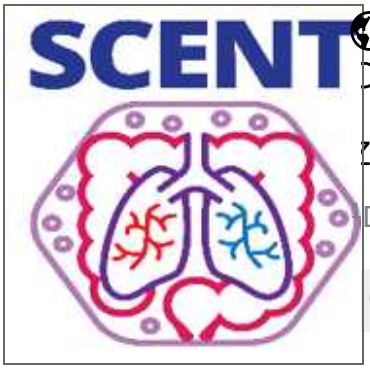


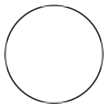
U54 SCENT T/NK Immunosenescence Profiling Flow Cytometry Panel

Zachary R Healy[1], David Murdoch[1], Alicia Cooper-Volkheimer[1]

[1]Duke University, Division of Pulmonary and Critical Care Medicine

Cellular Senescence Network (SenNet) Method Development Community

AUG 17, 2023



valerie.bekker

ABSTRACT

This protocol describes the T/NK Immunosenescence Profiling Flow Cytometry Panel

MATERIALS

Reagents & Materials:

- **Pipettes and Tips for 1-1000uL**
- **96 Well Round Bottom Plates:** Costar Cat #3799
- **Bullet Tubes:** Costar Cat #4401
- **BSB Plus:**BD Cat #566385
- **TruStain FcX:** BioLegend Cat #422302
- **dPBS:** Invitrogen Cat #: 41190-250

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Protocol status: Working
We use this protocol and it's working

Created: Aug 17, 2023

Last Modified: Aug 17, 2023

PROTOCOL integer ID: 86607

FBS Aliquot Prep

1 **FBS** (hiFBS): Gemini Bio Products Cat #100-106 1L
Prepare FBS Aliquots:

1.1 Thaw heat-inactivated FBS (hiFBS):
- Thaw a 500mL Bottle of FBS at 4°C. This may require more than overnight so the 500mL Bottle may be removed 2 or 3 days prior to use. Do not leave the FBS at room temperature overnight.

1.2 Aliquot the 500mL FBS Bottle in 50mL aliquots (a total of ten 50mL hi-FBS Aliquots)

1.3 Label the aliquots with Batch#, Expiration Date, Aliquot Date

1.4 Store 50mL aliquots @ -20ºC until expiration date or for up to 2 freeze/thaw cycles

FACS Wash

2 **FACS Wash w/ EDTA (D-PBS with 0.5% FBS + 2 mM EDTA):** Invitrogen Cat # 41190-250

Remove 4.5mL PBS from a 500mL bottle

2.1

2.2 Add 2.5mL of thawed FBS

2.3 Add 2mL of 0.5M EDTA solution (Catalog #: E7889-100ML)

2.4 Label the bottle with preparer's initials and expiration date (one month from preparation)

2.5 Store @ 4°C

Pen-Strep-Glut (PSG)

3 **Pen-Strep-Glut (PSG) (L-Glutamine-Penicillin-Streptomycin Soln):** Sigma Cat#: G6784-100ML

3.1 Thaw 100mL bottle

3.2 Aliquot into 10mL into 15mL conicals (total of ten 15mL conicals)

3.3 Label the aliquots with Batch#, Expiration Date, Aliquot Date

3.4 Store @ -20°C

R10FBS Media Preparation

4 R10FBS Media Preparation (“R10”)

4.1 Remove 55mL RPMI from a 500mL bottle of RPMI

4.2 Add 50mL aliquot of thawed, hiFBS

4.3 Add 5mL aliquot of thawed Pen-Strep-Glut

4.4 Label the bottle with preparer's initials and expiration date (one month from preparation)

Formaldehyde Solution

5 1% Formaldehyde Solution (“1% Fix”) (“PFA”)

Reagents:

- 10% Formalin
- PBS

5.1 Add 5 mL 10% Formalin to a sterile 50 mL centrifuge conical tube

5.2 Add 45 mL PBS

5.3 Label the bottle with reagent name, initials & expiration date (one month from preparation)

5.4 Store 1% Fix at RT (18-25°C) for up 1 month

Protocol

6 **Overview**

Thaw cells and distribute to plates. Stain immediately, then acquire flow data.

6.1 **Thawing**

1. Prepare 20ml of R10 in 50mL conical tube per sample (1-4 vials per tube)

2. Warm the R10 for 30 min at 37C prior to use

3. Place cryovials in a 37C bath for 3-5 sec at a time. Withdraw, examine and repeat (usually 3-4 rounds) until small, pea-sized amount of ice remains

4. Spray with 70% EtOH and wipe off before returning to the hood

5. To each cryovial, add 1ml of R10 dropwise to each cryovial

6. Transfer the 2ml PBMC sample from the cryovials into the 50ml conicals

7. Invert 3x to mix

8. Centrifuge at 350g for 10 min

9. Pour off the supernatant, do not shake to allow some volume to remain

10. Gently swirl the 50mL conical in remaining volume to loosen pellet

11. Add 10mL pre-warmed R10 and resuspend by pipetting 10 times. Mix sample carefully but thoroughly to break up any cell clumps.

12. Centrifuge at 350g for 10 min

13. Pour off the supernatant, do not shake to allow some volume to remain

14. Gently swirl the 50mL conical in remaining volume to loosen pellet

15. Add 10mL pre-warmed R10 and resuspend by pipetting 10 times. Mix sample carefully but thoroughly to break up any cell clumps

16. **COUNTING & VIABILITY:**

a) Perform a cell count using the Countess II to determine PBMC viability & recovery
b) Add 10uL Trypan Blue to well of mixing plate
c) Add 10uL Cells, pipette up and down
d) Remove 10uL of cell mix and dispense into Countess slide
e) Wait 30 sec
f) Insert into Countess II to calculate total cells and viability

17. Centrifuge 350g x10 min

18. Decant supernatant and resuspend at $10x10^6$ viable cells/mL R10

19. Aliquot 100uL of cells per well to plate ($1x10^6$ cells/well) for immediate staining

6.2 Staining

- Keep everything as cold as much as possible
- Keep everything covered as much as possible; work in dark or incandescent light

1. Centrifuge (400 x 3 min)

2. Flick off supernatant and vortex gently

3. Add 47.5uL FACS wash to each well

4. Add 2.5uL TruStain FCX blocking to each well

5. Incubate at 4C for 15 min

6. Prepare experiment-specific surface stain antibody mix in PBS with 10uL BSB Plus, set aside

7. Add 100uL of antibody mix to each well and gently vortex

8. Incubate in 4C fridge for 30 min

9. Add 100uL FACS wash to each well

10. Centrifuge (400g x 3 min)

11. Flick off supernatant and vortex gently

12. Add 200uL FACS wash to each well

13. Centrifuge (400g x 3 min)

14. Flick off supernatant and vortex gently

15. Add 200uL FACS wash to each well

16. Centrifuge (400g x 3 min)

17. Flick off supernatant and vortex gently

18. Add 200uL 1% PFA

19. Transfer samples to bullet tubes, cover with aluminum foil, store at 4°C, & acquire within 6 hours

	A	B	C	D	E	F	G	H	I

	A	B	C	D	E	F	G	H	I
	Specificity	Fluor	Vendor	Cat#	Clone	Isotype	Conc ug/mL	MRA uL	Titered uL
	KLRG1	BV421	BioLegend	367706	SA231A2	IgG2a k	100	5	1.25
	CD45RA	Pacific Blue	BioLegend	304118	H100	IgG2b k	500	1	0.5
	CD4	BV480	BD	566104	SK3	IgG1 k	50	5	1.25
	CD8	BV570	BioLegend	301038	RPA-T8	IgG1 k	100	5	2.5
	CD45RO	BV605	BioLegend	304238	UCHL1	IgG2a k	100	5	5
	CD56	BV650	BioLegend	362532	5.1H11	IgG1 k	100	5	1.25
	CX3CR1	BV711	BioLegend	341630	2A9-1	Rat IgG2b k	100	5	2.5
	CCR7	BV785	BioLegend	353230	G043H7	IgG2a k	200	5	5
	PD1	VioBright515	Miltenyi	130-120-386	REA1165	rHu IgG1		2	2
	CD3	AF532	Thermo Fisher	58-0038-42	UCHT1	IgG1 k	50	5	5
	CD127	PE	BioLegend	351304	A019D5	IgG1 k	500	5	2.5
	NKG2A	PE-Vio615	Miltenyi	130-120-035	REA110	rHu IgG1		2	1
	CD27	PE-Cy5	BioLegend	356438	M-T271	IgG1 k	50	5	1.25
	CD16	PerCP-Cy5.5	BioLegend	302028	3G8	IgG1 k	200	5	2.5
	CD38	PerCP-eFluor710	TF	46-0388-42	HB7	IgG1 k	120	5	5
	CD25	PE-Cy7	BioLegend	356108	M-A251	IgG1 k	100	5	5

	A	B	C	D	E	F	G	H	I
	CD14	APC	BioLegend	367118	63D3	IgG1 k	200	5	2.5
	CD19	APC	BioLegend	363006	SJ25C1	IgG1 k	50	5	2.5
	CD28	AF647	BioLegend	302954	CD28.2	IgG1 k	100	5	5
	Zombie nIR	Zombie nIR	BioLegend	423105	-	-	-	1	0.4
	CD95	APC-Fire810	BioLegend	305663	DX2	IgG1 k	200	5	1.25