January 12th, 2023

Cord Blood #2

70
1030..
70µl
890c/µl
60µl
840c/µl
60µl
810c/µl
40µl

Specimen	Status	Location	Conc	Date	Tasks	Volume	Ly (1:100)	Ly+Mon	Total	3E+6	.3E+6
a-3 Inf374-4	HEU-10		13.27µl			1.5	4.09	6.32	13.3	900µl	
Inf374	HEU-10		17.01µl			2	2.99	4.74	3.33 cells/ml		
Inf694	HU		1802µl			2	3.95	6.38	15.28	884µl	
Inf694	HU		18.7µl			2	3.69	6.26	3.39 cells/ml		
ND006	Adult		14E6	02/24/2021	03/13/20..	2	1.34 ~~1.35~~	1.82			

Specimen pull @ 9:28am

1st spec @ 9:41 am) 15 minute period @ -80°C //

10:09 2nd centrifuge out

Inf 694 white pellets

694 dnase @ RT @ 10:13

Inf374 ND006 10:16
  L2

✓ not alive?

ND006 leftovers:

135 c/µl

160 c/µl

93µl

☆ Thaw issue ☆ ~~water~~ rethawed

☆ going on ND006 @ 9:50 am
☆ stopped again
→ water bathed

10:33 am → single stain for count

☆ 11:01 am (ND006 count screwed up) . . . sigh . . .

7.35
5.98
13.33 ÷ 4 = 3.33

7.9
7.38 ÷ 4.5 = 3.39
13.28

3M → 10.33 = 3
3M → 12.28 = 4 wells

500µl

2.8 million ND006 → Ella.

cord into incubator @ 11:37 am 1.5M

= 900µl
= 884µl

[everything control]

2nd ND006 thaw @ 11:44 am (6ml total R10 for wash)
11:56 dnase

~ Guava glitched

// Either Guava is misbehaving or all these vials are dying //

246 c/µl

27.98µl

57 MAITs
547 NKTs
2.3 M cells

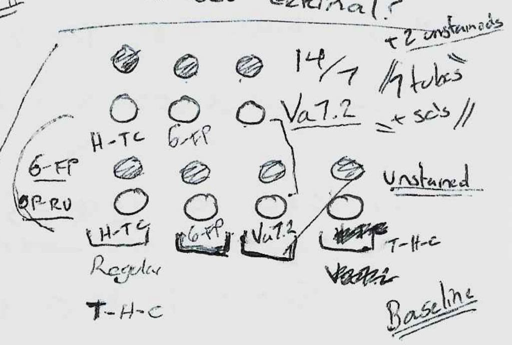
combined both ND006 tubes, divided into 3 mls added transport inhibitor. Not much else I can do about it. @ 12:16 pm

? loss suction due to low sheath external?

1µl

Abs prepped @ 4:26 pm
Reagents @ 4:11

3 M ~ 2.3 M acquired
~1.1 M Live RBC-
~550K CD3 cells
~0.5% NKT
~0.05% MAIT?



VS cd

135 mls 5% FBS-PBS

cord

Cells out @ 5:22pm

5:33 spin + 2 vials turned -AE spacers

5:47 L/O

@21

Adults out @ 5:52

T: 1pm blue 88/94

9E/6 x 3 = 27?

6:05pm In

6:18 pm NOOG spin down

T-H-C @ 6:22 tot one → 7:02pm

Hot @ 6:26 → 56pm

Scs PBS spin down after aliquot → 6:37pm

→ Obviously heavily cell heavy by PBS in 1ml

→ 6:59pm hotsout

Scs Hots In @ 6:59 → 7:29pm

Cold scs @ 7:10 → 7:40 pm

Biotin & L/O @ 7:12 pm → 7:42

→ 7:27 pm

RBC lyse 7:36 →

Tet-Hot-cold @ 7:16 pm → 7:46 pm

H-TC 10min @ 7:21 pm

7:34 → 8:04 pm

(250)

All scs RBC lysed & spun 2ml's 5% FBS @ 7:45pm

+ Tet-Hot's wash

RBC lyse @ 8:06 pm (250)

Spin @ 8:11 pm

Streptavidin @ 8:00 - 8:15

FixPerm (holding for strep)

All FixPermed @ 8:30 pm

→ 40 → 50

42

Cold stain @ 7:57pm

→ 8:27 pm

RBC lyse @ 8:27pm

Spin @ 8:31 pm

FixPerm @ 42 ✓

2nd @ 53

1st perm wash @ 8:59pm

9:22 sorted scs + T-Hc into 2nd PermWash

Intracellular @ 9:25 pm

→ 10:05pm sc → 10:14

Scs + T-Hc @ 9:34pm PDI got screwed up

January 11th, 2023 Cord Blood #2

CD56 ran out, using myeloids
PD1 sc got ruined @ last step

#	Filter	Single color (ul)	Ref ctrl	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	7	L/D 15 min (RT)	Tetramer 40 min @ RT	HotStain 30min @37C	7	ColdStain 30min @4C	7	RBC Lyse, then Fix/Perm	Intracellular Stain 40 min @RT	7
1	UV2				BUV395	CD62L	(DREG-56)												
					AF	AF-UV6									1.2	8.4			
2	UV7				BUV496	CD8	(RPA-T8)												
3	UV9				BUV563	CD69	(FN50)								0.7	4.9			
4	UV10				BUV615	CCR4	(1G1)								0.5	3.5		<1>	######
5	UV11				BUV661	Vδ2	(B6)						2.0	14					
6	U14				BUV737	CXCR3	(1C6/ CXCR3)								0.7	4.9			
7	UV16				BUV805	CD4	(SK3)								2.5	17.5			
8	V1				BV421	CD127	(A019D5)						1.5	10.5					
9	V3				Pacific Blue	CD14	(M5E2)						1.5	10.5				0.5	3.5
	V3				Pacific Blue	CD19	(HIB19)								2.0	14.0			
10	V5				BV480	CD161	(REA631)								2.0	14.0			
11	V7				BV510	CD45RA	(Hi100)						2	14					
12	V10														0.7	4.9			
13	V11				BV605	CD56													
28	V13				BV650	CCR7							1.0	7					
14	V14				BV711	CD7							~~1.5~~	~~10.5~~				~~0.5~~	~~3.5~~
15	V15				BV750	IFNγ	(B27)						1	7					
16	B2				BV786	CCR6	(11A9)											<1.5>	######
17	B3				AlexaFluor 488	hCD1d							1.5	10.5					
18	B4				Spark blue 550	CD3	(SK7)					<: >()			<: >()				
19	B6				PE	NKG2D									1.2	8.4			
20	B8				PE-CF594	CD26	(M-A261)						1.5	10.5				0.5	3.5
21	B10				PE-Cy5	CD25	(M-A251)						1.2	8.4					
22	B13				PerCP-Cy5.5	TNF	(MAB11)						1.2	8.4				0.5	3.5
29	R1				PE-vio770	PD1												<2.5>	######
23	R2				APC	CD16									1.5	10.5		0.5	3.5
24	R4				AlexaFluor647	hMR1									0.7	4.9			
25	R6				APC-R700	CD107a	(H4A3)		<2>	####		<: >( )			<: >( )				
26	R7				Zombie NIR	L/D					<1:2500>								
27	R8				APC/Fire 750	CD27	(O323)						2	14					
					APC/Fire 810	CD38	(HIT2)						1.6	11.2					
And UNSTAINED CONTROLS !!!							Antibody Total		0.0	0	Antibody Total		19.5	137	13.7	95.9		2.5	17.5
							R10 Media		20.5	144	Brilliant Stain		50	350	50.0	350		50	350
							Pippette draw volume /sample		19.5		Pippette draw volume /sample		67		60.7			49.5	

Notes: No cytokines or CD107a today

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

bulk
SCs done @ 9:42pm
(upcetty about PD1)
10:05pm
10:14 pm final spin @ 10:17pm

Just 1 2ml Permwash @ end

1400 rpm 6 min

Done @ 10:30 pm

ILT & NK SFC Panel

1/11/2023

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	V6									
525				V7	BV510	[1.5] CD45RA	B2	AF488	[1.5] hCD1d				
542	UV8			V8	BV570		B3	SparkBlue 550	[1] CD3				
582	UV9	BUV563	[3] CD69	V9			B4	PE	[4] NKG2D				
598				V10	BV605	[3] CD56	B5						
613	UV10	BUV615	[3] CCR4	V11	BV650	[3.5] CCR7	B6	PE-CF594	[4] CD26				
664	UV11	BUV661	[3] Vd2				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													
783	UV15			V15	BV786	[3] CCR6	B12			R6	Zombie NIR	[ ]	L/D
812	UV16	BUV805	[1] CD4	V16			B13	Pe-Vio770	[3] PD1	R7	APC-Fire 750	[2]	CD27
							B14			R8	APC-Fire 810	[3]	CD38

PeCy5 → Lower has ∆/ll influx.

BV510 gate on the average cell, ~~brightest~~ ≠ spectral viewer
[0.97]

APC → average bright 0.96
CD7 → average bright 0.97

PE-CF594 → 0.98
BV-605 → 0.9~~8~~
BUV-496 → 0.99

CD27 → brightest 0.98

Pe-Cy5 → bright (0.92)

☆ Are these different due to underlying culture conditions from their reference? ☆

[8 colors]

CD69 / PD1 ... Just noticed the click drag still adds why the axis changing ...

Nada in PD1

biotin dyed so no cause concern beyond mean shift

CCR4 a bit off

For NKG2 & MAITs gd's H5 is off in coord

tiny bit off NKG20 PE

InP394 H-TC ✓

APC-Fire 750

26,000 CD~~8~~ CD26+ Pacelap

9 is an NKT cluster!

27+ 26+ NKG2D CD3+ CCR6+ CD7+ CD161+ CD127+
(CD25)

CCR7 - CD45RA+

~~37~~ 393,347

7,560

This is truly bizzare ...

1.14 between 2 & 4 5995
2.61

328,806

4.49 & 13 % between 2 & 4
2863
2483

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Cord Blood #2

374-4 Box 12A Row A, col 7 ≠ two empty spaces
→ col immediately past was it.
694-9 Box 20A Row C, col 12 ≠ continue on next line
← 2, 1' aliquots.

Start after QC was at

5:27pm running 15,000 events
Less living in Inf694's unstained (or more RBCs?)
→ less living (more gray matter?)
20-21,000 events
24-25K //

Done @ 7:34pm

01/14/2023

Initial unmix [9 GB file] [113 GB data]
Since last Thesis Com meeting

PaCMAP
1.3 M cells @ 10:36am
↘ 11:06 am
(½ an hour)

PDI (old experiment) + 2 sc's cord unstained CCD8/CD45RA)
initial unmixing using internal neg
☆ Starting off w/ adult CD45 & CD8 for unmixing
(computer struggling PaCMAP, spotify, 2 unmixing)

☆ No Δ in AFs across PoPs that's significant beyond dead Ho has some red.

☆ SC gating → Universal
→ Internal ← subtraction
requires pop be consistent, ie Lym use Lym
→ Brightest bright
→ Average

Swapped Round to make hiding unstained's easier & have Internal appended at end.

So.... FlowJo SC Template

Drag my SC files, place into a "New Folder"

Want my Groups to automatically populate for every SC,

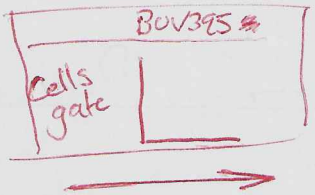
And have a default template assigned on a by group basis down to the pop level.

Intermediate | Average bright | Brightest

→ cycle through your new folder edit pop gates

→ Also do batch reports for JPEGs?

Into a LayOut



Designing this to eventually be run on FlowJo Command line

"At 17 "templated by group" @ 1:51 pm"

"SSC-A * B2-A for tetramers"
swapping monocyte position

No CD69 staining @ surface

Export only ~~$FIL~~ name, uncomp parameters

→ generated 51 files ✓

Flawless export ✓

- Zombie NIR + PacBlue CD14

→ success

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Cord Blood #2

AF488 ~ bit flotation

No effects: CD3

BUV661 → 611 in the template...

Unmixing ~ Optimal?

APC, hMR1, LD all screwed up

hCD1d unloaded is latched onto by monocytes? or...

BV421 PacBlue 480 510

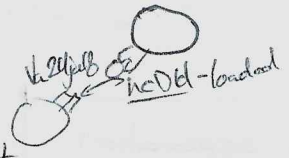
AF488 Spark550

AF spillovers

only one shared

By Rspectra:

AF488 unloaded spectra significant UV/V peaks to it



AF647 Lym ~ Mon 0.02

OP-RU vs G-FP identical on same cell type.

AF488 unloaded on CD3 ≠ spectra @ 0.94 similarity

AF488 unloaded ~ loaded Lym ~ Mon

~~Only debris gating~~

2nd Unmix:

CD69 internal ≠, merged in CordBlood1 unstained w/CD69 for artificial negative pop.

PD1 using internal.

- leaving CD45RA on an internal cat around average
- Swapping CD7 over to brightest
- pushing CD16 to brightest bright
- moved Zombie NIR over abit to left

(✡) Would unmixing coral benefit from tag approach to AF extraction rather than leave up to chance? (✡)

on a sample / sample basis? unmixing ~ red signal dying cells ~ Zombie?

✡ Just because a reference peak looks like that ≠ actual cells will but if system has actual "signature" it will resolve them...

Some improvements... 0.76

0.71

APC – AF647 – BV661 ~ 1/5

0.93

BV711 ↑

APC Fire 750

⟷ Swapping to less bright APC? For third ummm

grabbing middle helps

Centroid vs Periphery ILTs:

	Centroid		Periphery
Myeloid	57,000	Myeloid	23,193
NK	329,000	NK	15507
T	285,000	T	14231

AF AF647 APC

⟷ ☆ Unmixing issue ☆

CD3* CD45RA, ~~86~~ for NK separation

Vd2: CD62L
½ CD8+/-
Some CCR4

NKTs

CCR4+
CD4
RA-

CCR4 –
CD8 mid
DN
161+
RA+
CCR6+
NKG2D+

☆ Not sure what screwed up ☆ on APC ☆

ILTs: Not AF 48,408 by PaCMAP @ 4:57pm (includes DNs)

Not 16 308,895 by PaCMAP for CD8 @ 5:02pm

// cluster 1 & cluster 5 are NKTs // ?
→ CCR4, CD69 (cluster 1)
→ MAIT cluster & CD8 (cluster 5)

7 expresses 16 highly, CD8,

cluster 2 CD4

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CD8's #3 #2 #1

27 high →

CD38+ ← → CD38-

CD8 h →

CD69+ might be artefact.... of intrac & off chm PPD?

127 mid ← → mid → low

Low CXCR3 →

Neg for CD161 →

CD45RA high ← → lower

No S6 →

CCR7 mid | CCR7 high | CCR7 -

CD7 high →

No/low CD26 →

30% GITR CD20+ →

CD26+ ← → CD26-

PD1+

CCR7 & CD69 other

CD8
CD38-
CD127 low
~ CD45RA-
~ CCR7-
+ CD26-
PD1+

CD4's

CD27 high →

mostly 38+ (w/ 2 exceptions)

2 CD62L+

More CD69+ clusters

1 CCR4 cluster

CXCR3-

S6-

~ Cord ~

Shear brightness of Zombie is my issue.

↳ If adjust charges the NMR1/APC, then we are clear.

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Cord Blood #2