquoted +FBS wash @ 9:40pm
22:01 Hot samples → 31pm spin @ 10:36pm
Sc's hot @ 10:12 pm → 42 pm
Sc's cold @ 10:21 pm → 51 pm
Tcts @ 10:50pm
Va's 11:01 → 11:31pm
FixPerm Sc's @ 11:01 → 22 → 31
Cold stain 52 @ 11:48pm → 00:18 am (RBC lyse!!)
@ 00:21 → 24
23:57 2nd PermWash of sc's
Scs intra @ 12:18 am → 12:58 am

~~PBS~~	
162.9 PBS	176.5 Perm
9.6 Va7.2	4
12.0 Va24ja18	4

RBC FBS spin @ 12:27pm
FixPerm @ 12:39 → 49 → 59
1:04 am combined FixPerm spin
1:30 am Intracellular → 2:10 am
2:12am Final Spin
Done @ 2:25 am

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1:09 pm: yesterdays QC's failed on UV16
↳ water had cells in it, throwing off QC?

QC attempt @ 1:19 pm

Atta girl! - QC passed

Start @ 01:31 pm (check volume)

27 µl/min 6,600 event rate

~28 µl

1 hr

1:05

Unmixing at cell by cell level

matrix generation is @ population level define your scs

2000 cells

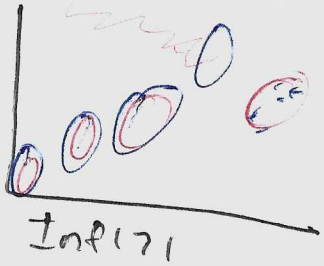
18 infants

2 - 3

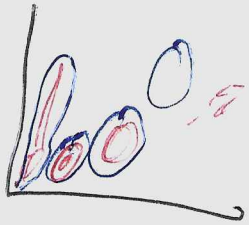
36 48

Zombie cord (50µl obt)

~ 2800 events ~600k cells

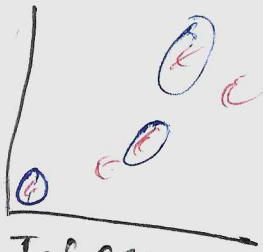


Inf171



Inf 248

~3M



Inf023

Done @ 3:28 pm

Sample	Total	Volume	Concentration	3M	2.75M	2.5M	2.25M	2.0M	1.75M	1.5M	1.25M	1.0M	0.75M	0.5M
INF171	12.52	2	6.26	0.48	0.44	0.40	0.36	0.32	0.28	0.24	0.20	0.16	0.12	0.08
INF248	8.82	2.5	3.53	0.85	0.78	0.71	0.64	0.57	0.50	0.43	0.35	0.28	0.21	0.14
INF023	11.07	2.5	4.43	0.68	0.62	0.56	0.51	0.45	0.40	0.34	0.28	0.23	0.17	0.11
ND050	10.23	1.5	6.82	0.44	0.40	0.37	0.33	0.29	0.26	0.22	0.18	0.15	0.11	0.07
ND060	12.5	1.5	8.33	0.36	0.33	0.30	0.27	0.24	0.21	0.18	0.15	0.12	0.09	0.06

R10

Sample	3M	2.75M	2.5M	2.25M	2.0M	1.75M	1.5M	1.25M	1.0M	0.75M	0.5M
INF171	0.52	0.477	0.434	0.39	0.347	0.303	0.26	0.216	0.173	0.13	0.087
INF248	0.15	0.137	0.124	0.11	0.099	0.087	0.07	0.062	0.050	0.04	0.025
INF023	0.32	0.295	0.268	0.24	0.214	0.188	0.16	0.134	0.107	0.08	0.054
ND050	0.56	0.513	0.466	0.42	0.373	0.326	0.28	0.233	0.186	0.14	0.094
ND060	0.64	0.586	0.533	0.48	0.426	0.373	0.32	0.266	0.213	0.16	0.107

PMA

Sample	3M	2.75M	2.5M	2.25M	2.0M	1.75M	1.5M	1.25M	1.0M	0.75M	0.5M
INF171	2	1.83	1.66	1.5	1.32	1.16	1	0.83	0.66	0.5	0.33
INF248	2	1.83	1.66	1.5	1.32	1.16	1	0.83	0.66	0.5	0.33
INF023	2	1.83	1.66	1.5	1.32	1.16	1	0.83	0.66	0.5	0.33
ND050	2	1.83	1.66	1.5	1.32	1.16	1	0.83	0.66	0.5	0.33
ND060	2	1.83	1.66	1.5	1.32	1.16	1	0.83	0.66	0.5	0.33

CD107a

Sample	3M	2.75M	2.5M	2.25M	2.0M	1.75M	1.5M
INF171	6	5.5	5	4.5	4	3.5	3
INF248	6	5.5	5	4.5	4	3.5	3
INF023	6	5.5	5	4.5	4	3.5	3
ND050	6	5.5	5	4.5	4	3.5	3
ND060	6	5.5	5	4.5	4	3.5	3

March 24th, 2023   2023_ILT_06

#	Filter	Single color (ul)	Ref ctrl	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	14	L/D 15 min (RT)	Tetramer 40 min @ RT	HotStain 30min @37C	14	ColdStain 30min @4C	14	RBC Lyse, then Fix/Perm	Spiked 30 min @RT	14
1	UV2				BUV395	CD62L	SK11								1.2	16.8			
					AF	AF-UV6													
2	UV7				BUV496	CD8	RPA-T8								0.7	9.8			
3	UV9				BUV563	CD69	FN50								0.5	7.0		1	14.0
4	UV10				BUV615	CCR4	1G1						2.0	28					
5	UV11				BUV661	Vδ2	B6								0.7	9.8		0.5	7.0
6	U14				BUV737	CXCR3	1C6/CXCR3								1.3	18.2			
7	UV16				BUV805	CD4	SK3						1.5	21				0.5	7.0
8	V1				BV421	CD127	A019D5						1.5	21					
9	V3				Pacific Blue	CD14	M5E2								2.0	28.0			
	V3				Pacific Blue	CD19	SJ25C1								2.0	28.0			
10	V5				BV480	CD161	HP-3G10						2	28					
11	V7				BV510	CD45RA	HI100								0.7	9.8			
12	V10				BV605	CD56	5.1H11						1.0	14					
13	V11				BV650	CCR7	G043H7						1	14				1	14.0
14	V13				BV711	CD7	M-T701						1	14					
15	V14				BV750	IFNγ	B27											1.5	21.0
16	V15				BV786	CCR6	11A9						1.5	21					
17	B2				FITC/AF488	Va24/hCD1d	6B11												
18	B3				Spark blue 550	CD3	SK7								1.2	16.8		0.5	7.0
19	B4				PE	NKG2D	1D11						1.5	21				0.5	7.0
20	B6				PE-CF594	CD26	M-A261						1.2	16.8					
21	B8				PE-Cy5	CD25	M-A251						1.2	16.8				0.5	7.0
22	B10				PerCP-Cy5.5	TNFα	MAb11											2.5	35.0
23	B13				PE-vio770	PD1	PD1.3.1.3								1.5	21.0		0.5	7.0
24	R1				APC	CD16	3G8								0.7	9.8			
25	R2				AlexaFluor647	Va7.2/hMR1	3C10												
26	R4				APC-R700	CD107a	H4A3		6.0	84									
27	R6				Zombie NIR	L/D	N/A				<1:2500>								
28	R7				APC/Fire 750	CD27	O323						2	28					
29	R8				APC/Fire 810	CD38	HIT2						1.6	22.4					
							Antibody Total		6.0	84	Antibody Total		19.0	266	12.5	175.0		9	126
And UNSTAINED CONTROLS !!!							R10 Media		14.5	203	Brilliant Stain		50	700	50.0	700		50	700
							Pippette draw volume /sample		19.5		Pippette draw volume /sample		66		59.5			56	

64   57.5   54

37°C   4°C

	37°C	4°C
PMA × 2	1	3½
Ctrl × 2	3(+Va7.2)	(4) ND050 + ctrl unstained
Ctrl × 1	9	8

+ ND006 ctl/PMA unstained s

+ CD107a (ND050 PMA +)

+ Zombie Cord (Inf Hu) 50 μl of volume

Vd2 CD14 PD1 CCR3

Simplified Protocol

Thaw cells, DNAse, count.
Collect, count, aliquot cells 2-3.0E+6 Cells R10 / 5ml polystyrene tube
Bring volume upto 1 ml R10, add 2 ul PMA/Ctrl and CD107a
Cap and incubate at 37°C for 6 hours

Wash with 2 ml PBS, spin down 1300rpm 8min
800 ul of LiveDead mix (1:2500) @RT for 15min
Wash 2 ml 5% PBS-FBS, spin 1300 rpm, 8min

Add HotStain mix, incubate @37C for 30 min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6 min

Add Tetramers, incubate @RT for 10 min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6 min

Add ColdStain mix, incubate @ 4C for 30min
Add 300-500 ul 1x RBC Lysis for 3 minutes
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

300 ul BD FixPerm, incubate @ 4C for 20min
(vortex every 10 minutes)

First PermWash: 1 ml PermWash 1500 rpm 6 min
Second Perm Wash: 1 ml PermWash 1500 rpm 6 min

Add Intracellular Stain, incubate @ RT for 40min
First PermWash: 2 ml PermWash 1500 rpm 6 min

Resuspend in 70 ul 0.4% PFA-PBS
Cap tubes, wrap rack in foil, store at 4°C

1.5/1.2 antibody

ILT & NK SFC Panel

3/24/2023

Spectrum	UV			Violet			Blue			Red			
373	UV1												
388	UV2	BUV395	[2] CD62L										
428	UV3			V1	BV421	[4] CD127							
443	UV4			V2									
458	UV5			V3	PacBlue	[1] CD14/19							
473	UV6	AF		V4									
508				V5	BV480	[3] CD161	B1						
514	UV7	BUV496	[2] CD8										
525				V6			B2	FITC/AF488	[1.5] Va24/hCD1d				
542	UV8			V7	BV510	[1.5] CD45RA	B3	SparkBlue 550	[1] CD3				
582	UV9	BUV563	[3] CD69	V8	BV570		B4	PE	[4] NKG2D				
598				V9			B5						
613	UV10	BUV615	[3] CCR4	V10	BV605	[3] CD56	B6	PE-CF594	[4] CD26				
664	UV11	BUV661	[3] Vd2	V11	BV650	[3.5] CCR7	B7			R1	APC	[3.5]	CD16
679							B8	PE-Cy5	[4.5] CD25	R2	AF647	[3.5]	Va7.2/hMR1
697	UV12			V12			B9	PerCP-Cy5.5	[2] TNFa	R3			
717	UV13			V13	BV711	[4] CD7	B10			R4	APC-R700	[3]	CD107a
738							B11			R5			
750	UV14	BUV737	[3] CXCR3	V14	BV750	[2.5] IFNg							
760							B12						
783	UV15			V15	BV786	[3] CCR6	B13	Pe-Vio770	[3] PD1	R6	Zombie NIR	[ ]	L/D
812	UV16	BUV805	[1] CD4	V16			B14			R7	APC-Fire 750	[2]	CD27
										R8	APC-Fire 810	[3]	CD38

Unmixing: deleted Fitc/AF488 respectively from tet/antibody subfile. ✓
reassigned background for Vd2 (ND050) CXCR3, PD1, CD16 ✓
to ND050_unstained_ctrl_sc.
Inf171_unstained_ctrl for dead ✓
ND050_unstained_PMA for CD107a ✓

~~And screen is not visible~~ ...

Va7.2 own color ⇅ swap

PD1 might be an issue

↑ eliminated ctrl unstaineds

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readded unstaineds to groups, still have two rows. ←

Would help if you removed abs samples from tet experiment & vice versa.

Abs: Unmixing w/ general AF extract on live T cells for initial unmix using AF647 CD3 (not Va7.2) for now.

two tell upper boundary for junk screen.

80% ND050 PMA DP ✓

Inf248 few NKTs
ditto ND050