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T cell purification and activation V.3

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We use this protocol and it's working

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Abstract

This protocol details the purification and activation of Mouse Naïve CD8+ T Cell from mouse spleens using a purification kit. The cells are then activated by culturing them in plates coated with anti-CD3 antibodies and adding soluble anti-CD28 antibodies. The activation process occurs over 72 hours, with IL-2 added after the first 24 hours to support T cell proliferation and survival. The isolation kit from STEMCELL is designed to isolate naïve CD62L+CD44-CD8+ T cells from single-cell suspensions of splenocytes by negative selection. Unwanted cells are targeted for removal with biotinylated antibodies that are directed against non-naïve CD8+ T cells (CD4, CD11b, CD11c, CD19, CD44, CD45R/B220, CD49b, TCR γ/δ , TER119) and streptavidin-coated magnetic particles. Labeled cells are separated using an EasySep™ magnet without the use of columns.

Materials

Reagents and solution:

A	B	C
Reagent or solution	Supplier	Catalogue #
Anti-mouse CD3 (Clone: 145-2C11)	BioXcell	BE0001
Anti-mouse CD28 (clone: 37.51)	Leinco	C379-5.0 mg
RPMI media	VWR (Corning)	CA45000-396
Phosphate Buffer saline (PBS)	Gibco	14190144
EasySep buffer	STEMCELL	20144
EasySep™ Mouse CD8+ T Cell Isolation Kit	STEMCELL	19858
ACK (Ammonium-Chloride-Potassium) Lysing Buffer	ThermoFisher	A1049201
L-glutamine 200 mM	VWR	CA45000-676
HEPES 1M	Fisher	MT25060CI
Sodium Pyruvate	VWR	CA45000-710
2-Mercaptoethanol	ThermoFisher	21985023
Non-Essential Amino acids	VWR (Corning)	CA45000-700
Fetal Bovin Serum	Gibco	12483020
24 wells plate flat bottom suspension plates	Sarstedt	83.1836.500
96 wells plate flat bottom suspension plates	Sarstedt	82.1581.001
Cell strainers (70um)	Fisher	08-771-2

⊗ InVivoMAb anti-mouse CD3 **BioXcell Catalog #BE0001**

⊗ Anti-Mouse CD28 (Clone 37.51) **Leinco Technologies Inc. Catalog #C379**

⊗ RPMI **VWR International (Avantor) Catalog #45000-396**

⊗ Phosphate buffered saline (PBS) without Ca/Mg **Thermo Fisher Scientific Catalog #14190144**

⊗ EasySep™ Buffer **STEMCELL Technologies Inc. Catalog #20144**

⊗ EasySep™ Mouse Naïve CD8 T Cell Isolation Kit **STEMCELL Technologies Inc. Catalog #19858**

⊗ ACK Lysing Buffer **Thermo Fisher Scientific Catalog #A1049201**

⊗ L ()-Glutamine solution 200 mM **VWR International (Avantor) Catalog #45000-676**



⊗ Sodium pyruvate solution 100 mM **VWR International (Avantor) Catalog #45000-710**

⊗ 2-mercaptoethanol **Gibco - Thermo Fisher Scientific Catalog #21985023**

⊗ Non essential amino acids **VWR International (Avantor) Catalog #45000-700**

⊗ Fetal Bovine Serum, qualified, Canada **Thermo Fisher Catalog #12483020**

⊗ Falcon™ Cell Strainers **Fisher Scientific Catalog #08-771-2**

RPMI complete (RPMIc):

	A	B
	RPMI	500 mL
	FBS 10%	50 mL (inactivated)
	L-Glutamine	5 mL
	Sodium pyruvate	5 mL
	Antibiotic (Pen-Strep)	5 mL
	Non-essential amino acids	5 mL
	HEPES	5 mL
	2-Mercaptoethanol	50 mmol/L (final)

Note

Note! Very important, 2-Mercaptoethanol is an essential growth factor for mouse T-lymphocytes.



Purification and activation

4d 0h 30m

- 1 Dilute CD3 antibody (145-2C11) (clone KT3 can also be used) to  1 μL in PBS.
- 2 Coat plates with anti-CD3 antibody.
- 3 Use 24 well (Sarstedt, cat# 83.1836.500) or 96 well (Sarstedt, cat# 82.1581.001) plates. If these plates are not used, there is a risk of partial stimulation due to low absorbance of the antibody on the plate.
- 3.1 Add  100 μL of antibody/well if 96 wells or  1 mL per well if 24 wells. 
- 3.2 Incubate the plate for  24:00:00 at  4 °C or  01:00:00 at  37 °C . 
1d
- 4 Remove the antibody (aspirate) and wash 2 times with PBS. 
- 5 Add  100 μL of RPMIc if 96 wells or  1 mL if 24 wells and incubate the plate at  37 °C for  00:15:00 . See complete recipe for RPMIc in Materials section.  
15m
- 6 Collect spleen from mice 6-8 weeks in complete RPMI media (RPMIc), sex matched with recipient mice.

Note: using spleens from 6-8 week old mice ensures that the isolated T cells are from young adult mice with a fully developed immune system, and the sex-matching helps prevent potential complications in downstream applications or experiments.
- 7 Purify CD8+ T cells using EasySep mouse naïve purification kit (STEMCELL, catalo # 19858) as follow:
 - 7.1 Use a frosted microscope slide to homogenize spleens in PBS or Hanks' Balanced Salt Solution (HBSS) containing 2% fetal bovine serum (FBS).
 - 7.2 Remove aggregates and debris by passing cell suspension through a 70 μm mesh nylon strainer. Collect cells in a 15 mL tube.



- 7.3 Centrifuge at  1300 rpm, 00:05:00 and discard the supernatant. 
- 7.4 Red blood cells lysis is done by adding  5 mL /spleen of 0.83% ammonium chloride and incubate for  00:05:00 (or  00:02:00 for ACK lysing buffer) at  Room temperature while continuously shaking tubes. 

- 7.5 Quench by filling up tube with RPMIc.
- 7.6 Centrifuge at  1300 rpm, 00:05:00 and discard supernatant. 

- 7.7 Resuspend in EasySep buffer at 1×10^8 cells/ml (Easysep mouse naïve CD8+ T cell isolation kit (#19858A) and follow the protocol provided by STEMCELL.
- 8 Wash cells with RPMIc medium. 
- 9 Resuspend cells in  5 mL of RPMIc, count cells and adjust the concentration to 2×10^6 cells/mL
- 10 Add  100 μ L of cells (2×10^5 cells) per well if 96 wells or  1 mL of cells (2×10^6 cells) per well if 24 wells). 
- 11 Add purified anti-CD28 antibodies to reach a concentration of  5 μ L . 
- 12 Incubate the plate at  37 °C and 5% CO₂ for  24:00:00 . 

- 13 Add 20 Unit/ml of IL-2. 



14 Incubate the plate at  37 °C and 5% CO₂ for another  48:00:00 .

2d



15

Note

*** **Don't forget the non-stimulated controls.**

- Resuspend the cells well throughout the experiment (the cells quickly settle to the bottom of the tube)
- Check purity of CD8+ T cells after STEMCELL isolation by staining with anti-CD8 antibody followed by flow cytometry.
- Check activation after stimulation using anti-CD8 and anti-CD44 staining followed by flow cytometry.