

Sep 23, 2024

Mitochondrial antigen presentation (MitAP) to primary 2C CD8 T (proliferation and suppression) assay

DOI

dx.doi.org/10.17504/protocols.io.dm6gpzk25lzp/v1

Alexandra Kazanova¹, Samantha Gruenheid¹

¹McGill Research Centre on Complex Traits and Department of Microbiology and Immunology, McGill University



Alexandra Kazanova

McGill University

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

[Create free account](#)

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.dm6gpzk25lzp/v1>

Protocol Citation: Alexandra Kazanova, Samantha Gruenheid 2024. Mitochondrial antigen presentation (MitAP) to primary 2C CD8 T (proliferation and suppression) assay. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.dm6gpzk25lzp/v1>

Manuscript citation:

Modeling gene-environment interactions in Parkinson's Disease: *Helicobacter pylori* infection of *Pink1*^{-/-} mice induces CD8 T cell-dependent motor and cognitive dysfunction
Alexandra Kazanova, Jacqueline Sung, Nathalia Oliveira, Christina Gavino, Sherilyn Recinto, Hicham Bessaiah, Jessica Pei, Lindsay Burns, Willemein Miller, Morgane Brouillard-Galipeau, Lei Zhu, Lucia Guerra, Moustafa Nouh Elemeery, Adam MacDonald, Joel Lanoix, Pierre Thibault, Heidi McBride, Michel Desjardins, Jo Anne Stratton, Nathalie Labrecque, Samantha Gruenheid
bioRxiv 2024.02.25.580545; doi: <https://doi.org/10.1101/2024.02.25.580545>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: September 17, 2024

Last Modified: September 23, 2024

Protocol Integer ID: 108061

Keywords: ASAPCRN, protocol details the mitochondrial antigen presentation, mitochondrial antigen presentation, activation of mitochondria, mitochondria, mitap in an assay, mitap, exposed bmdc

Funders Acknowledgements:

Aligning Science Across Parkinson's (ASAP)

Grant ID: ASAP 000525

Abstract

This protocol details the mitochondrial antigen presentation (MitAP) to primary 2C CD8 T (proliferation and suppression) assay. In order to assess MitAP in an assay that incorporated all signals for naïve T cells activation in vivo, we tested whether H.pylori-exposed BMDCs could trigger activation of mitochondria-reactive primary CD8 T cell.

Materials

Complete RPMI 1640:

	A	B
	RPMI 1640 with Glutamax (Gibco)	with 10% FBS
	sodium pyruvate	1%
	Hepes	1 mM
	penicillin/streptomycin	1%

MACS buffer: PBS, 2% FBS and 2mM EDTA

StemCell Isolation Buffer: PBS, 2% FBS and 1 mM EDTA

ACK (Ammonium-Chloride-Potassium) red blood cell lysis buffer: 8.29g NH₄Cl, 1g KHCO₃, 0.0367g EDTA, distilled water to 1L)

☒ 090-150- FBS, Premium Canadian Origin, 500mL **Wisent Bioproducts Catalog #090-150**

PBS 10x (Sigma; Cat# P5493-4L)

☒ Phosphate buffered saline **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P5493-4L** without calcium/magnesium

☒ Recombinant Mouse GM-CSF (carrier-free) **BioLegend Catalog #576308**

☒ Purified anti-mouse CD3ε Antibody **BioLegend Catalog #100302**

☒ EasySep™ Mouse CD8+ T Cell Isolation Kit For processing 1×10^9 cells **STEMCELL Technologies Inc. Catalog #19853**

☒ EasySep™ Magnet For isolating 2.5×10^8 cells **STEMCELL Technologies Inc. Catalog #18000**

☒ CD4 CD25 Regulatory T Cell Isolation Kit, mouse **Miltenyi Biotec Catalog #130-091-041**

☒ CFSE Cell Division Tracker Kit **BioLegend Catalog #423801**

☒ eBioscience®; Fixable Viability Dye eFluor®; 780 **Thermo Fisher Catalog #65-0865-18**

Anti-mouse CD8a; -FoxP3; CD4; CD25 conjugated to selected fluorochromes (except PE).

☒ e-Bioscience Foxp3 / Transcription Factor Staining Buffer Set **Invitrogen - Thermo Fisher Catalog # 00-5523-00**

MACS® LD and LS Columns Catalog #130-042-401, Catalog #130-042-901

QuadroMACS™ Separators

Tag-it Violet Biolegend Cat#455101

H. Pylori PMSS1 strain sonicate

70 μ m cell strainers

Paraformaldehyde 1%

1M glycine in PBS

Mice

Pink1^{+/+} (n=6) and Pink1^{-/-} mice (n=6). 2C TCR transgenic mouse (n=1-2). OT-1xRAG1^{-/-} (n=1-2).

Troubleshooting

Bone Marrow-derived Dendritic cells (BMDC, 0-9 days of experiment):

1m

- 1 Femurs from three Pink1^{+/+} and three Pink1^{-/-} mice are cleaned manually from soft tissues and flushed with ice cold PBS.
- 2 Cells are pelleted and  2 mL of ACK RBC lysis buffer are added for  00:01:00 and mixed gently with 1 ml pipette tip.  
 
- 3 Lysis buffer is blocked with  13 mL of complete RPMI and pelleted with centrifugation.
- 4 Cells are resuspended, counted with hemocytometer and brought to 200,000 cell/ml in complete RPMI.
- 5  10 ng/ml of mr-GMCSF is added to BMDC for differentiation. 
- 6 Cells are plated in non-tissue culture treated plasticware (6 well plate, sarstedt) for  72:00:00 at  37 °C .  3d
 
- 7 On day 3 equal volume of the starting culture of complete RPMI with  10 ng/ml mr-GMCS is gently added on top of the well (avoid any additional shaking of the cells).
- 8 On day 6 replace 50% of the culture media with new complete RPMI with  10 ng/ml mrGMCSF.
- 9 On day 9 collect all non-adherent and loosely adherent cells and count. Bring to 500,000 cell/ml in complete RPMI.
- 10  3 mL of BMDC cells/ per well of a 6-well plate. Add sonicate of *H. pylori*  100 µg/ml (300 µg/well) and incubate for  06:00:00 at  37 °C . Leave at least two wells without bacterial sonicate for a control.  6h
  
- 11 After 6 hours collect BMDC gently using cell scraper and wash. 

12 Pellet cells by spinning down at  800 x g, 00:05:00 (Eppendorf 5810 – 2000 RPM).

5m



13 Remove supernatant completely and add  2 mL of  Room temperature (RT) 1% PFA and mix gently with a pipette. Leave at RT for 9 minutes.



14 Quench with 13 ml of 0.1M Glycine in complete RPMI (1 1M Glycine : 9 Complete RPMI).

15 Pellet cells by spinning down at  800 x g, 00:05:00 and dumping the supernatant.

5m



16 Repeat  [go to step #13](#) -  [go to step #15](#) two more times.

17 Resuspend in complete RPMI count and bring to 1×10^6 cells/ml .

2C CD8 T cells (day 9)

18 Collect spleens from 2C TCR transgenic mice of 6-8 weeks old, verify no presence of thymoma in the mouse.

19 Mash spleen through a PBS pre-primed 70 μ m cell strainer using a 3 ml syringe plunger. Wash strainer with additional  10 mL of PBS.



20 Add  3 mL of ACK RBC lysis buffer to the splenocyte pellet and leave for  00:01:00 , mix gently.

1m



21 Block lysis buffer with  12 mL of complete RPMI, count cells and take  100 μ L aliquot for purification control (a) into a separate Eppendorf tube.

22 Proceed with EasySep™ Mouse CD8+ T Cell Isolation Kit (Stemcell; Cat#19853) according to the manufacturer protocol. After spinning resuspend cell pellet in Stemcell Isolation Buffer 10^8 /ml.

- 23 Transfer cell to a 5 ml round bottom conical tube and add  20 μL of Fc Blocking reagent per 1 ml of cell suspension.
- 24 Add 50 $\mu\text{l/ml}$ of Isolation cocktail to cell suspension and incubate  00:20:00 20m
 On ice .   
- 25 Add 125 $\mu\text{l/ml}$ of pre-vortexed for  00:00:30 Rapid spheres and incubate 5m 30s
 00:05:00 at  Room temperature   
- 26 Top to  2.5 mL with Stemcell isolation buffer, transfer to EasySep™ Magnet and let stand for  00:03:00 in the hood. 3m

- 27 Dump negatively selected CD8 T cells from the falcon tube in the magnet to a new 15 ml conical tube. Take a  50 μL aliquot of cell suspension for purification control (b).
- 28 Top the 15 ml tube with PBS and spin down at  450 x g,  00:05:00 (Eppendorf 5810 – 1500 rpm). 5m

- 29 Stain pelleted 2C CD8 T cells with  2 mL 2.5 μM cell tracker Tag-it Violet in PBS 12m
 00:12:00 at  37 °C in the dark.  
- 30 Add  2 mL of the FBS and incubate an additional 5 min in the dark at  Room temperature to efflux and quench excessive Tag-it violet dye.
- 31 Wash cells three times with 10 ml of complete RPMI. 
- 32 Resuspend in complete RPMI count and bring to 1×10^6 cells/ml.
- 33 Take an aliquot of  500 μL Tag-it Violet-2C CD8 T cells and store in the fridge (a undiluted Tag-it violet signal, one of undivided cells control for flow cytometry). 

34 Stain purification controls (a) and (b) with antiCD8 and viability to access CD8 T cell purification quality.

OT-1 CD8 T cells (TCR Tg CD8 T cell control)

35 collect inguinal, popliteal, axillar, and neck subcutaneous lymph nodes from OT-1 x Rag1 -/- mice.

Note

Make sure not to take any fat tissue.

36 Mesh lymph nodes through a pre-primed with PBS 70 µm 1 ml syringe plunger.

37 Wash the strainer with  10 mL of PBS. Take  500 µL aliquot for CD8 frequency assessment using flow cytometry. 

38 Spin cells down  450 x g, 00:05:00 . 

39 Stain pelleted OT-1 CD8 T cells with 2 ml 2.5 µM cell tracker Tag-it Violet in PBS  00:12:00 at  37 °C in the dark. 


40 Add  2 mL of the FBS and incubate an additional  00:05:00 in the dark at  Room temperature to efflux and quench excessive Tag-it violet dye. 


41 Wash cells three times with complete RPMI. 

42 Resuspend in complete RPMI count and bring to 1×10^6 cells/ml.

CD4CD25- (CD4 conv) and CD4CD25 T (Tregs) (day 9)

3h 40m

- 43 Collect spleens of three Pink1^{+/+} and three Pink1^{-/-} mice.
- 44 Mash spleen individually through the 70 μm cell strainers and lyse RBC (as in step 20-21).
- 45 Pull three spleens of each genotype together and count cells with hemocytometer and take a  100 μL aliquot for purification control (c). (Expected spleen count for B6.129 mixed genetic background mouse spleen is 5×10⁷).
- 46 Spin cells down at  450 x g, 00:05:00 , resuspend pellets in  600 μL of MACS buffer per 1.5×10⁸ (adjust the volume for more cells) and transfer to a new labeled 15 ml conical tube.
- 47 Add  150 μL of the CD4+CD25 regulatory T cell Biotin-Antibody Cocktail (100 μl/400μl cell suspension) and incubate  On ice  00:15:00 .
- 48 Wash cells by adding  10 mL of MACS buffer and spinning it down  450 x g, 00:05:00 . Resuspend pellets in  1200 mL of MACS buffer (800/ 10⁸) and add  300 μL of anti-Biotin MicroBeads (200/10⁸ cells) incubate  00:15:00  On ice .
- 49 Immediately transfer the QuadroMACS separator with two LD columns into the hood and prime columns with  2 mL of MACS buffer.
- 50 Transfer cells to the columns and collect negatively selected unlabeled total CD4 T cells to a new 15 ml labeled conical tubes.
- 51 Wash columns three times with  1 mL MACS buffer (passing through LD columns take a long time, make sure collection tubes under the columns is placed  On ice).
- 52 Take a  50 μL aliquot of total CD4 T cells in a separate Eppendorf cup – control (d). Spin cells down and add to the pellet  600 μL of MACS buffer with 15μl of anti CD25-PE.



5m

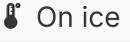
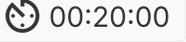
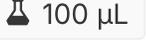
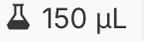
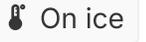
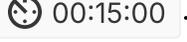
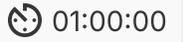
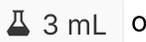


15m



20m



- 53 Incubate  On ice  00:20:00 in the dark. 20m 
- 54 Top with 10 of MACS buffer, mix and take  100 μ L aliquote (control e). 
- 55 After spinning cells down  450 x g, 00:05:00 aspirate supernatant and resuspend cells in 1350 of buffer ($900/10^8$). 5m 
- 56 Add  150 μ L of anti-PE microbeads (100/108) and place  On ice for another  00:15:00 . 15m 
- 57 Place 2 LS columns on the magnet and prime them with 3 ml buffer and discard the pass through.
- 58 Transfer samples to the columns and collect the pass through into a new 15 ml conical tube on ice labeled CD4+CD25-.
- 59 Wash column with 3 ml buffer three times and collect flow through. Take a  500 μ L aliquot from the flow through for a control (f). 
- 60 Spin CD4 CD25- cells down  450 x g, 00:05:00 and resuspend in 2 ml of complete RPMI with 1 μ g/ml anti-CD3 (145-2c11) and place in the CO₂ incubator to culture for  01:00:00 at  37 °C . 1h 5m 
- 61 Simultaneously with the  [go to step #60](#) transfer LS columns from the magnet to the new conical tube labeled CD4+CD25+.
- 62 Add  3 mL of buffer to the LS column and use column plunger to isolate CD25+ cells. 
- 63 Replunge dry columns 5 times to completely recover the cells.

64 Add  10 mL MACS buffer and spin down the cell. 

65 Completely remove the supernatant containing unbound microbeads and resuspend in  1 mL of complete media (take  30 μ L aliquot for a control (g).

66 Immediately acquire control f and g (10 μ L only) on a cytometer, using PE channel if purity of control (g)- PE+ cells are lower than 85% repeat steps [ go to step #57 -  go to step #59 and  go to step #61 -  go to step #63] one more time. If control f has CD25+ cells more than 1% repeat steps [ -  go to step #60] one more time.

67 Transfer controls d-g to a plate for staining with FoxP3 antibody.

68 Spin plate down and stain viability with FVS780 in PBS dilution 1:2000 50 μ L per well,  00:15:00  On ice in the dark.  

15m

69 Wash viability stain with  300 μ L of MACS buffer and leave cells for fixation in FoxP3 fixing buffer in the fridge 120 μ L/well. 

70 After an hour take CD4+CD25- cells from the incubator and top the tube with PBS.

71 Spin CD4+CD25- cells down and resuspend in 2 ml of CFSE 1.25 μ M stain in the same manner as B12-B14.

72 While staining CD4+CD25- count CD4CD25+ and bring them to the concentration of 10^6 cells/ml in complete RPMI.

73 Resuspend CFSE-CD4+CD25 in 3 ml complete RPMI, take  100 μ L aliquote for undilute CFSE staining control and place it in the fridge. 

74 using CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotech; Cat #130-091-041) according to manufacturer's protocol. CD4 conv T cells were resuspended in complete RPMI 10% FBS and incubated for  01:00:00 at  37 $^{\circ}$ C with 1 μ g/ml of anti-mouse   

1h

CD3 (145-2c11 clone; Biolegend; Cat#100363) or left untreated. Cells were washed in PBS and stained with CFSE 1.25 μ M in the same manner as Tag-it violet staining.

- 75 Purity of magnetic selection of 2C CD8, CD4+CD25+ and CD4+CD25- cells was determined with flow cytometry prior to co-culture. Regulatory CD25+CD4 T cells were stained with anti-FoxP3 and acquired with flow cytometry.
- 76 Co-culture was performed in 10% FBS complete RPMI in 96-well round bottom plates for suspension cells (Sarstedt; Cat#83.3925.500) at a following ratio: 50,000 2C to 50,000 CD4 conv cells to 100,000 fixed BMDC. For suppression assay Tregs were added to the co-culture at (1-0.25):1 2C CD8 T ratio. Each condition was performed in technical replicates. At 24- and 72-hours cells were collected and assessed by flow cytometry for Activation Induced Markers expression and proliferation.
- 77 Co-culture conditions for proliferation assay (excluding single cell type controls, and cell tracker controls from the fridge):

	A	B	C	D	E	F	G	H	I
	BMD C	<i>H. pylori</i> for BMDC	CD 4 conv	aCD3 for CD4	OT-1 CD8 T	2C CD8 T	SIINFE KL	SYIRYY GL	Function
	+	-	-	-	+	-	+	-	OT-1 proliferation – positive control
	+	-	-	-	+	-	-	-	OT-1 proliferation – negative control
	+	-	-	-	-	+	-	+	2C proliferation – positive control
	+	-	-	-	-	+	-	-	2C proliferation – negative control
	+	+	+	+	-	+	-	-	2C proliferation – experimental

	A	B	C	D	E	F	G	H	I
									condition
	+	+	+	+	+	-	-	-	TCR 2C specificity control
	+	-	+	+	-	+	-	-	2C No proliferation – infection effect on MitAP control
	+	+	+	-	-	+	-	-	CD4 help effect control
	+	+	-	-	-	+	-	-	CD4 help effect control
	-	-	+	+	+	-	-	-	Bystander activation control OT1
	-	-	+	+	-	+	-	-	Bystander activation control 2C
	+	+	+	+	-	-	-	-	CD4 proliferation
	+	-	+	+	-	-	-	-	CD4 proliferation
	-	-	+	+	-	-	-	-	CD4 proliferation
	-	-	+	-	-	-	-	-	CD4 proliferation negative control

78 Suppression of proliferation was calculated using the following formula:

% suppression = $100 \times (1 - \% \text{ of divided 2C in the presence of Treg} / -\% \text{ of divided 2C at No Treg condition})$