January 18th, 2023

Cord Blood - PMA Timing

Specimen	Status	Location	Conc	Date	Tasks	Volume	Ly (1:100)	Ly+Mon	Total	3E+6	.3E+6
70µl 1680 — IN8085-3 a-7	Cord	Box 33 6,? RXEK			15.6µl	2ml	4.45	6.98			
70µl 15000µl — " a-8		RXEK			×	2ml	3.40	6.03			
70µl 1250cm³ — " a9					24.87µl	2ml	3.41	5.41			
40 ← 340c " — NY062			20E6	08-12-23	21.9µl	2ml	4.57	7.59			

Thaw 9:10am

Pull from −80c to ice bath
Styrofoam freezing pan R
closed lid in freezer −20°c
2nd vial 9:14 am → still vaporing
3rd vial 9: left @ 9:20am ← still vapor

10 minutes

9:37 am DNAse for cord> complete
9:43 for NY062 40µl

Stain count @ 9:51 am

Count start @ 10:04 am

NY062 water bath thawed @ 9:28am, 3min wait for centrifuge

Incubation start @ 11:02 AM

	4	5	6	No spike	DirectIntra	Va7.2	
cust PMA	○	○	○	○	○	○	
cust ctrl	○	○	○	○	○	○	NMT

+ ○ ○ ○ 3 adult scs

+ 2ambic + 3 timepoints CD103 — 4µl of CD103 in 1ml

13 tubes
H+C
- CD3 spiked 11
- No CD3 spike 2)
- ~~DirectIntra~~

A488/F/c & AF647 will be added independently

cytokines 11 (10 minute head start) 2022 (12)
2 added w/ mix 3409 (14)

13 × 4 = 52

Total 11 Adult scored scs

2M cells
11 tubes

8.90
6.80
6.82
22.52 → 7 tubes EG cells
9.14 EG Adult
35×.3 = 10.5

	4	5	6	no spike	poly mix	
	○	○	○	○	○	½
	○	○	○	○	○	
Va7.2						½

~~Lets go~~ w/ the H-TC protocol

22.52 ÷ 7 = ~~3.217~~

22.52 ÷ 8 = 2.815

12:12pm unstained PMA received ~~PMA~~
Conterfuge as also got 2µl regular stop)
Is what it is.

- 9 tubes Intra w/ CD3
- 9 tubes Permwash Cytokines
- 2 tubes regular w/o CD3 spike
- 2 tubes permwash cytokines
- 2 tubes w/ intra spiked in a tube w/ cytokines

13 adult
6 56
3 44

533µl 266µl
467µl
× 5
23:0

2410µl R0 + 10µl

// unstained PMA 2µl + 2µl ctrl //

Abs prepped @ 2:35 pm

Reagents prepped @ 2:53 pm //

Note: No adult PMA sc's, talks from cord PMA unstained
CD69, TNFα, IFNγ

4hr: L/D @ 15:16 pm
(sits CD107a chillin) → after FBS wash into 4°c to wait for other sc's

CD107a from 3 cord timepoints

↳ FBS spin @ 3:33 pm

↳ 37°c @ 3:44 → 4:14 pm

5hr: spin @ 4:03 pm

5hr L/D @ 4:13 pm → 4:28 pm

2ml FBS wash @ 4:28 pm → 4:36 pm at

Thawed → spin, next up tetramers.

4hr @ 4:26 pm → 36 pm 10 min tetramer head start

59.7 cold

4:40

3 37° 65f 4:40 → 5:10 pm 5:10 pm

ALL cords spin @ 5:13 pm (coincided)

Adult aliquoted

Thawed FBS for additional wash

5's: 10 min tetramer @ 5:29 → 39

4's FkPerm @ 5:31 → ~~5:41~~ → 5:51 pm

(also CD107a's 4 & 5hr no RBC by sc!)

5 into 4°c 30 min

Perm wash @ 5:42 →

6's L/D @ 5:37 → 52 pm (FBS wash)

6:12 pm for RBC lysis

Cord sc's in hood waiting for adult sc's

Adult sc spin @ 5:45 → 53 pm (to hood)

↳ Perm Wash 1 in direct @ 1400 6 min
↳ 2nd w/ L/D wash @ 1300 8 min

Adult sc's worked //

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6:12pm 5. cold at needs RBC lysc

6:03pm 2nd Perm 4, 4/0 FBS spin for 6. (10 minute delayed, but 7 min in wash FBS)

6:07pm Scs moved to 4°c for time being (uncertain when I can get to them and rather let them stay cold)

5. RBC lysc wash @ 6:17pm

Mini centrifuge dead, cytoMix mix stuck inside

6:27pm ~~6~~ into 37° stain → 6:57pm. Didn't happen

5 into FxPerm @ 6:30 → 40 → 50

4° ~~PFA~~ CytoMine 10 @ 6:37 → 47 + 49.5µl spiked

4.5 COCIa 50µl 0.4% ~~PFA~~ @ 6:39pm

Making 10% FBS-PBS @ 6:43

4. Spiking Intes @ 6:43pm → 6:18pm oct

5. First ~~Fix~~ PermWash @ 6:51pm

→ 2nd Perm wash & FBS Wash

[scs start]

Scs cold in @ 7:07pm → 7:39pm

5. cyt @ 7:12 → 27 spiked → @9:23

6. Hot stand in ~~an~~ extra 10 minutes !!

In @ 7:10pm

Tetramers & Ab's added into hood @ 7:21pm

6. Tet start @ 7:32pm → 42pm

4. 7:18 Final Wash w/ 2nd perm/0.4% ~~PFA~~ 70µl @ 7:27pm

scs cold 7:37 RBC lysc ~~@7:0~~ @7:35

5.- Intracellular done @ 7:53pm → into 7th PFA @ 8:04pm

6. 4°c stain @ 7:46 → 8:16pm //

Hot SC's in @ 8:00AM → 8:30pm //

Tet Steps in @ 8:05 → 8:20pm
RBC lysed the unstained etc for sc's card

SC's FixPerm 8:26 → 36 → 46

6 samples FixPerm @ 8:34 → 44 → 54

combined perm wash spin @ 8:54pm
2nd Perm wash @ 9:08pm

Hot RBC lyse @ 8:39pm //
SC's
spin w/ FBS @ 8:42
↳ FixPerm @ 8:55 → 05 → 15

@ 9:35 intracyt → 45

Intracellulars in @ 9:49pm (Spiked components & SC's) ~35 min
→ 10:19pm

SC's organized @ 9:55pm

Final spin @ 10:24pm

Done @ 10:42pm

January 18th, 2023 Cord Blood - PMA Timing

#	Filter	Single color (ul)	Ref ctrl	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	13	L/D 15 min (RT)	Tetramer 40 min @ RT	HotStain 30min @37C	13	ColdStain 30min @4C	13	RBC Lyse, then Fix/Perm	Cytokine Stain 10 min @RT	11	Spiked 30 min @RT	9
1	UV2				BUV395	CD62L	(DREG-56)								1.2	15.6					
2	UV7				AF	AF-UV6															
3	UV9				BUV496	CD8	(RPA-T8)								0.7	9.1					
4	UV10				BUV563	CD69	(FN50)								0.5	6.5		1	11.0		
5	UV11				BUV615	CCR4	(1G1)						2.0	26							
6	U14				BUV661	Vδ2	(B6)								0.7	9.1					
7	UV16				BUV737	CXCR3	(1C6/CXCR3)								2.5	32.5					
8	V1				BUV805	CD4	(SK3)						1.5	19.5	1.3	16.2					
9	V3				BV421	CD127	(A019D5)						1.5	19.5						0.5	4.5
	V3				Pacific Blue	CD14	(M5E2)								2.0	26.0					
10	V5				Pacific Blue	CD19	(HIB19)								2.0	26.0					
11	V7				BV480	CD161	(REA631)						2	26							
					BV510	CD45RA	(Hi100)								0.7	9.1					
12	V10				BV605	CD56															
13	V11				BV650	CCR7							1.0	13							
28	V13				BV711	CD7							1	13						1	9.0
14	V14				BV750	IFNγ	(B27)						1	13							
15	V15				BV786	CCR6	(11A9)											1.5	16.5		
16	B2				AlexaFluor 488	hCD1d							1.5	19.5							
17	B3				Spark blue 550	CD3	(SK7)								< : > ( )						
18	B4				PE	NKG2D									1.2	15.6				0.5	4.5
19	B6				PE-CF594	CD26	(M-A261)						1.5	19.5						0.5	4.5
20	B8				PE-Cy5	CD25	(M-A251)						1.2	15.6							
21	B10				PerCP-Cy5.5	TNFα	(MAB11)						1.2	15.6						0.5	4.5
22	B13				PE-vio770	PD1												2.5	27.5		
24	R1				APC	CD16									1.5	19.5				0.5	4.5
23	R2				AlexaFluor647	hMR1									0.7	9.1					
26	R4				APC-R700	CD107a	(H4A3)		<4>	####					< : > ( )						
25	R6				Zombie NIR	L/D					<1:2500>										
26	R7				APC/Fire 750	CD27	(O323)						2	26							
27	R8				APC/Fire 810	CD38	(HIT2)						1.6	20.8							
							Antibody Total		0.0	0	Antibody Total		19.0	247	13.7	178.1		5	55	3.5	31.5
And UNSTAINED CONTROLS !!!							R10 Media		20.5	267	Brilliant Stain		50	650	50.0	650		5.5	60.5	50	450
							Pippette draw volume /sample		19.5		Pippette draw volume /sample		66		60.7			10		50.5	

Notes:

↗ To avoid spreading?

Simplified Protocol

Thaw cells, DNAse, count.
Collect, count, aliquot cells 3.0E+6 Cells R10 / 5ml polystyrene tube
Bring volume upto 1 ml R10, add 2 ul PMA/Ctrl and DuringStim antibody
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 tetramers for 40 minutes at RT>
Wash with 2 ml PBS, spin down 1300rpm 8min

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

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

Resuspend in 100 ul 0.4% PFA-PBS
Cap tubes, wrap rack in foil, store at 4°C

On at around ~ 8:34 AM

QC @ 8:57 am

Placed 70µl, collecting 80µl samples.

Cord unstained control ~ 7000 events @ 9:03 am ↗ factoring loss of 1/5th for CD107a controls

☆ closer to 1 million events / tube, so yeah, math was indeed off ☆

no harm, no foul, given = 1, it's @ PMA experiment

so even if not great MAIT it's now fair mal ~ timing ~ CD3 spiking ~ cytokine timing

¿ No CD3 spike looks FSC/SSC a bit different? (or is it a 6 hr trend?)

PMA unstained does look a bit different, but is it the PMA? ... or the extra block

SC's start @ 9:53 am

CD107a 4 & 5 ugly → due to no RBC lyse?

/CD27 → odd staining // (something is up....)

CD38 also issue, SC's @ 37°C?

David Rach, HaoTong, Ngina, Godfrey, Felix, Osward, Ingrid

Frank, Morcel, Miriam, Lyle & Cairo

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#	Filter	Single color (ul)	Ref ctrl	Unmixing ctrl name	Fluorochrome	Marker	All in One	2	No CD3 spike	2
1	UV2				BUV395	CD62L				
					AF	AF-UV6				
2	UV7				BUV496	CD8				
3	UV9				BUV563	CD69	1	2.0		
4	UV10				BUV615	CCR4				
5	UV11				BUV661	Vδ2				
6	U14				BUV737	CXCR3				
7	UV16				BUV805	CD4	0.5	1.0	0.5	1.0
8	V1				BV421	CD127				
9	V3				Pacific Blue	CD14				
	V3				Pacific Blue	CD19				
10	V5				BV480	CD161				
11	V7				BV510	CD45RA				
					BV570					
12	V10				BV605	CD56				
13	V11				BV650	CCR7	1	2.0	1	2.0
28	V13				BV711	CD7				
14	V14				BV750	IFNγ	1.5	3.0		
15	V15				BV786	CCR6				
16	B2				AlexaFluor 488	hCD1d				
17	B3				Spark blue 550	CD3	0.5	1.0		
18	B4				PE	NKG2D	0.5	1.0	0.5	1.0
19	B6				PE-CF594	CD26				
20	B8				PE-Cy5	CD25	0.5	1.0	0.5	1.0
21	B10				PerCP-Cy5.5	TNFα	2.5	5.0		
22	B13				PE-vio770	PD1	0.5	1.0	0.5	1.0
29	R1				APC	CD16				
23	R2				AlexaFluor647	hMR1				
24	R4				APC-R700	CD107a				
25	R6				Zombie NIR	L/D				
26	R7				APC/Fire 750	CD27				
27	R8				APC/Fire 810	CD38				
							8.5	17	3	6
And UNSTAINED CONTROLS !!!							50	100	50	100
							55.5		50	

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Second Perm Wash: 1 ml PermWash 1500 rpm 6 min

Resuspend in 100 ul 0.4% PFA-PBS
Cap tubes, wrap rack in foil, store at 4°C

Merged Tetramer adult Act Unst +TNFa
+IFNγ as the standards

CD69 is using activated → PMA-unstained Cocktail (6hr?)
CD107a's 4, 5, 6 ↗ But... extra step

PMA/Cocktail ctrl Zombie using 3dead ↙ (may need to break individually)

Unst 1: PMA zombie 6hr] VE2 needs swapped
& CD107a 6hr]

ILT & NK SFC Panel

1/18/2023

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-UV6		AF	V4											
508					V5	BV480	[3]	CD161	B1							
514	UV7	BUV496	[2]	CD8												
525					V6				B2	AF488	[1.5]	hCD1d/Va24Ja18				
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]	hMR1/Va7.2
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				R6	Zombie NIR	[ ]	L/D
783	UV15				V15	BV786	[3]	CCR6	B13	Pe-Vio770	[3]	PD1	R7	APC-Fire 750	[2]	CD27
812	UV16	BUV805	[1]	CD4	V16				B14				R8	APC-Fire 810	[3]	CD38

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