

December 5th, 2022

Staining Temps - ILT antibodies

Specimen	Status	Location	Conc	Date	Tasks	Volume	Ly i:100	Ly+Mon	Total	3E+6	.3E+6
NJ 006	Adult		1SEG	11/14/22		5	7E6	9.47E6	35EG	42841	
K	11		7.5 +1.5 EG +7.5	11							

Stain for count @ 09:21 → 09:28 am
all combined into 1 tube

Into incubator @ 10:08 am → 4:08 pm

1:26 pm Ab prep begins

Main mixes done @ 2:49 pm
SC's done @

remembered we forgot CD107A again!
it & Fc Block bane my existence

< place it in @ tetramer stage, @ 1.5 μl >

→ Not ideal, but not worth wasting antibody,
and we need it in for a complete panel.

Reagents prepped @ 3:46 pm

4:09 pm cells out (thank goodness for Board SC tubes)
Spin down 4:26 pm (PMA prepped)

* 8 sps
24 μl
+ 8
8 μl
HMRI

Lost CO4 sc to drain,
measured 15 μl from offlist
150 μl

LDE @ 4:44 pm

Cold SC's @ 5:01 pm 5:04 spin start

Hot SC's @ 5:10 pm → 5:40 pm

Tetramers & LD @ 5:21 pm → 6:01 →

Rhe lysis 36/37.4%

39 31% SC spin @ 1300 RPM

// Find AT488/647 and add "streptavidins" @ 5:45 pm → 5:53
CD7 pellet really really small → 6:00 → 6:15

8 tubes ^{inst}
10 hot cold reg spiked

10 = 20 M cells //

30 * .3 = 9.0 M cells
x 2
≈ 18.0 M cells?
SC's

Ly 35 M
52.5M → 67% recovery

LyM 47.35 M
52.5M → 90% recovery

8x 3 = 24
+ 2
10
135 million → 13 for each + 1.5

14.5 ~ 5 PMA tubes
21 ~ off tubes

572
4004 ml
14/1 cell
572
2360
10/1 PMA

1
2.8 .1

GOPS II

④ CD107 not added
CCP6/CD25 substituted
CD4 dumped
B721 CDR falquid

Steps: 6:15 pm spin out @ 6:24 pm

tetramer wash @ 6:03 pm

6:07 pm → 7:17 → 6:17, 6:27

< Grandma passed away >

- combine
&
Fix Perm

Hot SC's samples
Hot SC's @ 6:21 pm → 6:51 pm

107A rbc lysis @ 6:22 pm → spin @ 6:26

SC's 1st perm wash @ 6:35

Spin 1500 rpm 6 min

2nd perm wash @ 6:44 pm

Fix Perm @ 6:34 → 44 → 84

↓ Hot sample wash
+ strept/107A 1st perm ✓
end @ 7:01 pm

67.9 reg
80.7 cold

sds intra @

Cold sham samples @ 7:10 pm → 7:40 pm Rbc lysis @ 7:43

sds intracellular @ 7:24 pm → 8:04 pm

Samples cold wash @ 7:48 pm

Samples Fix Perm @ 7:57 → 8:07 → 8:17 pm

SC's 3rd Perm Wash @ 20:09 pm

Samples 1st perm wash (SC's final perm wash) @ 20:25 pm

SC's done @ 8:46 pm 30µl 4% PFA + residual

SL 5/6 3.5 Samples intra @ 20:50 → 9:30 pm

Samples 3rd perm wash @ 9:32 pm

Done @ 9:51 pm

W. 2. 2. 2. 2.

#	Filter	Single color (all)	Ref ctrl	Umixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stain!!	2	LID 15 min (RT)	Tetramer 40 min @ RT	HostStain 30min @37C	2	ColdStain 30min @4C	2	RBC Lyse, then FixPerm	Intracellular Stain 40 min @RT	2
1	UV2			BUV395		CD28L	(DRG-56)												
2	UV7			AF	AF-UV6														
3	UV9			BUV495		CD8	(RPA-T3)												
4	UV10			BUV563		CD69	(F750)												
5	UV11			BUV615		CD94	(16G)												
6	UV14			BUV651		VE2	(B6)												
7	UV16			BUV737		CXCR3	(15G)												
8	V1			BUV805		CD4	(SL3)												
9	V3			BV421		CD27	(A019505)												
10	V5			Pacific Blue		CD14	(M5E2)												
11	V7			Pacific Blue		CD19	(H519)												
12	V10			BV480		CD161	(BEA631)												
13	V11			BV510		CD45RA	(H410)												
14	V13			BV650		CD56													
15	V15			BV731		CD7													
16	B2			BV750		IFNγ	(B27)												
17	B3			BV786		CCR6	(1169)												
18	B4			AlexaFluor-488		ICD1d													
19	B6			Spark blue 550		CD3	(SK2)												
20	B8			PE		NKG2D													
21	B10			PEC-F594		CD26	(MA261)												
22	B13			PE-Q5		CD25	(MA251)												
23	R1			PE-Cy5.5		TNFα	(MA811)												
24	R2			PE-Q70		PD1													
25	R4			AlexaFluor647		CD16													
26	R6			APC-C7070		CD107a	(H4A3)												
27	R7			Zombie NIR		VE2	(H72)												
28	R8			APC/Fire 750		CD27	(O323)												
And UNSTAINED CONTROLS (1)				Antibody Total				6.0	12	Antibody Total				33.7	67.4	0.0	0.0	4.5	9
R10 Media				14.5	29	Brilliant Stain				50	100	50.0	100	50	100				
Pipette draw volume /sample				19.5		Pipette draw volume /sample				81		47.0		51.5					

Notes:

Simplified Protocol

Thaw cells, DIVAse, count.
Collect, count, aliquot cells 3.5E-6 Cells R10 5ml polystyrene tube
Bring volume upto 10 ml R10, add 2 ul PwActrl and DuringStim antibody
Cap and incubate at 37°C for 6 hours

Add tetramers for 40 minutes at RT
Wash with 2 ml PBS, spin down 300 rpm 8 min

Add HostStain mix, incubate @37C for 30 min
Wash 2 ml 15% 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 15% PBS-FBS 1400 rpm, 6min

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

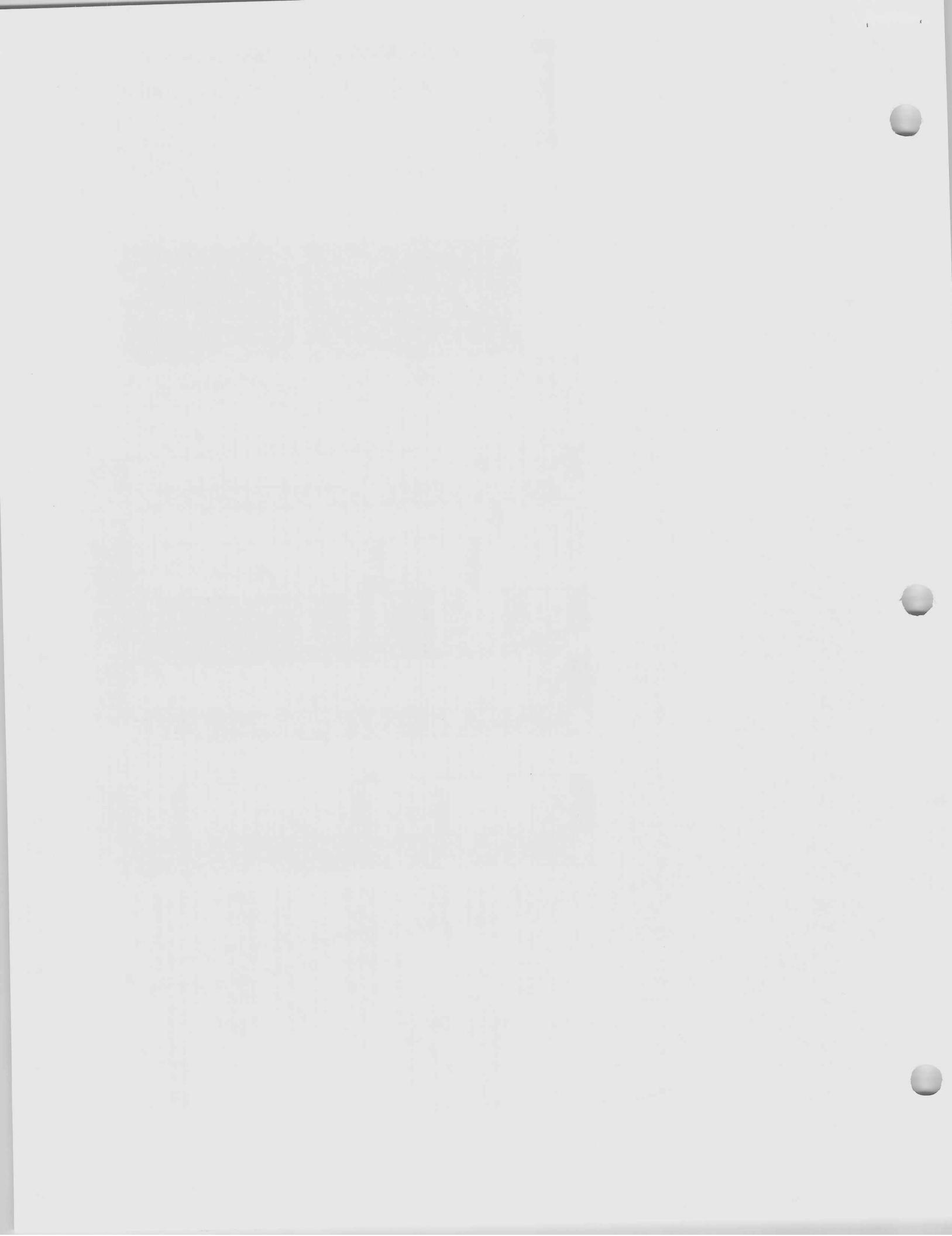
First Perm/Wash:
1 ml PermWash 1500 rpm 6 min

Second Perm Wash:
1 ml PermWash 1500 rpm 6 min

Add Intracellular Stain, incubate @ RT for 40min
First Perm/Wash:
1 ml PermWash 1500 rpm 6 min

Second Perm Wash:
1 ml PermWash 1500 rpm 6 min

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



#	Filter	Single color (ul)	Ref ctrl	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial lot #	During stain!!!	L/D 15 min	Tetramer 40 min @ RT	HotStain 30min @37C	2	ColdStain 30min @4C	2	RBC Lysed then FixPerm	Intracellular Stain 40 min @RT	2	
1	UV2			BUV395	CD62L	(DREG-56)													
2	UV7			AF	AF-UV6														
3	UV9			CD8	[RPA-8]														
4	UV10			CD69	[EN5D]														
5	UV11			CCR4	(1G1)														
6	U14			CD82	(B65)														
7	UV16			BLV937	CXCR3	(1G6)													
8	V1			BLV905	CD4	[IS63]													
9	V3			BV421	CD127	[A0195]													
10	V5			Pacific Blue	CD14	(ME2)													
11	V7			Pacific Blue	CD19	(HB19)													
12	V10			BV480	CD61	[BE653]													
13	V11			BV605	CD56														
14	V13			BV650	CCR7														
15	V15			BV750	IFNY	[B27]													
16	B2			BV786	CBR6	(11A9)													
17	B3			AlexaFluor 488	ICD10d				<1XX> [1.2]										
18	B4			Spark Blue 550	CD3	[SK2]													
19	B6			PE	NKG2D														
20	B8			PE-CF594	CD26	(MA256)													
21	B10			PE-Cy5	CD25	(MA251)													
22	B13			PerCP-Cy5.5	TNFz	[MAb11]													
23	R1			PE-41070	PO1														
24	R2			APC	CD16														
25	R6			AlexaFluor647	hmMRL	[H443]													
26	R7			Zombie NIR	CD107a	[H443]													
27	R8			APC/Fire 750	CD27	[O323]													
And UNSUSTAINED CONTROLS!!!				Antibody Total		6.0	12	Antibody Total		0.0	0	33.7	67.4	4.5	9				
				R10 Media		14.5	29	Brilliant Stain		50	100	50.0	100	50	100				
				Pipette draw volume /sample		19.5		Pipette draw volume /sample		47		80.7				51.5			

Notes:

1.5 ~~CD45b-780~~ for APC-41070

First Perm/Wash:
1 ml PermWash 1500 rpm 6 min
1 ml PermWash 1500 rpm 6 min
Second Perm Wash:
1 ml PermWash 1500 rpm 6 min
1 ml PermWash 1500 rpm 6 min
Add Intracellular Stain, incubate @ RT for 40min
First Perm/Wash:
1 ml PermWash 1500 rpm 6 min
Second Perm Wash:
1 ml PermWash 1500 rpm 6 min
Reuspend in 100 ul 0.4% FFA-PBS
Cap tubes, wrap rack in foil, store at 4C

Simplified Protocol
Thaw cells, DNase, count.
Collect, count, aliquot cells, 3.0E-6 Cells RT/0.5ml polypropylene tube
Cap and incubate at 37°C for 6 hours

1927
M. A.
D. M. C.

#	Filter	Single color (ul)	Ref ctrl	Unmixing ctrl	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	LUD	Tetramer 40 min @ RT	HotStain 30min @37C	2	ColdStain 30min @4C	2	RBC Lysed, then FixPerm	Intracellular Stain 40 min @RT	2
1	UV2				BLUV395	CD62L	(DRG5-56)											
2	UV7				AF	AF-UW6												
3	UV9				BLUV396	CD8	(RPA-13)											
4	UV10				BLUV397	CD69	(HS52)											
5	UV11				BLUV623	CCR6	(1G1)											
6	U14				BLUV651	CCR4	(B6)											
7	UV15				BLUV737	CKR8B	(16/15G3)											
8	V1				BLUV805	CD4	(5G3)											
9	V3				BLV421	CD27	(A0195)											
10	V5				Pacific Blue	CD34	(M1E2)											
11	V7				Pacific Blue	CD19	(HIB19)											
12	V10				BV480	CD161	(RE653)											
13	V11				BV510	CD45RA	(H11.0)											
14	V14				BV605	CD26												
15	V15				BV650	CCR7												
16	B2				BV750	IFNγ	(BZ2)											
17	B3				EVN786	CCR6	(11A9)											
18	B4				AlumFluor 488	HOXA10												
19	B5				Sparl blue 550	CD9	(5K7)											
20	B8				PE	NGK2D												
21	B10				PE-FC594	CD26	(MA251)											
22	B13				PE-Cy5.5	CD25	(MA251)											
23	R1				PerCP-Cy5.5	TRФx	(MA251)											
24	R4				PE-vio770	PD1												
25	R6				APC	CD16												
26	R7				AlumFluor 647	IRMR1												
27	R8				APC-R700	CD107a	(H443)		6.0	12	<1:500> (5)							
And UNSTAINED CONTROLS III																		
Antibody Total																		
R10 Media																		
Pipette draw volume /sample																		
19.5																		
Pipette draw volume /sample																		

Notes:

Simplified Protocol

Draw cells, DIVAse, count.

Collect, count, aliquot cells 3.0E+6 cells 101/1ml polystyrene tube

Bring volume upto ml R10, add 2 ul PHA-Ct and DuraStim antibody

Cap and incubate at 37°C for 6 hours

Wash with 2 ml PBS, spin down 1300rpm 5min

800 ul of LiveDead mix (1:2500) @RT for 15min

Wash 2 ml 5% PBS+BS, spin 1300 rpm, 8min

Add tetramers for 40 minutes at RT

Wash with 2 ml PBS, spin down 1300rpm 5min

Add HotStain mix, incubate @37C for 30 min

Wash 2 ml 5% PBS+BS 1400 rpm, 6min

Add ColdStain mix, incubate @ 4C for 30min

Add 300-500 ul 1x RBC Lysis for 3 minutes

Wash 1 ml 5% PBS+BS 1400 rpm, 5min

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

First PermWash:

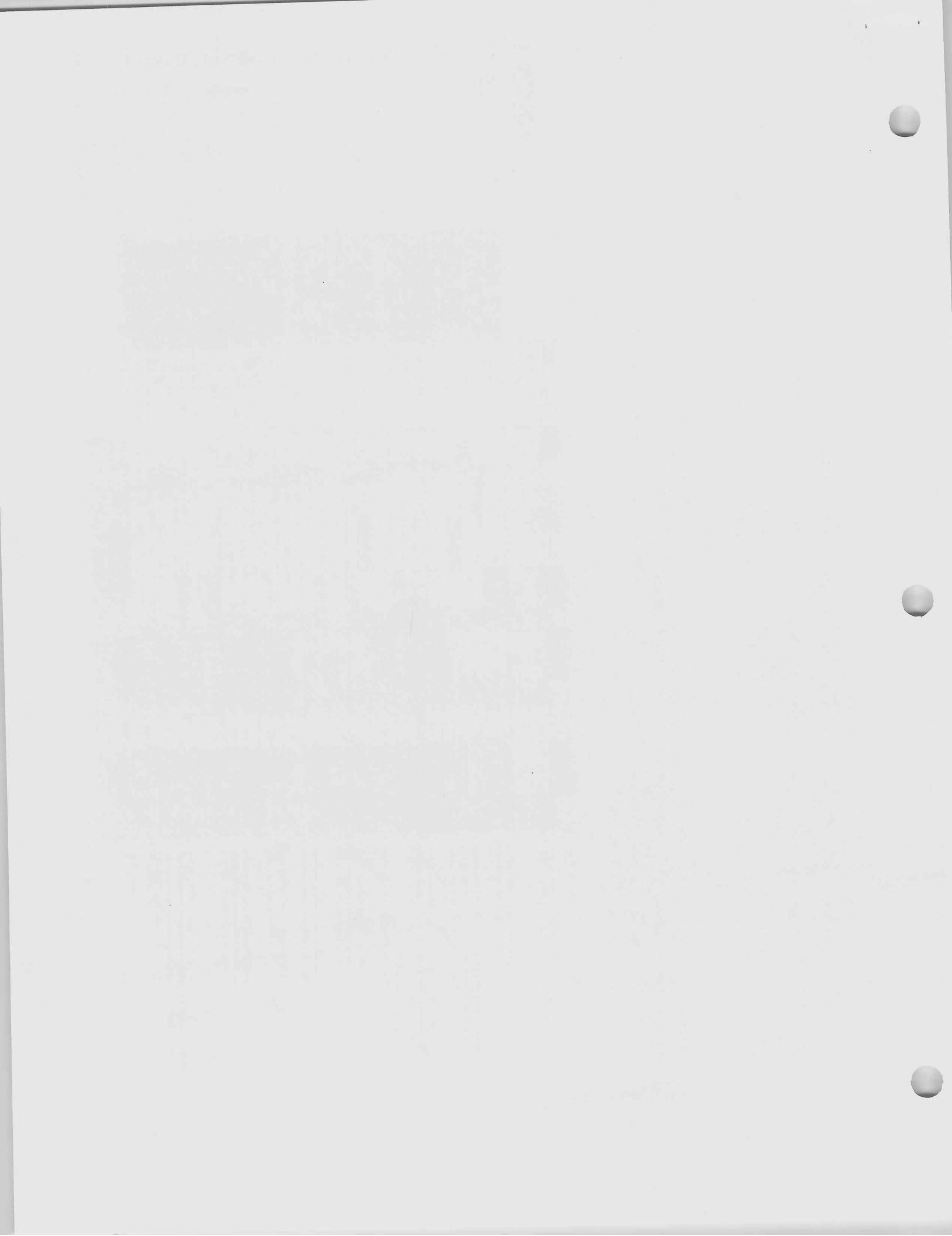
1 ml PermWash 1500 rpm 6 min

Second Perm Wash:

1 ml PermWash 1500 rpm 6 min

Resuspend in 100 u 0.4% PFA+PBS

Cap tubes, wrap rack in foil, store at 4C



ILT SFC Panel

Spectrum

UV

Violet

Blue

Red

12/6/2022

Spectrum	UV	Violet	Blue	Red
373	UV1			
388	UV2	BUV395	[2] CD62L <i>cold</i>	
428	UV3		V1	BV421
443	UV4		V2	
458	UV5		V3	PacBlue
473	UV6		V4	[1] CD14/19
508	UV7	BUV496	V5	BV480
525	UV8			[3] CD161 <i>hot</i>
542	UV9			B1
582	UV10	BUV563	V6	AF488
598	UV11		V7	BV510
613	UV12	BUV615	V8	BV570
664	UV13	BUV661	V9	[1.5] CD45RA <i>cold</i>
679	UV14		V10	SparkBlue 550
697	UV15		V11	PE
717	UV16		V12	BV605
738			V13	[3.5] CD56 <i>hot</i>
750			V14	PE-CF594
760			V15	CD26 <i>hot</i>
783			V16	PerCP-Cy5.5
812				[4.5] CD25 <i>hot</i>
		BUV737		TNFa
		[3] CXCR3 <i>cold</i>		R1
		V14		R2
		BV750	[2.5] IFNg <i>hot</i>	R3
			B10	APC
			B11	[3.5] CD16 <i>cold</i>
			B12	APC-R700
			B13	R4
			B14	R5
		BV786	[3] CCR6 <i>hot</i>	R6
				R7
				R8
		BUV805	[1] CD4	Zombie NIR
				[1] APC-Fire 750
				[2] APC-Fire 810
				[3] CD38 <i>hot</i>

*Spotted**Spotted**more spreading erosion
hot walls*

0.04

0.06

1 AF488
2 AF647
3 CD3

4 ED14

5 AF647 CD3
6 AF647 CD3

0.09

0.01



December 5th, 2022

Staining Temps - ILT antibodies

Early morning start: Aurora on 8:04 AM (May Kahlil last user)

- PMT set unstained 500 cells/sec

- 30 μ l resuspension is too low
Strong Vd2 signal today N.D.

Buv's 8:58 am
Bvs \sim 9:20 am

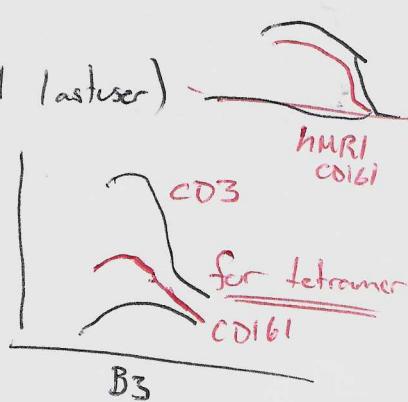
(CD161 stains cells weak)
BLs @ 9:32 am ✓

Sc's done @ 9:48 pm [1 hour]

QC @ 8:34 am
8:38 QC passed ✓

Sc's @ 8:43 pm start AF488

2 1/2 hours booked



will need to check normalized spectra. use CD3 biotin

it then literally gives a single island

sample PMT Unstained \sim 4,800 event rate,

Brain sample + 10 = 30 minutes - 40 min

PMTs done @ 10:04 am \sim 20 min

ctrls done @ 10:22 am

10:22 + 22:00
8:43 + 1:17:00
1:39:00

ctrl looks to have the V12-V15 plateau AF
9400 events / sample

Unmixing w/ AF extract; CD14 ProBlue, biotin AF488, biotin AF647,
EBV screenshots of everything sc, placed into Powerpoint

At the level of sc's

CD62L everything shifts, marginal + gain

CCR4 shift marginal gain

CXCR3 marginal @ best

CD8 shift but resolvable (no fail)
Vd2 shift but resolvable
CD4 shift no fail

[CD127]

CCR6 absolute never

CD161 loose resolution ↑
CCR7 ↑ (NKG2D // ↑ CCR1)

CD40 nada notable

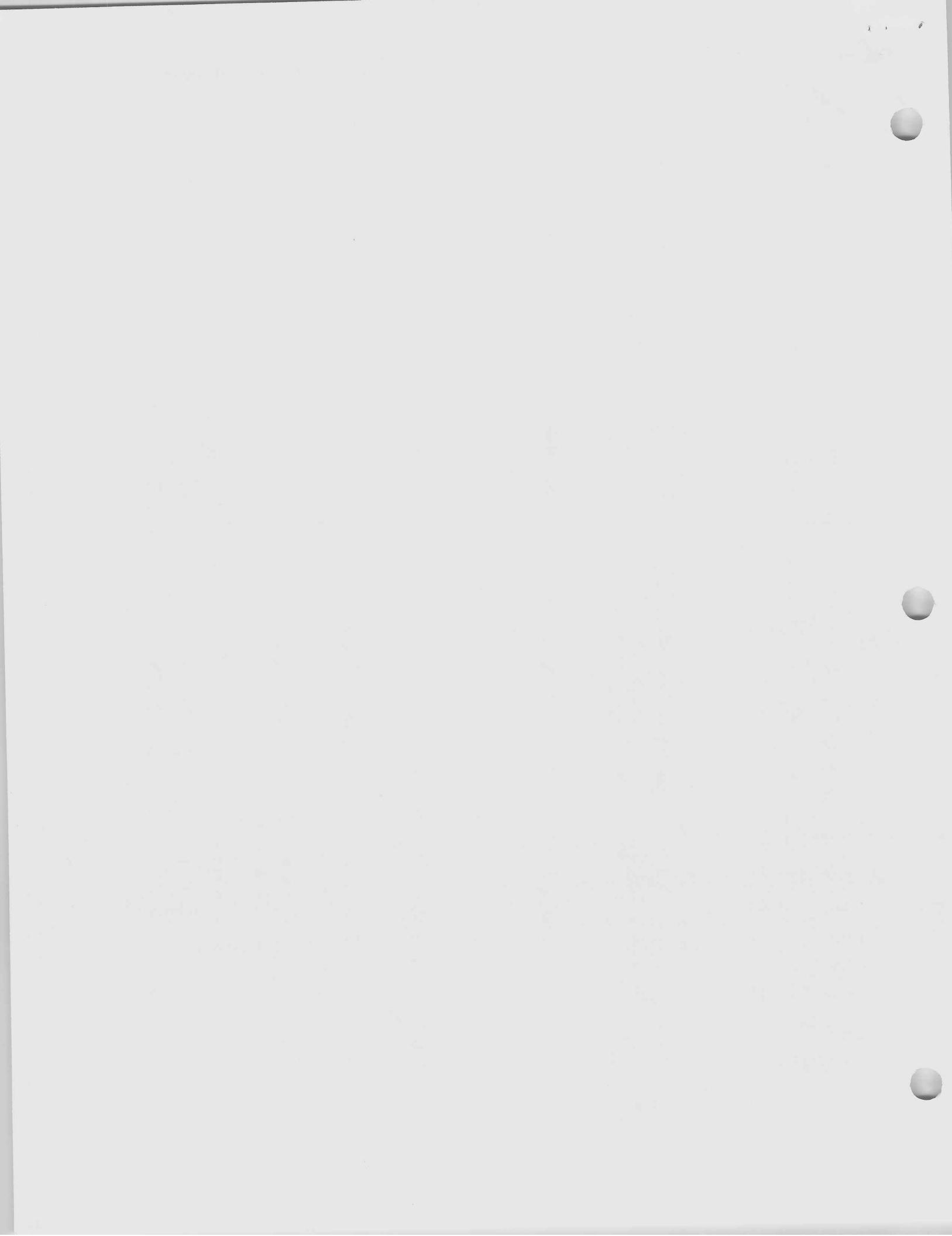
CD69 = NA

PMA well ~ spilling striking effect on CD3

Single Colors

December 5th , 2022

Staining Temps – ILT antibodies



December 5th , 2022

Staining Temps – ILT antibodies

CD3? spike/no spike

actR3 cold

- spiked? no spike

CD5c hot
CD7 hot
NM2D

- Spiked no spike

CD25 hot

CD127 I
CD61

- Spiked no spike

actR7 spiked

CD25 spiked

PDI spiked