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

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SOP for Immune cells isolation mouse adult brain with CD45 beads

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ABSTRACT

SOP for Immune cells isolation mouse adult brain with CD45 beads

MATERIALS

Material:

Miltenyi GMHO machine (ref 130-096-427).
Adult brain dissociation kit mouse and rat (ref 130-107-677; 50 digestions).
GentleMACS C tubes (ref 130-093-271 for 25 tubes or 130-096-334 for 100 tubes).
D-PBS with calcium, magnesium, glucose, and pyruvate. (ref 14-287-080)
PB buffer (always prepare fresh): Prepare a solution containing D-PBS, pH 7.2, and 0.5% bovine serum albumin (BSA) by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with D-PBS (+/+).
70 um filters.
Small petri dishes and scalpels to cut mouse brains.
Double-distilled water (ddH_2O) or MilliQ.
MACS MultiStand and magnetic field.
LS columns (ref 130-042-401 for 25 columns).

Columns' size depends on the amount of cells (MS $2x10^8$ total cells and 10^7 expected

CD11b+ cells; LS $2x10^9$ total

cells and 10^8 expected CD11b+ cells)
Miltenyi mouse CD45 beads (#ref 130-052-301).
Beads Buffer (always prepare fresh): MACS BSA Stock Solution (#130-091-376) 1:20 with AutoMacs Rinsing Solution (#130-091-222).
Ice Buckets

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Before:

1 EVERYTHING DONE ON ICE!!!

2 Label all the tubes and make sure all the material is available.

3 Make sure all the buffers are prepared and cold!! Can be done the day before, or day of.

4 Set up centrifuge at 4°C (use centrifuge on the right; close to the external entrance of the lab).

5 Prepare the hood and check if there is enough material in the tissue culture room.

6 Prepare enzyme mixes and store in the fridge:

*Tip: buffer Z can be added first in C-tubes-then start cutting brains-add them in C-tube-add enzyme P.
Enzyme mix 1: 50 ul of enzyme P and 1900 ul of buffer Z per sample/C tube.*
Enzyme mix 2: 10 ul of enzyme A and 20 ul of buffer Y per sample/C tube.

A. Brain dissociation

7 Remove mice brains and put them in pre-prepared 15mL falcon tubes filled with 3mL cold D-PBS.

8 Add 1900 ul of buffer Z in each C-tube.

9 Cut brain in ~8 equal pieces and add them in C-tubes (do not cut too much or viability will be affected).

10 Add 50 ul of enzyme P in each C-tube.

11 Add 30 ul of enzyme mix 2 in each C-tube.

12 Close lids tightly until lid clicks into place

13 Place C-tubes in GMHO machine-make sure all the brain pieces are at the bottom of the tubes; otherwise, they will not be properly dissociated.

14 Start program: 37_ABDK_01 for 1 brain >100 mg; 37_ABDK_02 for smaller pieces 20-100 mg

Tip: if any piece is stuck in the upper part, stop the program, mix brain pieces and start the program again.

15 Centrifuge briefly or mix up and down manually to ensure all the sample is at the bottom of the tube.

16 Prepare 50 ml tube with 70 um filters. Tip: add 2ml of PBS to the filter before.

17 Add the cell suspension into the C-tube.

18 Add 10 ml of cold D-PBS to the C-tubes, close the C-tubes, shake gently, and apply the cell suspension into a 50 ml falcon with a 70 um filter.

19 Add an extra 10 ml of D-PBS in the C-tube to make sure everything is taken and transfer again to the 50 ml falcon tube with the 70um filter. Optional: Use an extra 10 ml of D-PBS to dissociate small pieces that remain in the filter as much as possible.

20 Centrifuge 300xg for 10 min at 4°C. B. Debris removal

B. Debris removal

21 Remove supernatant, resuspend the cell pellet with 3100 ul of D-PBS and add 900 ul of debris removal solution.

22 Transfer cell solution to 15 ml tubes. If there are a lot of samples to one at a time. Avoid having the samples with the debris removal solution sitting on ice for long periods of time.

23 Overlay with 4 ml of D-PBS (debris removal solution is critical to have the overlay). The overlay volumes vary when using 1 or 2 brains:

	Debris Removal Solution	D-PBS	Overlay (D-PBS)	Reagent tube
20–100 mg	450 µL	1550 µL	2 mL	5 mL
400–500 mg (~ 1 brain)	900 µL	3100 µL	4 mL	15 mL
800–1000 mg (~ 2 brains)	1800 µL	6200 µL	4 mL	15 mL

24 Centrifuge 3000xg for 10 min at 4°C with full acceleration and brake of 7. Tip1: use the centrifuge on the right, close to the external entrance of the lab. Place the tubes on the inside of the centrifuge, the positions close to the rotor. Tip2: if after the centrifugation the debris layer is in the bottom of the tube with the cells, remove the upper layers, add again 900 ul of debris removal solution, overlay with respective D-PBS, and centrifuge again. Optional: If centrifuges give suboptimal centrifugation, the acceleration and brake can be reduced.

25 Three phases should be formed; aspirate the two top phases and discard them.

26 Add D-PBS up to 15 ml and gently invert the tube three times- do not vortex!

27 Centrifuge 1000xg for 10 min at 4°C with full acceleration and full brake. C. Red blood cell removal

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28 Prepare red blood cell removal (RBCR; prepare fresh every time). RBCR: 1:10 RBCR solution (10x) with ddH2O (i.e., 1 ml of RBCR with 9ml of cold ddH2O).

29 Resuspend the cell pellet with the appropriate RBCR volume:

30 ml for 100-1000 mg (one mouse brain).

31 5 ml for 20-100 mg.

32 Incubate the cells for 10 min at 2°C -8°C (fridge at 4°C).

33 Add PB buffer:

34 ml for 100-1000 mg (one mouse brain)

35 ml for 20-100 mg

36 Centrifuge 300xg for 10 min at 4°C. If need to count cells: resuspend in 10 ml of Beads Buffer◊ take an aliquote to count cells◊ centrifuge 300g 10 min at 4°C. D. CD45 beads isolation

D. CD45 beads isolation

37 Prepare Eppendorf tubes to incubate cells with CD45 microbeads.

38 Resuspend the cell pellet in 90 ul of Beads Buffer and 10 ul of CD45 microbeads for 107 cells Tip: cells should be counted to resuspend the cell pellet with appropriate vol of beads buffer and beads; however, we never expect more than 107 total cells per brain, thus directly resuspend the pellet with min vol to speed up the protocol.

39 Incubate the cells in the fridge (4°C) for 15 min. Tip: mix up and down after 7 min.

40 Add 2 ml of Beads Buffer (for 107 total cells) to each sample and centrifuge 300xg for 10 min. Tip: no temperature recommended, but I would use at 4°C.

41 Prepare the MACS MultiStand and magnetic field under the hood.

42 Place the columns (make sure they are correctly placed) and 15 ml tubes for trash.

43 Pre-wash columns with 500 ul Beads Buffer (MS columns).

44 Resuspend cell pellet with 500 ul of Beads Buffer (up to 108 cells). Tip: make sure the columns don't dry from now on.

45 Transfer the cell suspension to the columns (CD45+ cells are attached in the magnetic part of the column).

46 Wash the columns x3 times with 500 ul of Beads Buffer, add buffer only when column is empty each time.

47 Place columns in new 15 ml tubes.

48 Add 1 ml (MS) or 5 ml (LS) of Beads Buffer to each column and flush out the columns by pushing the plunger into the column.

49 Take an aliquot to count cells.

50 Centrifuge 300xg for 10 min at 4°C.

51 Resuspend according to the follow-up experiment.