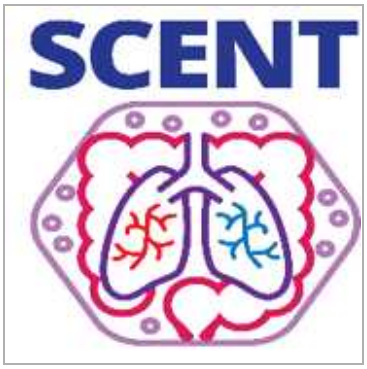

JUL 25, 2023

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

Created: Jul 25, 2023

Last Modified: Jul 25, 2023

PROTOCOL integer ID: 85485

U54 SCENT Transcription Factor (TF)-Phosflow 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

valerie.bekker

ABSTRACT

This flow cytometry panel was developed for simultaneous quantification of transcription factors, phosphorylated kinases, and endosomal β-galactosidase activity in human peripheral blood mononuclear cell (PBMC) subsets including T cells, B-cells, NK cells, and monocytes. This protocol begins immediately following cell thawing, counting, viability determination, and peptide stimulation. This is detailed in **SENNET Antibody Staining Protocol**.

MATERIALS

Protocol Reagents:

Conjugated antibodies and viability dyes: All antibody:fluorophore conjugates were titrated using the same conditions as
outlined below, including all staining, incubation, fixation, and permeabilization steps.

	A	B	C	D	E	F	G	H	I	J	K
	Target	Fluor	Clone	Isotype	Clonality	Pro. #	Manuf.	MRA	No Test @ MRA	Vol. Titrated	No. Actual Tests/Vial

	A	B	C	D	E	F	G	H	I	J	K
	ZnIR	ZnIR	NA	NA	NA	423106	Biolegend	0.5	100	0.1	500
	CD3	APC-Fire810	SK7	Mouse IgG1 kappa	Mono	344858	Biolegend	5	100	1.25	400
	CD14	BV570	M5E2	Mouse IgG2a kappa	Mono	301831	Biolegend	5	100	2.5	200
	CD19	BV605	HIB19	Mouse IgG1 kappa	Mono	302243	Biolegend	5	100	0.63	794
	CD56	BV510	HCD56	Mouse IgG1 kappa	Mono	318340	Biolegend	5	100	1.25	400
	CD39	R718	A1	Mouse IgG1 kappa	Mono	567674	BD	5	100	0.63	794
	CX3CR1	BV711	2A9-1	Rat IgG2b kappa		341630	Biolegend	5	100	2.5	200
	phospho-gH2Ax (Ser139)	BV421	N1-431	Mouse IgG1 kappa	Mono	564720	BD	5	50	2.5	100
	phospho-Bcl-2	PE-CF594	Bcl-2/100	Mouse IgG1 kappa	Mono	563601	BD	5	50	2.5	100
	phospho-NFkB (Ser529)	PerCP-efluor710	B33B4WP	Mouse IgG2b kappa	Mono	46-9863-42	TF	5	100	2.5	200
	phospho-p38 (Thr180, Tyr182)	PE-Cy7	4NIT4KK	Mouse IgG2b kappa	Mono	25-9078-42	TF	5	100	2.5	200
	p16	PE	REA973	HUMAN IgG1	Mono/R	130-116-137	Miltenyi	2	100	2	100
	p21	AF647	195720	Mouse IgG2b kappa	Mono	IC1047R	RnD	5	100	2.5	200

*****Intracellular targets*** *requiring FMOs for gating. This also includes* ***β-galactosidase*** *yielding a total of 7 FMO controls.*

Other Reagents:

	A	B	C	D
	Product Name	**Manufacturer**	**Pro. #**	**Notes:**
	CellEvent Senescence Green Flow Cytometry Assay Kit	TF	C10841 (200 assays)	Optimal Conditions: -Fix in 2% paraformaldehyde -10 min fix at RT in dark -1:750 dilution -90 minute incubation at 37C without CO2 in dark
	TrueNuclear Transcription Factor Buffer Set	Biolegend	424401 (120 tests)	Per protocol for 96-well plates, with 60-min fixation and 45 minute staining, all at RT in dark
	Human TruStain FCX	Biolegend	422302 (200 tests)	During surface staining
	Cell Staining Buffer	Biolegend	420201 (500 mL)	Can substitute FACSwash (PBS without Mg/Ca + 2% HI-FBS and 1 mM EDTA)
	Fixative Solution	Biolegend	420801 (100 mL)	Used prior for fixation during β-gal activity portion of protocol
	FluoroFix Buffer	Biolegend	422101 (200 tests)	Used for flow cytometry acquisition
	Spectroflow QC Beads (lot 2003)	Cytek	B7-10001	Used for daily Cytek Aurora QC.
	Brilliant Stain Buffer Plus	BD	566385	Used in all staining tubes during surface and ICS.
	CEF Pool Extended (MHC I)	JPT peptide	PM-CEF-E-1	Stored in 20-test aliquots at a concentration of 0.5 mg/mL), requiring 0.45 uL per 225 uL stimulation.
	CEFX Ultra SuperStim Pool (MHC II)	JPT peptide	PM-CEFX-3	Stored in 20-test aliquots at a concentration of 0.5 mg/mL), requiring 0.45 uL per 225 uL stimulation.
	BD CompBeads Anti-Mouse	BD	552843	We use single-cell stained controls for compensation/unmixing, however we run comp beads as well as cells for troubleshooting issues.

	A	B	C	D
	BD CompBeads Ant-Rat/Hampster	BD	552845	See above comment
	REA control antibody, human IgG1, PE, Reaffinity	Miltenyi	130-118-347 (100 tests)	Used for FMO controls prior to batch runs
	BV421 Mouse IgG1 k isotype control	Biolegend	400158	Used for FMO controls prior to batch runs
	AF647 Mouse IgG2b k isotype control	Biolegend	400330	Used for FMO controls prior to batch runs
	PE-Cy7 Mouse IgG2b k isotype control	TF	25-4732-81	Used for FMO controls prior to batch runs
	PerCP-efluor710 Mouse IgG2b k isotype control	TF	46-4732-82	Used for FMO controls prior to batch runs
	PE-CF594 Mouse IgG1 k isotype control	BD	562292	Used for FMO controls prior to batch runs
	Corning Costar 96-well PS U-bottom tissue culture-treated plates	Corning	3799	Plates for staining
	Corning Costar 96-well PP Cluster tubes (1.2 mL)	Corning	4401	Cluster tubes for acquisition

Misc Lab Items: Swinging bucket microplate centrifuge (Eppendorf 5430), Eppendorf pipettes (Eppendorf Research Plus), Eppendorf MixMate, reservoirs for multichannel pipettes (VWR), pipette tips, filtered (VWR), aluminum foil, deionized water, DPBS (Gibco).

General Notes

1 **Instrument QC, Single-Stain Controls, Fluorescence minus-one (FMO) controls, and General Protocol Notes** . We routinely perform single-stain controls (using both cells and compensation beads) as well as FMO for the intracellular targets (highlighted in **BOLD** in materials section) prior to any batch processing.

1.1 **Instrument QC**. The instrument, a Cytek Aurora NorthernLights, 3-laser (405nm, 488nm, 605 nm) undergoes daily QC prior to any experimentation. Experiments are only performed after the instrument passes QC. This is performed after a 30-minute laser warm-up period using 1 drop of Cytek SpectroFlow QC beads (current lot 2003) in 200 uL of DI water. All QC reports are maintained on a local server and the desktop hard-drive for monitoring. The instrument receives annual preventive maintenance is under an active service contract with Cytek.

1.2 **Single-Stain Controls**. Single stain controls are obtained using both cells (from a known batch control normal/healthy donor maintained in our laboratory repository following leukapheresis) and using BD compensation beads. All single stain controls undergo the entire protocol prior to acquisition, including all incubations listed below for the designated durations to ensure a reliable representation each step has on the fluorophore.

1.3 **FMO controls**. FMO controls are performed for each of the intracellular targets above prior to any batch processing of cells; however, due to the nature of targets in this panel, we do use isotype controls during the FMO experiments as recommended by the majority of the manufacturers above.

1.4 **General Protocol Notes**. First, we use BD Brilliant Stain Buffer Plus in all surface and intracellular staining steps of the protocol at 10 µL per 100 µL staining volume. As all our staining is performed at 50 µL, we use 5 µL per test. In addition, during surface staining, we use Biolegend TruStain FCX blocking agent at 5 µL per 100 µL volume (2.5µL in 50 µL). All stimulation and staining is performed in Costar 96-well U-bottom TC-treated PS plates at a starting concentration of 2 x 10^6 cells per well. Stimulations with overlapping peptides are performed for 6 hours at 37C in a 5% CO_2, humidified incubator environment. All centrifugation steps are performed in an Eppendorf 5430 swinging bucket centrifuge at room temperature (centrifugation speeds vary by step, please be aware). All experiments use a batch control.

Stimulation with CEF Peptide

2 Following thawing, viability assay, counting, and distribution of cells to the 96-well plate, stimulation using CEF extended pool I (MHC I) and CEFX Ultrastim Pool (MHC II) is performed as detailed in **SENNET Antibody Staining Protocol** using a final concentration of each peptide at 1.0 µg/µL in a 225 µL total volume with 2 x10^6 cells/well starting concentration. The DMSO concentration is maintained at a maximum of 1% during this process, and DMSO solvent control

is used for non stimulated cells as an equivalent concentration. The stimulation is performed for 6 hours after which the cells are transferred to a 4C refrigerator until surface staining is begun.

Surface Staining

3 Prepare surface staining cocktail using the following table in the indicated order, adding 10% of each reagent to account for pipetting errors (see table in 3.1)

3.1

	A	B
	Surface Stain	Amount per Test
	Cell Staining Buffer (base panel)	33.64
	BD Brillant Stain Buffer Plus	5
	TruFCX block	2.5
	(mix by pipetting)	
	ZnIR	0.1
	CD3 APC-Fire810	1.25
	CD14 BV570	2.5
	CD19 BV605	0.63
	CD56 BV510	1.25
	CD39 R718	0.63
	CX3CR1 BV711	2.5

3.2 Centrifuge the cells in the 96-well plate at 300xg for 3 minute (wash 1)

3.3 Decant supernatant, being careful not to disrupt cell pellet.

3.4 Wash the cells in 200 µL of cell staining buffer (wash 2).

3.5 Centrifuge the cells in the 96-well plate at 300xg for 3 minutes.

3.6 Decant supernatant, being careful not to disrupt cell pellet.

3.7 Place the cells in an Eppendorf MixMate to release the cell pellet for 30s at 600 rpm.

3.8 Pipette 50 µL of surface staining cocktail to each well.

3.9 **Cover with aluminum foil and incubate at 4C for 30 minutes.**

3.10 *During this incubation, we typically prepare the reagents for the next step of the protocol (*β-galactosidase activity assay*).*

3.11 Following incubation, add 150 µL of cell staining buffer to each well (wash 1).

3.12 Centrifuge the cells in the 96-well plate at 300xg for 3 minute.

3.13 Decant supernatant, being careful not to disrupt cell pellet.

3.14 Wash the cells in 200 µL of cell staining buffer (wash 2).

3.15 Centrifuge the cells in the 96-well plate at 300xg for 3 minutes.

3.16 Decant supernatant, being careful not to disrupt cell pellet.

3.17 Wash the cells in 200 µL of cell staining buffer (wash 3).

3.18 Centrifuge the cells in the 96-well plate at 300xg for 3 minutes.

3.19 Decant the supernatant, being careful not the disrupt the cell pellet.

3.20 Place the cells in an Eppendorf MixMate to release the cell pellet for 30s at 600 rpm.

β-galactosidase activity assay (Cell Event Senescence Gree...

4 Optimal β-galactosidase staining conditions were determined using a single-color high-throughput approach in human primary T cells with Palbociclib incubation (5 µM) in the presence of anti CD2/anti-CD3/anti-CD28 (StemCell) and high-dose IL-2 (500 IU/mL; Miltenyi IL-2 IS) for 7 days to induce β-galactosidase expression. The optimal conditions were 10 minute fixation at

0.5X BL Fixative solution, followed by 90 minute incubation at a concentration of 1:750 of the β-galactosidase Green Probe.

4.1 Resuspend the cell pellet in 100 µL of 0.5X Biolegend Fixative Solution (1:1 D-PBS w/o Mg, Ca and 1x Fixative solution).

4.2 Incubate the cells for 10 minutes at room temperature in the dark.

4.3 Following incubation, add 100 µL of cell staining buffer to each well (wash 1).

4.4 Centrifuge the cells in the 96-well plate at 300xg for 3 minutes.

4.5 Decant supernatant, being careful not to disrupt cell pellet.

4.6 Wash the cells in 200 µL of cell staining buffer (wash 2).

4.7 Centrifuge the cells in the 96-well plate at 300xg for 3 minutes.

4.8 While cells are centrifuging, prepare β-galactosidase Working Solution using the following table, including 10% extra of each component to account for pipetting errors/variability:

	A	B

	A	B
	Component per test (1:750 dilution)	
	βgal Green Probe	0.133 µL/test
	Senescence Diluent Buffer	100 µL/test

4.9 Incubate the cells in 100 µL of Working Solution for 90 minutes at 37C without CO_2 in the dark.

4.10 Following incubation, add 100 µL of cell staining buffer to each well (wash 1).

4.11 Centrifuge the cells in the 96-well plate at 300xg for 3 minutes.

4.12 Decant supernatant, being careful not to disrupt cell pellet.

4.13 Wash the cells in 200 µL of cell staining buffer (wash 2).

4.14 Centrifuge the cells in the 96-well plate at 300xg for 3 minutes.

4.15 Decant supernatant, being careful not to disrupt cell pellet.

4.16 Wash the cells in 200 µL of cell staining buffer (wash 3).

4.17 Centrifuge the cells in the 96-well plate at 300xg for 3 minutes.

4.18 Decant supernatant, being careful not to disrupt cell pellet.

4.19 Place the cells in an Eppendorf MixMate to release the cell pellet for 30s at 600 rpm.

Intracellular Staining for TF, Phosphorylated kinase targets..

5 First, it should be noted that the fixation and permeabilization conditions vary for cytokines, transcription factors, and phosphorylated targets. While mild permeabilization is necessary for cytokine staining in cytoplasm, harsher conditions are necessary for staining nuclear targets. In particular, methanol permeabilization is recommended for some transcription factors and phosphorylated targets. The downside to the use of methanol is that methanol is detrimental to many fluorophores (particularly protein-based and tandem dyes) and alters epitopes of may proteins. Many transcription factors and phosphorylated kinases can be successfully stained using paraformaldehyde fixation followed by more harsh permeabilization (typically involving triton-X or higher saponin concentrations than typically used in cytokine staining). This approach has the advantage of improved signal-to-noise ratio/reduced background staining and less negative effect on fluorophores. We tested the BL TruePhos (methanol buffer), the BL True Nuclear (detergent-based), and the BD PhosFlow buffer III (methanol) for optimal performance. Overall, the BL True Nuclear buffer provided the best performance overall. Although the staining of p16 was less optimal (although still able to be resolved), the staining of NFkB, p38, p21, and H2Ax was improved compared to the methanol-based fixative. In addition, this allowed for the inclusion of addition cell surface staining targets (CD39, CX3CXR1) which we would not have been able to include otherwise.

This protocol is based on the recommendations of Biolegend for the use of this kit in 96-well plate format. Please refer to the following for further details (https://www.biolegend.com/protocols/true-nuclear-transcription-factor-staining-protocol-for-96-well-u-bottom-plate/4246/). Note that the prior fixation performed during the β-galactosidase assay (section 4) was mild and it was necessary during optimization to further fix the cells after

the β-galactosidase assay to improve background staining.

5.1 Prepare 1x Biolegend TrueNuclear Fix Concentrate by mixing 1-part 4X Fix Concentrate with 3 parts Fix Diluent to allow for 200 µL of 1X Fix Concentrate per well (e.g., for 10 samples, one would need 2200 µL (including 10% additional) of 1x Fix Concentrate; therefore, this would require 550 µL of 4x Fix and 1650 µL of 4x Fix Diluent).

5.2 Resuspend the cell pellet in 200 µL of 1x Fix Concentrate for 60 minutes at RT in the dark.

5.3 Following incubation, centrifuge the cells in the 96-well plate at **500xg**. Note the increased speed which will be necessary to pellet the cells following permeabilization.

5.4 Decant supernatant, being careful not to disrupt cell pellet

5.5 Wash the cells in 200 µL of 1x PermWash (wash 1).

5.6 Centrifuge the cells in the 96-well plate at 500xg for 3 minutes.

5.7 Decant supernatant, being careful not to disrupt cell pellet

5.8 Wash the cells in 200 µL of 1x PermWash (wash 2).

5.9 Centrifuge the cells in the 96-well plate at 500xg for 3 minutes.

5.10 Decant supernatant, being careful not to disrupt cell pellet

5.11 Wash the cells in 200 µL of 1x PermWash (wash 3).

5.12 Centrifuge the cells in the 96-well plate at 500xg for 3 minutes.

5.13 Place the cells in an Eppendorf MixMate to release the cell pellet for 30s at 600 rpm.

5.14 Prepare the Intracellular Staining Mix/Cocktail using the following table, in the order listed:

	A	B
	ICS Panel	**Amount Per Test**
	1xPermWash	35.5
	BD Brilliant Stain Buffer Plus	5
	phospho-gH2Ax (Ser139) BV421	2.5
	Bcl-2 PE-CF594	2.5
	phospho-NFkB (Ser529) PerCP-efluor710	2.5
	phospho-p38 (Thr180, Tyr182) PE-Cy7	2.5
	p16 PE	2
	p21 AF647	2.5

5.15 Resuspend the cell pellets in 50 µL of the Intracellular Staining Mix.

5.16 Incubate at RT for 45 minutes in the dark.

5.17 Add 150 µL of 1x PermWash to each well (wash 1).

5.18 Centrifuge the cells in the 96-well plate at 500xg for 3 minutes.

5.19 Decant supernatant, being careful not to disrupt cell pellet.

5.20 Wash the cells in 200 µL of 1x PermWash (wash 2).

5.21 Centrifuge the cells in the 96-well plate at 500xg for 3 minutes.

5.22 Decant supernatant, being careful not to disrupt cell pellet.

5.23 Wash the cells in 200 µL of 1x PermWash (wash 3).

5.24 Centrifuge the cells in the 96-well plate at 500xg for 3 minutes.

5.25 Place the cells in an Eppendorf MixMate to release the cell pellet for 30s at 600 rpm.

5.26 Resuspend the cells in 200 µL of BL FluorFix Buffer and acquire the samples on the flow cytometer.