Miltenyi Biotec

Isolation and cultivation of astrocytes 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

This product is for research use only.

1.1 Background information

Astrocytes represent the most abundant type of glial cells in the central nervous system. They display a remarkable heterogeneity in their morphology and function. In the past years previously unrecognized functions of astrocytes have been revealed, including control of synapse formation, modulation of synaptic function, and neurogenesis.

This protocol has been developed to generate highly purified and viable astrocytes 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™ 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 a subsequent removal of erythrocytes using the Red Blood Cell Removal Solution (10×). The Anti-ACSA-2 MicroBead Kit is used to isolate astrocytes from the single-cell suspension.

During cell separation dead cells are removed using the AstroMACS Separation Buffer. Isolated cells can be cultivated in AstroMACS Medium, which is an optimized serum-free cell culture medium designed for the optimal growth and cultivation of isolated astrocytes.

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).
- AstroMACS Separation Buffer (# 130-117-336). Keep buffer cold (2–8 °C).
- MACS Columns and MACS Separators: Anti-ACSA-2$^+$ cells can be enriched by using MS Columns. Positive selection can also be performed by using the autoMACS Pro.

Column	Max. number of labeled cells	Max. number of total cells	Separator
MS	10^7	2×10^7	MiniMACS, OctoMACS, 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.

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

- (Optional) Pre-Separation Filters (70 µm) (# 130-095-823)
- Anti-ACSA-2 MicroBead Kit, mouse (# 130-097-678)
- Anti-ACSA-2 antibodies, mouse (clone IH3-18A3) conjugated to PE or APC. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.

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- (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 and immunocytochemical staining

- Double-distilled water (ddH_2O)
- Imaging Plate CG 1.5 (24 well) (# 130-098-263)
- AstroMACS Medium (# 130-117-031) containing:
  MACS Neuro Medium
  MACS NeuroBrew®-21
  AstroMACS Supplement
- 200 mM L-Glutamine
- Poly-L-Lysine (0.01%) and laminin
- Penicillin/streptomycin
- 2% paraformaldehyde (PFA) for the fixation
- FcR Blocking Reagent
- Anti-GLAST (ACSA-1) pure, human, mouse, rat (# 130-095-822) and anti-rat-IgG2b secondary antibody or Anti-ACSA-2 pure, mouse (# 130-099-138) and anti-mouse-IgG2a secondary antibody
- Staining buffer: Prepare a solution containing autoMACS® Running Buffer (# 130-091-221) with FcR Blocking Reagent, mouse (# 130-092-575) in a ratio 1:10, e.g., add 1 mL FcR Blocking Reagent to 9 mL autoMACS Running Buffer.
- Phosphate-buffered saline (PBS)
- autoMACS Running Buffer (# 130-091-221)

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 50 µL	Buffer Z 1900 µL	Buffer Y 20 µL	Enzyme A 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 AstroMACS Medium and cell culture dish

1. Start preparation of AstroMACS Medium by thawing MACS NeuroBrew-21 at 2-8 °C prior to use.
2. Reconstitute lyophilized Astro MACS Supplement with 1 mL MACS Neuro Medium.
   ▲ **Note:** Single-use aliquots of the reconstituted AstroMACS Supplement can be stored at –20 °C. It is recommended to prepare aliquots of 100 µL.
3. To obtain the complete medium add 0.2% of the reconstituted AstroMACS Supplement, 2% of the MACS NeuroBrew-21, 50×, and 0.25% L-Glutamine (0.5 mM) to the MACS Neuro Medium, e.g., 100 µL AstroMACS Supplement, 1 mL MACS NeuroBrew-21, and 125 µL L-Glutamine to 50 mL MACS Neuro Medium.
4. Use the complete medium within 2 weeks when stored at 2–8 °C. Do not freeze.

- Coat the culture dish (24-well plate) with 0.01% Poly-L-Lysine overnight at 37 °C. After three times washing with ddH_2O coat the plate overnight at 37 °C with 10 µg/mL laminin. Wash again three times with ddH_2O. 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.

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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 (max. 1 g) as starting material. 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

▲ 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 80 µL of AstroMACS Separation Buffer.
2. Add 10 µL of FcR Blocking Reagent.
3. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
4. Add 10 µL of Anti-ACSA-2 MicroBeads.
5. Mix well and incubate for 15 minutes in the dark in the refrigerator (2–8 °C).
6. Wash cells by adding 1 mL of AstroMACS Separation Buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
7. Resuspend up to 10^7 cells in 500 µL of AstroMACS Separation Buffer.
   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
8. (Optional) Take 20 µL for later flow cytometric analysis (original fraction).
9. Proceed to magnetic separation (2.3).

2.3 Magnetic separation

▲ Choose an MS Column and an appropriate MACS Separator. For details refer to the table in section 1.4.

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

▲ Degas buffer before use, as air bubbles could block the column.

Magnetic separation with MS Columns

1. Place 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 AstroMACS Separation Buffer before use.
3. Prepare column by rinsing with 500 µL of AstroMACS Separation Buffer.
4. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
5. Wash column with 3×500 µL of AstroMACS Separation Buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 3. This is the ACSA-2$^-$ cell fraction.
   ▲ **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 AstroMACS Separation Buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. This is the ACSA-2$^+$ cell fraction.
8. To increase the purity of ACSA-2$^+$ 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.
9. Take aliquots of positive and negative fraction and proceed to flow cytometric analysis (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 AstroMACS Separation 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: Possel
   Collect positive fraction in row C of the tube rack. This fraction represents the ACSA-2$^+$ cells.
4. (Optional) Collect negative fraction in row B of the tube rack. This fraction represents the ACSA-2$^-$ cells.
5. Take aliquots of positive and negative fraction and proceed to flow cytometric analysis (2.4).

2.4 Flow cytometric analysis

▲ The recommended antibody dilution for labeling of cells is 1:10 for up to 10^6cells/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 100 µL of positive and negative fraction. Include the 20 µL of the original fraction.
2. Resuspend up to 10^6 nucleated cells per 45 µL of D-PBS/BSA buffer.
3. Add 5 µL of Anti-ACSA-2-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 nonspecific 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 D-PBS/BSA buffer for analysis by flow cytometry, e.g. using the MACSQuant® Analyzer 10.

2.5 Cell culture

1. Plate 10^5 cells in 50 µL of pre-warmed prepared AstroMACS Medium as a drop in the middle of each well of a 24-well plate which has been coated overnight (refer to 2.1 "Preparation of cell culture dish").
2. Let the cells settle down for 30 minutes at 37 °C in the incubator.
3. Carefully add 450 µL of prepared AstroMACS Medium to each well.
4. Maintain the culture by replacement of 50% of prepared medium every other day.
   ▲ **Note:** Cells loosely attach after the first days of cultivation. Therefore, a cultivation period of approximately 1 week is recommended.

2.6 Immunocytochemical staining of cultured astrocytes

1. Wash cells 3× with PBS.
2. Fix cells with 2% PFA for 10 minutes at room temperature.
3. Wash cells 3× with PBS.
   ▲ **Note:** Fixed cells can be stored in azide-containing buffer at 2–8 °C for up to 1 week.
4. Add staining buffer and incubate for 10 minutes at room temperature.
5. Discard staining buffer.

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6. Add primary antibody in staining buffer to the cells with a final concentration of 1–5 µg/mL and incubate at room temperature in the dark for 10 minutes.
7. Wash cells 3× with autoMACS Running Buffer.
8. Add a corresponding secondary antibody in staining buffer to the cells and incubate at room temperature in the dark for 10 minutes.
9. Wash cells 3× with autoMACS Running Buffer.

   ▲ **Note:** For co-staining with additional antibodies repeat steps 6–9.
10. Store cells in autoMACS Running Buffer.
11. Cells are now ready for immunofluorescence microscopy.

    ▲ **Note:** Samples can be stored at 2–8 °C in the dark for up to one week.

    ▲ **Note:** When working with cells cultured on coverslips, the coverslips need to be mounted onto slides before imaging.

3. References

1. G. Kantzer, C. *et al.* (2017) Anti-ACSA-2 defines a novel monoclonal antibody for prospective isolation of living neonatal and adult astrocytes. Glia 65 (6): 990–1004.
2. Batiuk, M. Y. *et al.* (2017) An immunoaffinity-based method for isolating ultrapure adult astrocytes based on ATP1B2 targeting by the ACSA-2 antibody. J. Biol. Chem. 292 (21): 8874–8891.

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