Bone Marrow Harvesting & Hematopoietic Stem Cell Isolation Kit

10 Isolations

Catalog No. SCR051

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

Hematopoietic stem cells (HSC) are frequently located in close proximity to bone lining cells within the endosteum. Recent studies demonstrated the significance of the endosteal region for hematopoiesis, and in particular that it contains a high number of HSCs. Consequently, traditional methods for harvesting bone marrow (BM) from mice via flushing of the long bones do not maximize the recovery of all HSCs. The Bone Marrow Harvesting and Hematopoietic Stem Cell Isolation Kit permits the optimal recovery of total cells and HSCs from mouse bones, and uniquely the isolation of HSCs specifically from the endosteal region. A combination of mechanical fragmentation by grinding of bones and enzymatic digestion is used for this purpose. Using this method, HSCs isolated specifically from the endosteum have been demonstrated to have a greater hematopoietic potential than HSCs isolated from the central marrow core including a 1.8-fold greater proliferative potential, an almost 2-fold greater ability to home to the bone marrow following tail vein injection and a significantly greater long-term hematopoietic reconstitution potential (Haylock, D. *et al.* Stem Cells, 2007). Additionally, this method has been shown to produce a 6-fold greater recovery of primitive HSCs (Lineage$^-$ Sca-1$^+$ and c-Kit$^+$) than traditional flushing methods.

Product Description

Hematopoietic stem cells (HSCs) are frequently located in close proximity to bone lining cells within the endosteum. It is unlikely that traditional methods for harvesting bone marrow (BM) from mice by simply flushing long bones would result in optimal recovery of HSCs. The Bone Marrow Harvesting and Hematopoietic Stem Cell Isolation Kit provides the necessary tools for grinding bones and enzymatic digestion to harvest maximal numbers of BM cells from murine bones as well as the purification of HSC from the endosteal and central marrow regions.

Kit Components

Sufficient reagents are supplied in the kit for 10 separate isolations.

SCR051-1
Mortar and pestle: One (1) porcelain mortar and pestle.

SCR051-2 (Store at 2° to 8°C)
Dispase II: (Catalogue No. CS200649) Ten (10) single use vials of 40 mg powdered enzyme.
Collagenase: (Catalogue No. CS200650) Ten (10) single use vials of 30 mg powdered enzyme.

SCNY00040
Steriflip® 40 µm, nylon net, radio-sterilized: Twenty five (25) individually wrapped units with 40 µm nylon mesh.

Materials Not Supplied

1. Phosphate Buffered Saline (310 mOsm)
2. Heat inactivated (HI) fetal calf serum
3. PBS-2% HI serum
4. Sterile Centrifuge tubes
5. Pipettes

6. 1 mL syringes
7. 23 and 26 gauge needles
8. Steriflip® 40 µm, nylon net, radio-sterilized (Catalogue No. SCNY00040) (optional)
9. Standard 50 mL tubes that have double–lead threads. Steriflip units are compatible only with double-lead threaded tubes. These have two leads, 180° from each other that wind around the tube from its top. ***CAUTION:*** *Leakage may occur if Steriflip unit is attached to a single-lead tube.*

   The following chart lists the manufacturers and part numbers of Steriflip unit-compatible 50 mL tubes:

50 mL Tube	Catalogue Number
BD Falcon™	352070, 352098
Fisherbrand®	05-539-6, 05-539-7, 05-539-8, 05-539-9
Greiner®	210261, 210270, 227261, 227270
Iwaki®	2341-050
Nunc™	334959, 334940
Perfector Scientific	2650

Storage and Preparation

Mortar, pestle, and Steriflip filter units can be stored at room temperature.

Single use vials containing the enzymes Dispase II and Collagenase I should be stored at 2° to 8°C until ready to use. Each vial contains enough enzyme to make 10 mL of enzyme solution. To make enzyme solution, add the contents of one vial of Dispase II to 10 mL PBS. Shake until dissolved. Next, add the contents of one vial of Collagenase I and swirl until dissolved. Enzyme solution should be made fresh on the day of use and can not be stored.

Assay Procedure

The number of nucleated cells recovered from the bones of mice processed according to the kit protocol depends on the strain, age, weight and sex of the animals. As a guide, 2 femurs, 2 tibias and 2 iliac crests from a single 6-8 week old male C57Bl/6J mouse should yield approximately 80-100 x 10^6 nucleated cells. Accordingly, bones from 10 mice should yield approximately 80-100 x 10^7 nucleated cells. The same number of bones from 6-8 week old female mice will yield fewer cells.

The actual recovery of defined populations of HSC (for example Lineage$^-$ Sca-1$^+$ kit$^+$; LSK) depends on the methods used for pre-enrichment and HSC isolation.

I. Method for harvesting total bone marrow from mouse bones.

This method is designed for maximal recovery of nucleated cells from the bones of 10 mice. Volumes can be adjusted for larger or smaller numbers of animals.

1. Start with femurs, tibias and iliac crests devoid of muscle and connective tissue. For each set of 6 bones add approximately 2 mL PBS-2% HI serum. For example, for 10 mice, start with 60 bones in approximately 20 mL PBS-2% HI serum.

2. Decant the tube containing bones into a sterile mortar.

3. Grind the bones thoroughly with the pestle so that the bones are opened and broken into small fragments.

   Note: The bones should not be ground to a fine powder as this may cause problems for subsequent HSC purification by flow cytometry.

4. Remove the cell supernatant with a pipette and transfer the cell sample to a sterile 50 mL double-lead threaded conical tube (not provided).

5. Attach the conical tube containing the cell supernatant to the Steriflip filtration unit (provided), invert and connect the filtration unit to a vacuum (see figure on page 4).

6. Turn on the vacuum to separate and filter the dissociated cells from the larger bone fragments.

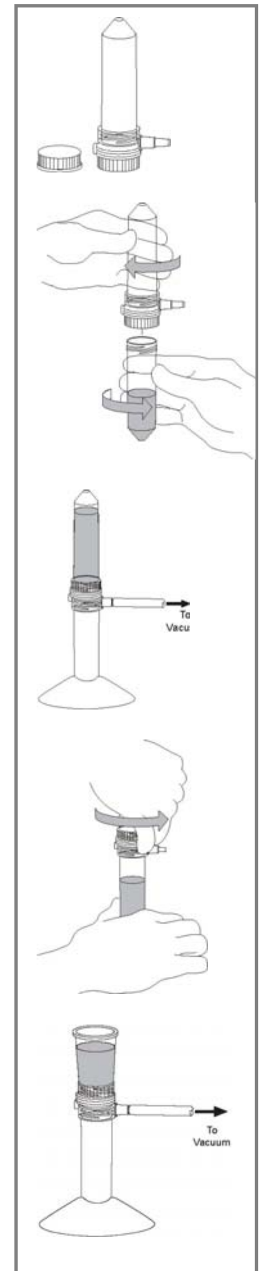
7. Remove the tube containing the dissociated filtered cells from the filtration unit. Being careful to maintain the orientation of filter, attach a fresh 50 mL double-lead threaded conical tube in its place to the filtration filter. Unscrew and discard the upper chamber (the used conical tube). Set the filtration unit aside for reuse in downstream steps.

8. Top up the centrifuge tube containing the freshly filtered cells with fresh PBS-2% HI serum. Set tube aside on ice until step 25.

9. Rinse the remaining crushed bone fragments with a further 50 mL PBS-2% HI serum.

10. Remove the cell supernatant with a pipette and transfer the cell sample to a sterile 50 mL conical tube (not provided).

11. Attach the conical tube containing the cell supernatant to the same 40 µm nylon net Steriflip filtration unit that was set aside in step 7. Invert and connect the filtration unit to a vacuum. Repeat steps 6-11 for tubes 3 and 4. At this point, there will be a total of 200 mL cell suspension. Note: The same 40 µm nylon net Steriflip filtration unit can be used for tubes 1-4. Set tubes aside on ice until step 25.



12. Prepare the enzyme solution as follows. Add one vial of Dispase II to 10 mL of PBS in a 50 mL tube. Mix by shaking then add one vial of Collagenase I and swirl until dissolved.

13. Transfer the crushed bones from the mortar into a 50 mL conical centrifuge tube.

14. For each set of 6 bones (i.e. 2 femurs, 2 tibia, and 2 iliac crests) add 2 mL of enzyme solution to the 50 mL centrifuge tube. For 60 bones use 10 mL of the enzyme mixture. For different numbers of bones add sufficient volume of the enzyme solution to ensure that the bones are covered.

15. Agitate the tube containing the bone fragments for 5 minutes at 37°C in an orbital shaker at 250 rpm.

    Note: Incubation of bone fragments in the enzymes for longer periods at 37°C may result in cleavage of cell surface antigens used for identification and purification of HSC. For example, c-kit is sensitive to cleavage by collagenase and dispase or other proteinases that might contaminate collagenase and dispase.

16. Add 25 mL PBS to the bone fragments and shake the tube vigorously for 10 seconds.

17. Attach the conical tube containing the cell suspension to a fresh 40 µm nylon net Steriflip filtration unit (provided). Invert and connect the filtration unit to a vacuum (see figure on page 4).

18. Turn on the vacuum to separate and filter the dissociated cells from the larger bone fragments.

19. Remove the tube containing the dissociated filtered cells from the filtration unit. Being careful to maintain the orientation of filter, attach a fresh 50 mL conical tube in its place to the filtration filter. Unscrew and discard the upper chamber (the used conical tube). Set the filtration unit aside for reuse in downstream steps.

20. Top up the centrifuge tube containing the freshly filtered cells with fresh PBS-2% HI serum. Set tube aside on ice until step 25.

21. Add a further 25 mL PBS to the bone fragments and shake the tube vigorously for 10 seconds.

22. Attach the conical tube containing the cell suspension to the same 40 µm nylon net Steriflip filtration unit that was set aside in step 19. Invert and connect the filtration unit to a vacuum (see figure on page 4). Note: The same Steriflip filtration unit that was set aside in step 19 can be used or alternatively a fresh Steriflip filtration unit can be used (not provided in kit).

23. Turn on the vacuum to separate and filter the dissociated cells from the larger bone fragments.

24. Remove the tube containing the dissociated filtered cells from the filtration unit. Top up the tube with 25 mL PBS-2% HI serum.

25. At this point, centrifuge all of the cell suspensions (6 x 50 mL tubes) at 400 xg for 5 minutes at 4 °C.

26. Decant supernatant and pool the cell pellets in PBS-2% HI serum. The volume that cells are resuspended in depends on the subsequent method for pre-enrichment and isolation of HSC.

27. Conduct a viable cell count. As a guide, 2 femurs, 2 tibias, and 2 iliac crests from a single 6-8 week old male C57Bl/6J mouse should yield 80-100 x 10^6 viable nucleated cells.

28. Proceed with HSC isolation strategy of choice.

II. Method for harvesting bone marrow from the endosteal region of murine bones

1. Start with femurs, tibias and iliac crests devoid of muscle and connective tissue. For easier flushing, cut the ends of the bones. The ends will be added to the endosteal tube and ground with the flushed bones.

2. Using a 1 mL syringe attached to a 21-gauge (femurs and tibias) or 23-gauge needle (iliac crests) gently flush the bones with PBS-2% HI serum into a 50 mL centrifuge tube. When harvesting endosteal bone marrow, the flushed marrow is discarded, however it may be put aside for HSC isolation if required.

3. Collect the flushed bones and ends into approximately 20 mL PBS-2% HI serum.

4. Decant the tube containing bones into a sterile mortar.

5. Grind the bones thoroughly with the pestle so that the bones are opened and broken into small fragments.

   Note: The bones should not be ground to a fine powder as this may cause problems for subsequent HSC purification by flow cytometry.

6. Remove the cell supernatant with a pipette and transfer the cell sample to a sterile 50 mL conical tube (not provided).

7. Attach the conical tube containing the cell supernatant to the Steriflip filtration unit (provided). Invert and connect the filtration unit to a vacuum (see figure on page 4).

8. Turn on the vacuum to separate and filter the dissociated cells from the larger bone fragments.

9. Remove the tube containing the dissociated filtered cells from the filtration unit. Being careful to maintain the orientation of filter, attach a fresh 50 mL conical tube in its place to the filtration filter. Unscrew and discard the upper chamber (the used conical tube). Set the filtration unit aside for reuse in downstream steps.

10. Top up the centrifuge tube containing the freshly filtered cells with fresh PBS-2% HI serum. Set tube aside on ice until step 27.

11. Rinse the crushed bone fragments with a further 50 mL PBS-2% HI serum.

12. Remove the cell supernatant with a pipette and transfer the cell sample to a sterile 50 mL conical tube (not provided).

13. Attach the conical tube containing the cell supernatant to the same Steriflip filtration unit that was set aside in step 9. Invert and connect the filtration unit to a vacuum. Repeat steps 8-13 for tubes 3 and 4. At this point, there will be a total of 200 mL cell suspension. Note: The same 40 µm nylon net Steriflip filtration unit can be used for tubes 1-4. Set tubes aside on ice until step 27.

14. Prepare the enzyme solution as follows. Add one vial of Dispase II to 10 mL of PBS in a 50 mL tube. Mix by shaking then add one vial of Collagenase I and swirl until dissolved.

15. Transfer the crushed bones from the mortar into a 50 mL conical tube.

16. For each set of 6 bones (i.e. 2 femurs, 2 tibia, and 2 iliac crests) add 2 mL of enzyme solution to the 50 mL centrifuge tube. For 60 bones use 10 mL of the enzyme mixture. For different numbers of bones add sufficient volume of the enzyme solution to ensure that the bones are covered.

17. Agitate the tube containing the bone fragments for 5 minutes at 37°C in an orbital shaker at 750 rpm.

    Note: Incubation of bone fragments in the enzymes for longer periods at 37°C may result in cleavage of cell surface antigens used for identification and purification of HSC. For example, c-kit is sensitive to cleavage by collagenase and dispase.

18. Add 25 mL PBS to the bone fragments and shake the tube vigorously for 10 seconds.

19. Attach the conical tube containing the cell suspension to a fresh 40 µm nylon net Steriflip filtration unit (provided). Invert and connect the filtration unit to a vacuum (see figure on page 4).

20. Turn on the vacuum to separate and filter the dissociated cells from the large bone fragments.

21. Remove the tube containing the dissociated filtered cells from the filtration unit. Being careful to maintain the orientation of filter, attach a fresh 50 mL conical tube in its place to the filtration filter. Unscrew and discard the upper chamber (the used conical tube). Set the filtration unit aside for reuse in downstream steps.

22. Top up the centrifuge tube containing the freshly filtered cells with fresh PBS-2% HI serum. Set tubes aside on ice until Step 27.

23. Add a further 25 mL PBS to the bone fragments and shake the tube vigorously for 10 seconds.

24. Attach the conical tube containing the cell supernatant to the same Steriflip filtration unit that was set aside in step 21. Invert and connect the filtration unit to a vacuum.

25. Turn on the vacuum to separate and filter the dissociated cells from the large bone fragments.

26. Remove the tube containing the dissociated filtered cells from the filtration unit. Top up the centrifuge tube with fresh PBS-2% HI serum.

27. At this point, centrifuge all of the cell suspensions (6 x 50 mL tubes) at 400 xg for 5 minutes at 4°C.

28. Decant supernatant and pool the cell pellets in PBS-2% HI serum. The volume that cells are resuspended in depends on the subsequent method for pre-enrichment and isolation of HSC.

29. Conduct a viable cell count. As a guide, 2 femurs, 2 tibias, and 2 iliac crests from a single 6-8 week old male C57Bl/6J mouse should yield 10-20 x 10^6 viable nucleated cells.

Analysis of Results

HSCs within the endosteal region have significantly increased homing efficiency to the bone marrow *in vivo*

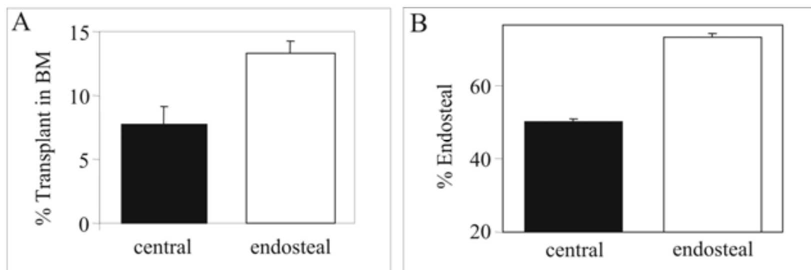


Figure 1. HSC isolated from the endosteal region and transplanted into non-ablated recipients had a significantly higher homing efficiency to the bone marrow after 15 hrs compared to HSC isolated from the central marrow core ($p<0.05$) (A). In addition, HSC isolated from the endosteal region and transplanted into non-ablated recipients had a significantly higher affinity to re-lodge within the endosteal region of the bone marrow after 15 hrs compared to HSC isolated from the central marrow core ($p<0.05$) (B). Data are the mean±SEM from both endosteal and central marrow HSC isolated from 15 animals and transplanted into 3 individual recipients.

HSCs within the endosteal region have significantly increased proliferative potential *in vitro*

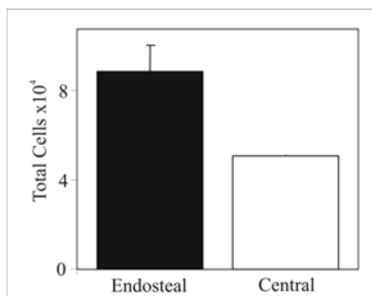


Figure 2. Sorted HSC (100 per well) isolated from the endosteal region (black bar) and the central marrow core (white bar) were grown in serum free media in the presence of 4 stimulatory growth factors. After 6 days there was a significant ($p=0.02$, students t-test) increase in the number of cells generated from endosteal HSC compared to those isolated from the central marrow core. Data are from a representative experiment ($n=4$) showing the mean±SEM from quadruplicate wells.

References

1. Haylock, D. et al. (2007) Hemopoietic Stem Cells with Higher Hemopoietic Potential Reside at the Bone Marrow Endosteum. Stem Cells, 25: 1062-1069.

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Cat No. SCR051

May 2009
Revision E, SCR051MAN