APPLICATION PROTOCOL

Preparation of Adult Mouse Brain for LeviCell System

Overview

Successful enrichment of specific CNS cell types from adult mouse brains require gentle tissue dissociation with the LeviPrep™ Tissue Dissociation Kit, PN 1005001 (LeviPrep) followed by subsequent removal of red blood cells (RBCs) and myelin. The perfusion state of the brain tissue and the target cell type(s) all determine which protocol is best. Here, we break down how to process perfused versus non-perfused brain tissue to isolate and enrich microglial cells only, or all CNS cell types with the LeviCell™ system.

Materials Needed

- BSA
- FBS
- PBS
- P1000 pipette
- P1000 wide bore tips
- 50 mL conical tube
- 70 µm filter
- 0.2 µm sterile filter
- 3 mL syringe
- Ice
- Timer
- Razor blade or scalpel
- 2.0 mL low binding microcentrifuge tubes
- Cell culture dish for mincing the tissue
- Centrifuge with swinging bucket rotor
- Cell counter or cell counting method
- Analytical balance

Enzyme Preparation for Brain Dissociation

Resuspend lyophilized specific enzymes and mix them following the LeviPrep protocol.

Prepare Buffers

For each dissociation, you will need 15 mL of 1X PBS containing 10% FBS. This buffer is used to quench the enzymes and rinse the dissociated tissue through the filter.

You will also need 10 mL of 1X PBS containing 0.5% BSA for the final cell suspension and wash step.

LeviPrep Tissue Dissociation Protocol

The LeviPrep protocol has been evaluated for use with 50 - 250 mg adult brain tissue using 1.9 mL of enzyme volume. When working with larger tissue amounts, scale the total volume accordingly. Follow all steps in the LeviPrep Mouse Tissue Dissociation Kit (see Figure 1).

After the final wash step, proceed to protocols A, B or C based on the perfusion state of your brain sample, and your desired enrichment type (see Figure 2).

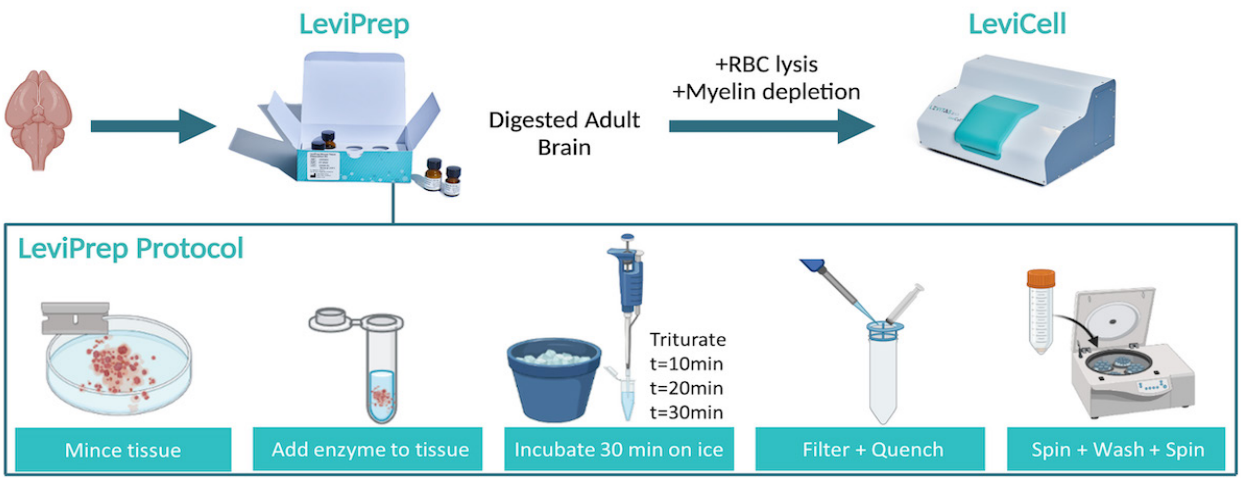


Figure 1. *Schematic representation of the LeviPrep™ system workflow.*

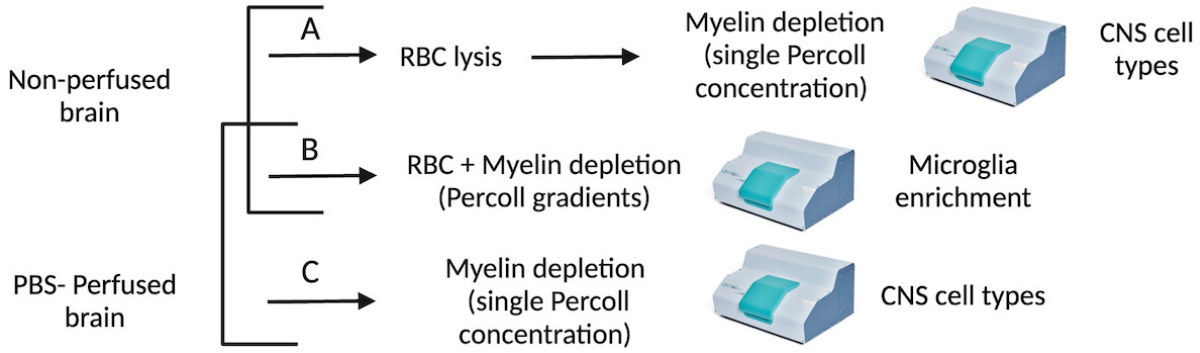


Figure 2. *Schematic representation of the workflow after LeviPrep digestion depending on the type of adult brain sample and the final desired enrichment of cells.* ***Note:*** *Protocol B can also be performed in Perfused Brain.*

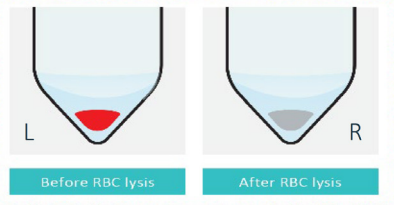
Protocol A - All CNS Cell Types from Non-Perfused Brains

Materials Needed

- 1X PBS and 10X PBS
- BSA
- Percoll
- Centrifuge with swinging bucket rotor
- Plastic Pasteur pipette
- 15 mL conical tubes
- RBC lysis buffer

When working with non-perfused adult mouse brains, removal of all RBCs and myelin before a LeviCell run is critical. In this protocol, RBC lysis is performed prior to myelin removal[1].

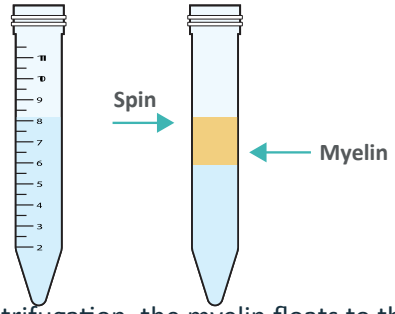
1. First, take the cell pellet previously prepared with the LeviPrep kit, and add an appropriate volume of RBC lysis buffer. After cell lysis, the plasma will change from red (left) to colorless (right), as indicated in the illustration below. LevitasBio has tested the RBC lysis buffer and protocol from Alfa Aesar, part of Thermo Fisher Scientific (Fisher Scientific PN AAJ62150AK).



2. Next, prepare a 50% Percoll solution for myelin removal.
   a. Mix 9 parts of Percoll with 1 part 10X PBS to make a 100% Stock Isotonic Percoll (SIP) solution.
   If you are working with a whole adult mouse brain, 5 mL 100% SIP is sufficient.
   b. Add 5 mL 1X PBS to 5 mL 100% SIP to dilute down to a 50% Percoll solution. Store at room temperature.
3. Place 4.5 mL of 50% Percoll solution into two 15 mL conical tubes and set aside.
4. Take your cell pellet in step 1 and add 6 mL of 1X PBS. If you are using less than a whole adult mouse brain, scale the volume down accordingly.
5. Take one of the 15 mL conical tubes from step 3, and gently transfer 3 mL of the resuspended cell pellet on top of the 50% Percoll. Repeat with the remaining 3 mL into the other prepared tube.

Critical: Be gentle when pipetting the sample on top of the Percoll. Prevent mixture of the two solutions by slowly dispensing the sample down the side of the 15mL conical tube with a P1000 pipette.

6. To isolate the myelin, centrifuge both 15 mL conical tubes at room temperature for 10 min, 700 x g with no brake.



7. After centrifugation, the myelin floats to the top and forms a band. Using a Pasteur pipette, carefully remove this myelin band along with any visible debris.
8. Add up to 14 mL 1X PBS to wash the residual Percoll.
9. Pellet the cells by centrifuging for 10 min at 700 x g/4˚C, with brake.

Amount	Resuspension Volume
¼ brain	300-500 µL
½ brain	500 µL- 1 mL

10. Resuspend the pellet in your preferred buffer (i.e 1X PBS + 0.5 % BSA or levitation buffer) and perform a cell count. A whole brain will typically yield 500,000 total cells.
11. Decide how many cells to load into the LeviCell system based on your cell counts.

For instructions on how to prepare your sample and run it on the LeviCell system, go to the LeviCell Viable Cell Enrichment section

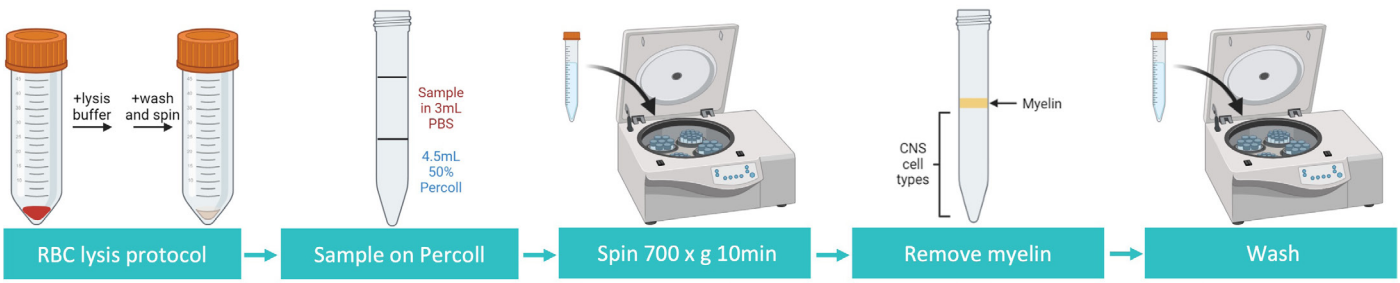


Figure 3. *Schematic representation of Protocol A. Entire protocol is 45 minutes long.*

Protocol B - Microglia Enrichment from Non-Perfused Brains

Materials Needed

- 1X PBS
- 10X PBS
- BSA
- Percoll
- Plastic Pasteur pipette
- 15 mL conical tubes
- Centrifuge with swinging bucket rotor

When working with adult mouse brains, removal of all RBCs and myelin before a LeviCell run is critical. In this protocol, RBCs and myelin are removed simultaneously while enriching for microglia cells. The Percoll gradient in this protocol[2] has been optimized for use on the LeviCell system.

1. First, prepare different Percoll gradients for myelin removal.
   a. Mix 9 parts Percoll with 1 part 10X PBS to make a 100% Stock Isotonic Percoll (SIP) solution.
   b. Prepare the following Percoll % gradients, and store at room temperature.

Percoll %	SIP Solution	1xPBS
70%	7 mL	2 mL
37%	3.7 mL	5.3 mL
30%	3 mL	6 mL

2. Resuspend the cell pellet in 4 mL of 37% SIP for each brain and transfer to a 15 mL conical tube.
3. Slowly underlay 4 mL of 70% SIP with a plastic Pasteur pipette as indicated in Figure 4. ***Caution: Do not use glass Pasteur pipettes. They can be easily broken.***
4. On top of the 37% layer, slowly pipette 4 mL of 30% SIP, followed by 2 mL of 1X PBS.
5. Centrifuge tube for 40 min at 300 × g / 18 °C with no brake.

   Critical: Make sure the centrifuge will stop with no brake so that the interphase is not disturbed.

6. After centrifugation, the myelin floats to the top and forms a band. Using a Pasteur pipette, carefully remove this myelin band along with any visible debris.
7. Collect the remaining liquid and split between 2 new 15 mL conical tubes.
8. Add up to 14 mL 1X PBS to wash the residual Percoll.
9. Centrifuge both tubes for 5 min at 300 × g/4 °C to pellet cells.
10. Resuspend the pellet in 500 µL buffer (i.e 1X PBS + 0.5 %BSA or levitation buffer) and perform a cell count. A whole brain will typically yield 200,000 - 300,000 total cells.
11. Decide how many cells to load into the LeviCell system based on your cell counts.

For instructions on how to prepare your sample and run it on the LeviCell system, go to the LeviCell Viable Cell Enrichment section.

Figure 4. *Schematic diagram of Protocol B for enrichment of microglia cells,which takes 60 minutes to complete.*

Protocol C - All CNS Cell Types from Perfused Brains

Materials Needed

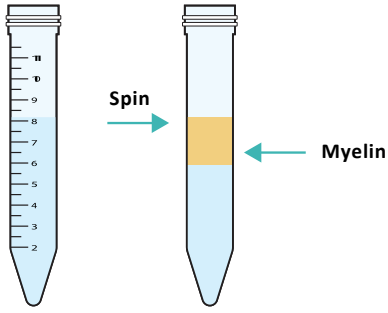
- 1X PBS
- 10X PBS
- BSA
- Percoll
- Plastic Pasteur pipette
- 15 mL conical tubes
- Centrifuge with swinging bucket rotor

When working with adult perfused mouse brains, removing all myelin before a LeviCell run is critical. Protocol C describes a quick and easy way to isolate and remove myelin from your sample[1]

1. Prepare a 50% Percoll solution for myelin removal.
    a. Mix 9 parts of Percoll with 1 part 10X PBS to make 100% Isotonic Stock Percoll (SIP) solution. If you are working with a whole adult mouse brain, 5 mL of 100% SIP is sufficient.
    b. Add 5 mL 1X PBS to 5 mL 100% SIP to dilute down to a 50% Percoll solution. Store at room temperature.
2. Place 4.5 mL 50% Percoll solution into two 15 mL conical tubes, and set aside.
3. Retrieve the cell pellet from the LeviPrep protocol and resuspend in 6 mL of 1X PBS. If you are using less than a whole adult mouse brain, scale the volume down accordingly.
4. Take one of the 15 mL conical tubes from step 2, and gently transfer 3 mL of the resuspended cell pellet on top of the 50% Percoll. Repeat with the remaining 3 mL into the other prepared tube.

Critical: Be gentle when adding the sample on top of the Percoll. Prevent mixture of the two solutions by slowly dispensing the sample down the side of the 15mL conical tube with a P1000 pipette.

5. To isolate the myelin, centrifuge both 15 mL conical tubes at room temperature for 10 min, 700 x g with no brake.



6. After centrifugation, the myelin floats to the top and forms a band. Using a Pasteur pipette, carefully remove the myelin band along with any visible debris.
7. Add up to 14 mL 1X PBS to wash the residual Percoll.
8. Pellet cells by centrifuging for 10 min at 700 x g/4˚C, with brake.
9. Resuspend the pellet in your preferred buffer (i.e 1X PBS + 0.5 % BSA or levitation buffer) and perform a cell count. A whole brain will typically yield 500,000 total cells.

Amount	Resuspension Volume
¼ brain	300-500 µL
½ whole brain	500 µL- 1 mL

10. Decide how many cells to load into the LeviCell system based on your cell count.

For instructions on how to prepare your sample and run it on the LeviCell system, go to the LeviCell Viable Cell Enrichment section.

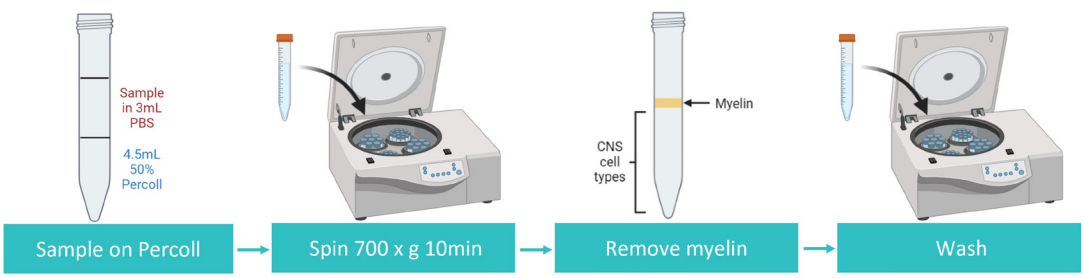


Figure 5. *Schematic diagram of Protocol C for enrichment of all CNS cell types, which takes 25 minutes to complete,*

Viable CNS Cell Enrichment with the LeviCell System

After resuspension, count your cells and decide how many cells to load into the LeviCell system.

1. Centrifuge cells for 5 min at 300 x g.
2. Resuspend the pellet in levitation buffer (PBS + 0.5% BSA + 150 mM Levitation Agent) prepared at room temperature.

Critical: Be gentle resuspending your cells.

NOTE: If clumping is seen, 125 U/mL of Enzyme C from the LeviPrep Tissue Dissociation Kit can be added to the sample. These samples will need to be washed prior to loading onto the LeviCell system if performing downstream sequencing.

3. Run CNS cell types using the standard LeviCell run protocol of 20 min.

For more information on viable cell enrichment and the use of the LeviCell system, please refer to the following documents:

- Quick Reference Guide for Viable Cell Enrichment #90-00213
- LeviCell Instrument User Guide #90-00204

Tips for Counting Brain Cells

If myelin is present during cell count, a 1:10 or 1:20 dilution is recommended.

Dual staining with Acridine Orange and Propidium Iodide (AO/PI) is recommended to resolve live and dead cells more easily.

References:

1. Nikodemova, M., Watters, J.J. Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. J Neuroinflammation 9, 147 (2012). https://doi.org/10.1186/1742-2094-9-147

2. Lee JK, Tansey MG. Microglia isolation from adult mouse brain. Methods Mol Biol. 2013;1041:17-23. doi:10.1007/978-1-62703-520-0_3

Enrichment of Perfused vs. Non-Perfused Brain Samples

Non-perfused brain digested following Protocol B:

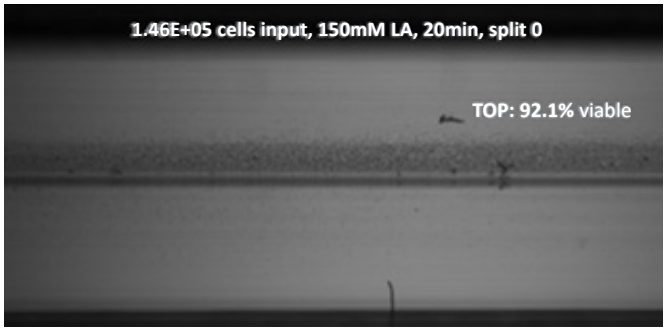


Figure 6. Levitation of cells from an adult mouse brain digested with LeviPrep and enriched for microglia cells using protocol B. *Approximately 150K cells were introduced into the cartridge. After 20 min of levitation, cells were collected and measured at 92% viability. Cells were dual stained with Acridine Orange and Propidium Iodide for cell counting purposes only. Levitation conditions: 150mM Levitation Agent in 1X PBS + 0.5% BSA.*

Perfused brain digested following Protocol C:

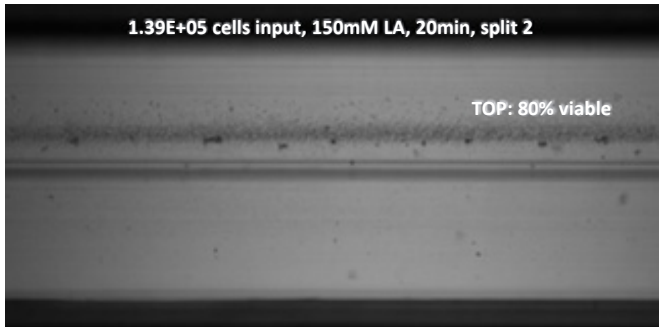


Figure 7. Levitation of cells from a PBS-perfused adult mouse brain digested with LeviPrep and enriched for all CNS cell types using protocol C. *Approximately 140K cells were introduced into the cartridge. After 20 min of levitation, cells were collected and measured at 80% viability. Cells were dual stained with Acridine Orange and Propidium Iodide for cell counting purposes only. Levitation conditions: 150mM Levitation Agent in 1X PBS + 0.5% BSA.*