PREPARATION OF MOUSE BONE MARROW NEUTROPHILS

We use Ca-Mg-free HBSS supplemented with 20 mM NA-HEPES (pH 7.4) and 0.5% FCS. This solution will be called HBSS-Prep. You can make up an 8x stock and dilute it from that. All solutions should be kept sterile and prepared in endotoxin-free water.

Sacrifice the mouse by LARC-approved protocol. (We usually use 10-15 wks old mice) Cut out the femurs and tibias and remove the muscles. Place the bones into HBSS-Prep to prevent dry-out. Continue with the next step before killing another mouse.

Cut the ends of the bones (it is recommended to cut as far towards the end as possible; in case of distal femur and proximal tibia, dislocation of the joint surface is sufficient). Flush the bone marrow into a 50 ml conical tube with HBSS-Prep using a short (5/8") 25 G needle and a 10 ml syringe.

Continue with the next animal. The bone marrow is fine in HBSS-Prep at RT until you finish all mice (we've done it with up to 10 mice). Suck the isolated BM through an 18 G needle to disaggregate larger BM pieces.

Pellet the suspension at 400xg (~1000 rpm on our Sorvalls) for 5 min and resuspend in 6 ml of 0.2%NaCl. After lysing for 45 sec, restore the osmolarity with 14 ml 1.2% NaCl. Pour the suspension into another 50 ml conical tube through a 70 micron cell strainer (Falcon #352350). This way you will get rid of the clot that appears and also of any bone remnants. Re-pellet.

In a 15 ml conical tube, pour 5 ml 62% Percoll (in HBSS-Prep). Resuspend the lysed BM in 5 ml HBSS-Prep (no Percoll) and carefully layer it over the 62% Percoll. Centrifuge for 30 min at 1000xg (~2200 rpm on our Sorvalls), brake off.

At the end of the gradient-centrifugation, you should see a sharp interface atop the 62% Percoll (these are immature cells and non-granulocytic lineages) and a more cloudy pellet (the neutrophils). Carefully remove and discard the cells at the interface, the HBSS-Prep, and the upper part of the 62% Percoll. I usually leave 2-3 mls of the 62% gradient to avoid disturbing the neutrophil pellet. Transfer the pellet to another 15 ml tube, wash twice with HBSS-Prep (or any other solution of your choice), resuspend in 1-5 ml of your assay medium and count. We usually keep the cells in Ca- and Mg-free medium until use and try to use them as soon as possible.

FURTHER NOTES

On average, you can expect 10-20 million neutrophils per mouse. At least 90% of the cells should be granulocytes based on the forward and side scatter and high GrI-staining. Most of the contaminating cells are from the B-lymphocytic lineage (forward/side scatter, B220-staining).

I recommend to do the preparation at RT. If you prefer, you can also do it at 4 degrees C from the gradient centrifugation step on. When we did it like that, the cells didn't respond to some of our stimuli.
It is essential to use reagents as clean as possible. We use endotoxin-free water from the cell culture facility and filter everything immediately after making up the solutions. Percoll contains endotoxin, but the cells can cope with that as long as there's no other source of endotoxin or other contamination. Pharmacia told me that newer stocks of Percoll contain much less endotoxin than previous ones. Another source of endotoxin can be BSA. This is why in this protocol HBSS-Prep contains FCS instead of BSA.