Miltenyi Biotec

Isolation and cultivation of endothelial cells from adult mouse brain

Contents

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

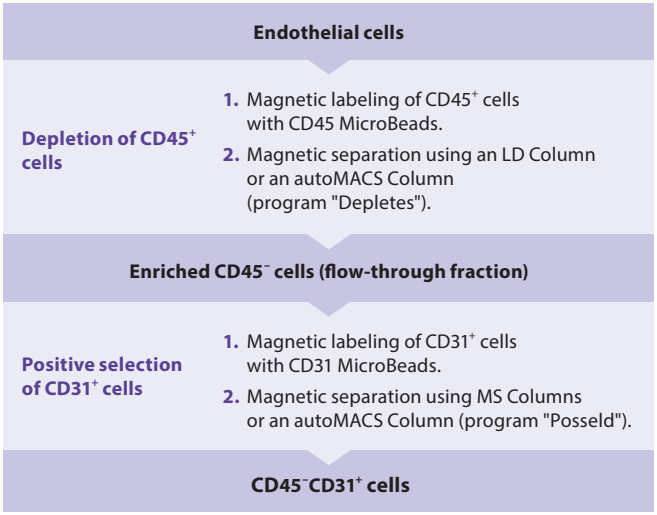
1. Description

1.1 Background information

Endothelial cells are one of the main components that form the blood brain barrier (BBB) in the central nervous system (CNS). Endothelial cells form the linings of the brain capillaries, and separate the blood from the brain parenchyma. The endothelial monolayer between blood and brain functions not only as a crucial interface for nutrients exchange, but also as a barrier for neurotoxic components from plasma. Additionally, the role of endothelial cells in tumor growth is of increasing interest.

This protocol has been developed to generate highly purified and viable endothelial cells from adult mouse brain tissue. Brain tissue from mice older than P7 is dissociated into single-cell suspensions using the Adult Brain Dissociation Kit. The extracellular matrix is enzymatically digested using the kit components, while the gentleMACS™ Octo Dissociator with Heaters is used for the mechanical dissociation steps during the on-instrument enzyme incubation. After the dissociation, the myelin and cell debris is removed using the Debris Removal Solution and is followed by an subsequent removal of erythrocytes using the Red Blood Cell Removal Solution. Endothelial cells are enriched by depletion of CD45$^+$ cells with CD45 MicroBeads followed by a positive selection using CD31 MicroBeads.

Overview



1.2 Reagent and instrument requirements

- Dulbecco's phopshate-buffered saline (D-PBS) with calcium, magnesium, glucose, and pyruvate. Keep buffer cold (2–8 °C).
- D-PBS/BSA buffer: Prepare a solution containing D-PBS and 0.5% bovine serum albumin (BSA) by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with D-PBS. Keep buffer cold (2–8 °C).
  Always use freshly prepared buffer. Do **not use** autoMACS Running Buffer or MACSQuant® Running Buffer as they contain a small amount of sodium azide that could affect the results.

  ▲ **Note:** BSA can be replaced by other proteins such as mouse serum albumin, mouse serum, or fetal bovine serum (FBS).
- MACS Columns and MACS Separators: Endothelial cells can be enriched by depletion using LD Columns followed by subsequent positive selection using MS Columns. Enrichment can also be performed by using the autoMACS® Pro Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
MS	10^7	2×10^7	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
LD	2×10^7	4×10^7	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
autoMACS	5×10^7	10^8	autoMACS Pro

▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ II Separators. For details refer to the respective MACS Separator data sheet.

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For preparation of brain dissociation

- Adult Brain Dissociation Kit, mouse and rat (# 130-107-677)
- gentleMACS™ Octo Dissociator with Heaters (# 130-096-427)
- gentleMACS C Tubes (# 130-093-237, # 130-096-334)
- 35 mm diameter sterile petri dish
- Sterile scalpel
- Sterile forceps
- (Optional) ART® 1000 REACH™ pipet tips (Molecular BioProducts, Inc.) for removal of dissociated material from the closed C Tubes.
- MACS SmartStrainers (70 µm) (# 130-098-462)
- 15 mL and 50 mL tubes
- Centrifuge with swinging bucket rotor

For cell isolation and flow cytometric analysis

- CD45 MicroBeads, mouse (# 130-052-301)
- CD31 MicroBeads, mouse (# 130-097-418)
- (Optional) Pre-Separation Filters (70 µm) (# 130-095-823)
- Fluorochrome-conjugated Labeling Check-Reagents to stain labeled cells for flow cytometric analysis, e.g., Labeling Check-Reagent-APC. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.

  ▲ **Note:** The use of CD31 antibodies (clone 390), is not recommended for analysis of cells that are labeled with CD31 MicroBeads.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells.
- (Optional) MACSQuant® Analyzer 10 (# 130-096-343)

For cell culture

- Double-distilled water (ddH_2O)
- Imaging Plate CG 1.5 (96 well) (# 130-098-265)
- Fibronectin, e.g. Human Fibronectin (Fragment) (# 130-109-393) for coating of cell culture dishes
- EBM-2 basal medium and all supplements (Lonza, EGM™-2-MV BulletKit™, CC-3202)

2. Protocol

2.1 Preparation of brain dissociation

▲ For subsequent cell separation and cultivation it is recommended to dissociate at least 800 mg of adult mouse brain tissue.

▲ Volumes given below are for one adult mouse brain (max. 500 mg) in 1980 µL enzyme mix. When working with less than 500 mg, use the same volumes as indicated. When working with higher tissue quantitities scale up all reagent volumes and total volumes accordingly. A maximum of 500 mg brain tissue per C Tube can be processed.

▲ A swinging bucket rotor is recommended for centrifugation, e. g., Heraeus® Multifuge 4KR by Thermo Fisher® Scientific.

- Enzyme P is ready to use. Prepare aliquots of appropriate volume to avoid repeated freeze-thaw-cycles. Store aliquots at –20 °C. This solution is stable for 6 months. Resuspend the lyophilized powder in the vial labeled Enzyme A with 1 mL Buffer A. Do not vortex. This solution should then be aliquoted and stored at –20 °C for later use. Avoid repeated freeze-thaw-cycles.
- Prepare enzyme mix 1 and enzyme mix 2 according to the table below.

Enzyme mix 1		Enzyme mix 2	
Enzyme P	Buffer Z	Buffer Y	Enzyme A
50 µL	1900 µL	20 µL	10 µL

Preparation of 1× Red Blood Cell Removal Solution

- Dilute the Red Blood Cell Removal Solution (10×) 1:10 with double-distilled water (ddH_2O), for example, dilute 0.1 mL of cold Red Blood Cell Removal Solution (10×) with 0.9 mL cold ddH_2O.

  ▲ **Note:** Do not use deionized water for dilution!
- Store the prepared 1× Red Blood Cell Removal Solution at 2–8 °C. Discard unused solution at the end of the day.

Preparation of cell culture dish

- The coating concentration should be 3 µg of Human Fibronectin (Fragment) per cm^2.
  For example, when coating a 96-well plate (0.32 cm^2 per well), use 48 µL of a 20 µg/mL solution per well. Dispense the appropriate volume of Human Fibronectin (Fragment) solution into each plate. Incubate overnight at 37 °C and wash three times with ddH_2O afterwards. Let the culture dish dry under sterile conditions.

2.1.1 Dissociation protocol

▲ For details on the use of the gentleMACS™ Octo Dissociator with Heaters, refer to the user manual.

▲ A maximum of one mouse brain (max. 500 mg) in 2 mL enzyme mix can be processed in one C Tube.

▲ For dissociation of small amount of neural tissue (<100 mg), refer to data sheet of Adult Brain Dissociation Kit, mouse and rat.

▲ For cell culture experiments subsequent to tissue dissociation, all steps should be performed under sterile conditions.

1. Remove the mouse brain. Wash the brain in cold D-PBS.
2. Prepare the appropriate volume of enzyme mix 1 (refer to table in chapter 2.1) and transfer it into a gentleMACS C Tube.
3. Place the brain on a petri dish and cut it into 8 sagittal slices using a scalpel.
4. Transfer the tissue pieces into the C Tube containing 1950 µL of enzyme mix 1.
5. Transfer 30 µL of enzyme mix 2 into the C Tube.
6. Tightly close C Tube and attach it upside down onto the sleeve of the gentleMACS Octo Dissociator with Heaters.
7. Run the gentleMACS Program **37C_ABDK_01**.
8. After termination of the program, detach C Tube from the gentleMACS Octo Dissociator with Heaters.
9. (Optional) Centrifuge briefly to collect the sample at the bottom of the tube.
10. Resuspend sample and apply it to a MACS SmartStrainer (70 µm) placed on a 50 mL tube.

▲ **Note:** Moisten MACS SmartStrainer with buffer before use.

▲ **Note:** When upscaling the reagent volume and total volumes, increase also the number of MACS SmartStrainers (70 µm). One MACS SmartStrainer (70 µm) can be used for one adult mouse brain.

▲ **Note:** Dissociated tissue can be removed from the closed C Tube by pipetting through the septum-sealed opening in the center of the cap of the C Tube. Use ART 1000 REACH 1000 µL pipette tips.

▲ **Note:** Cells with a diameter >70 µm may be lost. To obtain these cells within the flow through, use a cell strainer with an appropriate mesh size.

11. Apply 10 mL of cold D-PBS onto the MACS SmartStrainer (70 µm).
12. Discard MACS SmartStrainer (70 µm) and centrifuge cell suspension at 300×g for 10 minutes at 4 °C. Aspirate supernatant completely.
13. Proceed to 2.1.2 for debris and red blood cell removal.

2.1.2 Debris and red blood cell removal

▲ Volumes given below are for the cell suspension from up to two adult mouse brains as starting material (max. 1 g). When working with higher tissue quantities, scale up all reagent volumes accordingly.

▲ A maximum of cell suspension from two adult mouse brains (max. 1 g) can be processed in one 15 mL reagent tube.

▲ Always use pre-cooled buffers and solutions (4 °C).

	Debris Removal Solution	D-PBS	Overlay (D-PBS)
1 brain (400–500 mg)	900 µL	3100 µL	4 mL
2 brains (800–1000 mg)	1800 µL	6200 µL	4 mL

1. Resuspend cell pellet carefully with the appropriate volume of cold D-PBS according to the table above and transfer cell suspension to a 15 mL tube. Do not vortex.
2. Add appropriate volume of cold Debris Removal Solution.
3. Mix well.
4. Overlay very gently with 4 mL of cold D-PBS.
   ▲ **Note:** Pipette very slowly to ensure that the D-PBS phase overlays the cell suspension and phases are not mixed.
5. Centrifuge at 4 °C and 3000×g for 10 minutes with full acceleration and full brake.
   ▲ **Note:** If centrifuges give suboptimal centrifugation, the acceleration and brake can be reduced.
6. Three phases are formed. Aspirate the two top phases completely and discard them.
7. Fill up with cold D-PBS to a final volume of 15 mL.
8. Gently invert the tube three times. Do not vortex!
9. Centrifuge at 4 °C and 1000×g for 10 minutes with full acceleration and full brake. Aspirate supernatant completely.
10. Resuspend cell pellet from up to two adult mouse brains carefully in 1 mL of cold 1× Red Blood Cell Removal Solution. Do not vortex.
11. Incubate for 10 minutes in the refrigerator (2–8 °C).
12. Add 10 mL of cold D-PBS/BSA buffer.
13. Centrifuge at 4 °C and 300×g for 10 minutes. Aspirate supernatant completely.
14. Proceed to 2.2 for magnetic labeling.

2.2 Magnetic labeling of CD45$^+$ cells

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 70 µm nylon mesh (MACS SmartStrainer (70 µm), # 130-098-462) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

1. Resuspend cell pellet in 90 µL of cold D-PBS/BSA buffer per 10^7 total cells.
2. Add 10 µL of CD45 MicroBeads, mouse.
3. Mix well and incubate for 15 minutes in the dark in the refrigerator (2–8 °C).
4. Wash cells by adding 1 mL of cold D-PBS/BSA buffer per 10^7 cells and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
5. Resuspend up to 10^7 cells in 500 µL of D-PBS/BSA buffer.
   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
6. (Optional) Take 20 µL for later flow cytometric analysis (original fraction 1).
7. Proceed to magnetic separation (2.3).

2.3 Magnetic separation: Depletion of CD45$^+$ cells

▲ Choose an LD Column and an appropriate MACS Separator. For details refer to the table in section 1.2.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

Magnetic separation with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
2. (Optional) Place Pre-Separation Filter (70 µm) on top of the column to remove clumps which may clog the column.
   ▲ **Note:** Moisten Pre-Separation Filter with buffer before use.

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3. Prepare column by rinsing with 2 mL of D-PBS/BSA buffer.
4. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
5. Wash column with 2×1 mL of D-PBS/BSA buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 4. This is the target cell fraction ($CD45^-$ cells).

   ▲ **Note:** Perform washing steps by adding buffer aliquots only when the column reservoir is empty.
6. (Optional, if $CD45^+$ cells are needed) Remove column from the separator and place it on a suitable collection tube. Pipette 3 mL of D-PBS/BSA buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

   ▲ **Note:** (Optional) Take 100 µL of positive and negative fraction for later flow cytometric analysis (pos 1 and neg 1).
7. Centrifuge at 4 °C and 300×g for 10 minutes. Aspirate supernatant completely.
8. Proceed to magnetic labeling (2.4).

Magnetic separation with the autoMACS® Pro Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Use D-PBS/BSA buffer. Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:

   Depletion: Depletes

   Collect negative fraction in row B of the tube rack. This fraction represents the target cells ($CD45^-$ cells).

   ▲ **Note:** (Optional) Take 100 µL of positive and negative fraction for later flow cytometric analysis (pos 1 and neg 1).
4. Centrifuge at 4 °C and 300×g for 10 minutes. Aspirate supernatant completely.
5. (Optional, if $CD45^+$ cells are needed) Collect positive fraction in row C of the tube rack.
6. Proceed to magnetic labeling (2.4).

2.4 Magnetic labeling of $CD31^+$ cells

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for $2×10^7$ total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 70 µm nylon mesh (MACS SmartStrainer (70 µm), # 130-098-462) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

1. Resuspend cell pellet in 90 µL of cold D-PBS/BSA buffer per 10^7 total cells.
2. Add 10 µL of CD31 MicroBeads, mouse.
3. Mix well and incubate for 15 minutes in the dark in the refrigerator (2–8 °C).
4. Wash cells by adding 1 mL of cold D-PBS/BSA buffer per 10^7 cells and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
5. Resuspend up to 10^7 cells in 500 µL of D-PBS/BSA buffer.

   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
6. (Optional) Take 50 µL for later flow cytometric analysis (original fraction 2).
7. Proceed to magnetic separation (2.5).

2.5 Magnetic separation: Positive selection of $CD31^+$ cells

▲ Choose an MS Column and MACS Separator according to the number of total cells and the number of $CD31^+$ cells. For details refer to the table in section 1.2.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

Magnetic separation with MS Columns

1. Place MS Column in the magnetic field of a suitable MACS Separator. For details refer to the respective MS Column data sheet.
2. (Optional) Place Pre-Separation Filter (70 µm) on top of the column to remove clumps which may clog the column.

   ▲ **Note:** Moisten Pre-Separation Filter with buffer before use.
3. Prepare column by rinsing with 500 µL of D-PBS/BSA buffer.
4. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
5. Wash column with 3×500 µL of D-PBS/BSA buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 4.

   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
6. Remove column from the separator and place it on a suitable collection tube.
7. Pipette 1 mL of D-PBS/BSA buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. This is the $CD45^-CD31^+$ cell fraction.

8. To increase the purity of $CD31^+$ cells, it is recommended to enrich the positive fraction over a second MS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

   ▲ **Note:** (Optional) Take 100 µL of positive and negative fraction for later flow cytometric analysis (pos 2 and neg 2).

9. Centrifuge at 4 °C and 300×g for 10 minutes. Aspirate supernatant completely.

10. Proceed to flow cytometric analysis (2.6).

Magnetic separation with the autoMACS® Pro Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Use D-PBS/BSA buffer. Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

1. Prepare and prime the instrument.

2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.

3. For a standard separation choose the following program:

   Positive selection: Posseld

   Collect positive fraction in row C of the tube rack. This fraction represents the target cells ($CD31^+$ cells).

   ▲ **Note:** (Optional) Take 100 µL of positive and negative fraction for later flow cytometric analysis (pos 2 and neg 2).

4. Centrifuge at 4 °C and 300×g for 10 minutes. Aspirate supernatant completely.

5. (Optional, if $CD31^-$ cells are needed) Collect negative fraction in row B of the tube rack.

6. Proceed to flow cytometric analysis (2.6).

2.6 Flow cytometric analysis

▲ Fluorochrome-conjugated Labeling Check-Reagents are recommended to stain the isolated endothelial cells for flow cytometric analysis. The CD31 antibodies (clone 390) are not recommended for analysis of cells that are labeled with CD31 MicroBeads.

▲ The recommended antibody dilution for labeling of cells is 1:10 for up to 10^6 cells/50 µL of D-PBS/BSA buffer.

▲ Volumes given below are for up to 10^6 nucleated cells. When working with fewer than 10^6 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^6 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. (Optional) For analysis take positive and negative fractions (pos 1, neg 1, pos 2, neg 2). Include the original fractions 1 and 2. Centrifuge at 4 °C and 300×g for 10 minutes. Aspirate supernatant completely.

2. Resuspend up to 10^6 nucleated cells per 45 µL of D-PBS/BSA buffer.

3. Add 5 µL of Labeling Check-Reagent-APC.

4. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).

   ▲ **Note:** Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.

5. Wash cells by adding 1 mL of D-PBS/BSA buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

6. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry, e.g. using the MACSQuant® Analyzer 10.

2.7 Cell culture

1. Take 96-well plate which has been coated overnight (refer to 2.1 "Preparation of cell culture dish") and plate 10^5 cells in EBM-2 basal medium and all supplements.

2. After 24 hours in culture round compact cells are visible. Fix and stain cells for microscopy analysis at day 2.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols.