# Adoptive Transfer of Isolated Bone Marrow Neutrophils

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**[Abstract]** Adoptive transfer experiments of specific cell populations are widely used methods to assess the role of the injected population on an ongoing process. In the last years, new and unprecedented roles in the regulation of immune responses have been reported for neutrophils. The following protocol is to be used to isolate neutrophils from bone marrow and to inject them in an appropriate host to test the role of neutrophils during infection, inflammation or other pathological conditions.

## Material and Reagents

1. Mice: Donor 8-12 week-old mice and sex-matched receptors. We always used C57BL/6 mice but it should work for any mice strain.
2. Red blood lysing buffer Hybri-Max (Sigma-Aldrich, catalog number: R7757)
3. PBS (Life Technologies, Gibco®, catalog number: 10010-023)
4. EDTA (Life Technologies, Gibco®, catalog number: 15575-020)
5. Fetal Bovine Serum (FBS) (PAA Laboratories GmbH, catalog number: A15-201)
6. Anti-Ly-6G Microbead kit (Miltenyi Biotec, catalog number: 130-092-332)
7. 0.4% Trypan blue solution (Sigma-Aldrich, catalog number: T8154)
8. PE-Cy7 labeled anti mouse Ly-6G (eBioscience, catalog number: 25-5931)
9. FITC labeled anti mouse CD11b (eBioscience, catalog number: 11-0112)
10. Isoflurane
11. Flushing/washing buffer (see Recipes)
12. MACS buffer (modified) (see Recipes)

## Equipment

1. LS column (Miltenyi Biotec, catalog number: 130-042-401)
2. Laminar Flow Cabinet
3. MACS separators
4. Centrifuge Eppendorf 5810R (Eppendorf, model: 5811000.010)

5. Centrifuge rotor for plates A-4-62 (Eppendorf, model: 5810709.008)
6. Optic microscope
7. Counting Neubauer chamber
8. Flow cytometer BD FACS Canto II
9. Automatic pipettes (full range volumes)
10. Sterile forceps and scissors
11. Tips (full range volumes)
12. Microtubes (1.5 ml)
13. Tubes (15 and 50 ml)
14. Culture dishes (45 mm)
15. Conventional Insulin 1 ml syringes with detachable needle (25G) (BD Biosciences, catalog number: 329651)
16. Low-dead volume insulin 1 ml syringes (29G) (BD Biosciences, catalog number: 329410)
17. Cell strainer 40 µm (BD Biosciences, catalog number: 352340)
18. Bell jar
19. 0.2 µm filter

## Procedure

Work fast and always keep buffers and cell suspensions on ice. These will avoid neutrophil apoptosis and/or activation.

A. Bone marrow cell isolation

1. Euthanize donor WT mice by an approved method.
2. Clip and remove the skin from the arms and legs.
3. Remove as much as possible tissue from arms and legs using scissors and dissect the bones away from body. To this end, cut the arms at the levels of the shoulders and legs at the levels of the hips.
4. Separate the feet and hands by delicately twisting the joints with two forceps. Work carefully to avoid breaking the bones. Do not separate knees and elbows at this step.
5. Put the arms and legs on ice in 1.5 ml microtubes filled with washing buffer until all bones have been dissected. The washing buffer should completely cover the bones.
6. Transfer the bones to a 45 mm culture dish. If there are too much remaining tissues in the bones, remove it using scissors or scalpels as cells associated to the remaining tissue may contaminate marrow preparation.
7. Separate the bones from the arms and legs gently twisting the elbows and knees with two sterile forceps.
8. Cut off each end of the bone using sterile scissors and forceps.

9. Harvest the marrow from the bone by inserting a 25 g needle in one end of the bone and flushing 1 ml of washing buffer with the syringe into a 15 ml tube.
10. Repeat with the other end of the bone. Repeat for all the bones to obtain a cell suspension (typically 15 ml tube can collect bone marrow from 2 mice). While flushing the marrow, the 15 ml tube can remain at room temperature, but work fast and once the tube is filled, transfer it to ice.
11. Centrifuge cell suspension at 4 °C and 2,000 rpm for 5 min. Discard supernatant and keep the cell pellet.
12. Lyse red blood cell using 2.5 ml red blood lysing buffer per tube that contains the bone marrow cell pellet from 2 mice (approximately, 150-200 x $10^6$ cells). Scale-up as required. Gentle pipet up and down to disaggregate any clump. Incubate 5 min at room temperature.
13. Inactivate lysing buffer with 7.5 ml washing buffer and filter cell suspension into a 50 ml using a 40 μm cell strainer.
14. Centrifuge cell suspension at 4 °C and 2,000 rpm for 5 min. Discard supernatant and keep the cell pellet.
15. Resuspend pellet in 5 ml of MACS buffer and count viable cell number using 0.4% Trypan blue solution, a Neubauer chamber and an optic microscope. Record total cell number. Set aside 2 x $10^5$ cells for evaluating purification efficiency as described below.
16. Centrifuge cell suspension at 2,000 rpm for 5 min. Discard supernatant and keep the cell pellet.

B. Neutrophil purification from bone marrow using Miltenyi kit
1. Resuspend the cell pellet in 200 μl of MACS buffer per $10^8$ cells. Use total cell number determined in step A13 to scale-up the amounts of buffer, antibodies and beads.
2. Add 50 μl of Anti-Ly-6G-Biotin per $10^8$ total cells.
3. Mix well and incubate for 10 min in the refrigerator (2-8 °C).
4. Add 150 μl of MACS buffer per $10^8$ total cells.
5. Add 100 μl of Anti-Biotin MicroBeads per $10^8$ total cells.
6. Mix well and incubate for 15 min in the refrigerator (2-8 °C).
7. Wash cells by adding 10 ml MACS buffer and centrifuge cell suspension at 4 °C and 2,000 rpm for 5 min. Discard supernatant and keep the cell pellet.
8. Resuspend up to $10^8$ cells in 500 μl of buffer and proceed to magnetic separation.
9. Place an LS column in a suitable MACS Separator and wash the column by rinsing with 3 ml of MACS buffer.
10. Apply cell suspension from step B8 onto the column. Collect flow-through containing unlabeled cells.

11. Wash the column three times with 3 ml of MACS buffer.
12. Collect unlabeled cells that pass through and combine with the flow through from step B11. Centrifuge unlabeled cell suspension and resuspend in 5 ml washing buffer. Count viable cell number using 0.4% Trypan blue solution, a Neubauer chamber and an optic microscope. Set aside 2 x $10^5$ cells for evaluating purification efficiency as described below.
13. Remove the column from the separator and place it on a suitable collection tube (typically 15 ml tube).
14. Pipette 5 ml of MACS buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
15. Centrifuge cell suspension at 4 °C and 2,000 rpm for 5 min. Discard supernatant and keep the cell pellet.
16. Resuspend the cell pellet in 5 ml of washing buffer and keep on ice. Count viable cell number using 0.4% Trypan blue solution, a Neubauer chamber and an optic microscope. Record total cell number. Set aside 2 x $10^5$ cells for evaluating purification efficiency as described below.

C. Control of purification efficiency by flow cytometry
1. Stain 2 x $10^5$ total bone marrow cells (step A15), unlabeled bone marrow cells (step B12) and Ly-6G+ isolated bone marrow cell (step B16) with 20 µl of a suspension contained pre-titrated amounts of anti-mouse Ly-6G and anti-mouse CD11b.
2. Incubate 10 min at 4 °C and wash in 150 µl of washing buffer.
3. Centrifuge cell suspension at 4 °C and 2,000 rpm for 5 min. Discard supernatant and keep the cell pellet.
4. Resuspend in 200 µl of washing buffer and analyze in a flow cytometer.
5. Purity of neutrophils (Ly-6G high CD11b high) in the Ly-6G+ population should be higher than 95%.

   Percentages of neutrophils (Ly-6G high CD11b high) in total bone marrow cells as well as in unlabeled cells together with cell counts will allow calculation of the purification yield. Typically our yields are around 50%.

D. Neutrophil injection in the retro-orbital sinus
1. Centrifuge cell suspension from step B16 at 4 °C and 2,000 rpm for 5 min. Discard supernatant and keep the cell pellet.
2. Wash cell pellet twice in 5 ml PBS.
3. Resuspend cell pellet in PBS to a density of 40 - 50 x $10^6$ cells per ml.

 

4. Anesthetize the mouse using 30% V/V isoflurane in propylene glycol mixture in a bell jar of a size appropriate for the size of the animal. Work inside a chemical fume hood.
5. After removal from the anesthetic, place the mouse in lateral recumbancy with the eye to be injected facing up.
6. Retract the skin above the eye toward the body causing the eye to protrude.
7. Insert the needle bevel up into the medial canthus of the eye at a 45 °C angle to the nose into the vessels behind the eye ball. Insert the needle around 2-3 mm, avoiding reaching the bone behind the eye.
8. Gently inject up to 100 µl (maximum volume allowed) of the neutrophil cell suspension into the retro-orbital vessels using low dead volume syringes (29G).
9. Slowly withdraw the needle and apply light pressure to the eye to control bleeding.
10. If properly performed, only small amount of blood or liquid should come out from the eye at the site of the injection. Liquid or blood should never come out from the nose. No inflammation of the eye or peri-orbital area should be observed.
11. Only one injection of a maximum of 100 µl volume per day is allowed. If repeated adoptive transfer is required you should alternate the eyes for injection allowing at least one day of recovery and with a maximum of total 2 injections per eye (4 injections per mice).

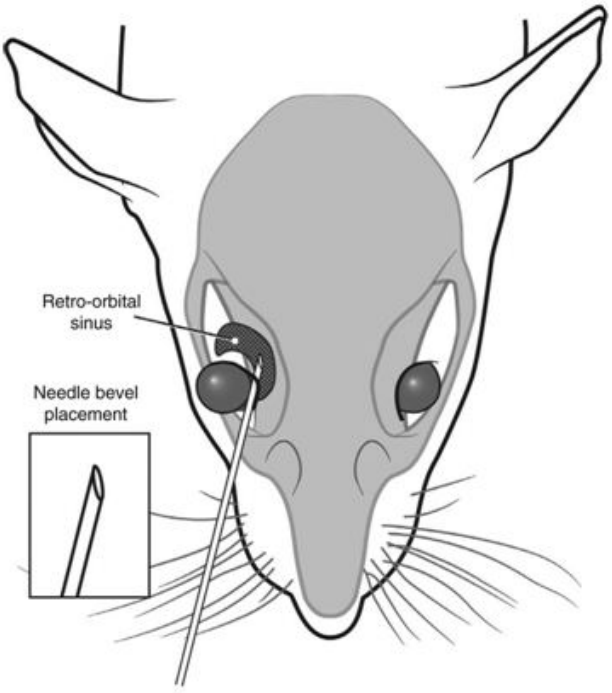


**Figure 1. Retro-orbital sinus injection.** The scheme shows the proper site of cell injection including the correct needle bevel placement.

## Recipes

Prepare the following buffer in advance and keep at 4 °C throughout the procedure:

1. Flushing/washing buffer
   2% FBS PBS
2. MACS buffer (modified)
   2 mM EDTA
   2% FBS
   PBS (pH 8.2) filtered through a 0.2 µm filter

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## References

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