Supplementary Material For:

# Primary Bone Cell Isolation

Protocol For:

## Isolation and culture of primary osteocytes from the long bones of skeletally mature and aged mice

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## Introduction

This technique facilitates the isolation of osteocytes from skeletally mature bone. The early digestions (where noted) can also be used for obtaining primary osteoblasts. Prior to starting the isolation, several solutions and media must be prepared.

### Collagenase Solution:

* *HINT: This solution must be prepared fresh the morning of the isolation.*

Prepare the collagenase solution using 300 active units/mL of collagenase type IA (Sigma-Aldrich, St. Lewis, MO) dissolved in α–minimal essential medium (αMEM) (Mediatech Inc., Manassas, VA). 50 mL is adequate for an isolation from one or two mice.

### EDTA Solution:

* *HINT: This solution can be prepared prior to the day of isolation and stored at 4°C.*

Prepare the 5 mM ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA) solution (Sigma-Aldrich, St. Lewis, MO) in magnesium- and calcium-free Dulbecco's Phosphate-Buffered Solution (DPBS) (Mediatech Inc., Manassas, VA) with 1% bovine serum albumin (Sigma-Aldrich, St. Lewis, MO). The solution will need to be brought to a neutral pH of 7.4. 30 mL is adequate for an isolation from one or two mice.

### Primary Bone Cell Culture Medium:

* *HINT: This solution can be prepared the day before the isolation and stored at 4°C.*

Prepare α–minimal essential medium (αMEM) (Mediatech Inc., Manassas, VA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO), 5% heat-inactivated calf serum (CS) (HyClone Laboratories, Logan, UT), and 1% penicillin and streptomycin. This culture medium was chosen based on the culture of the MLO-Y4 osteocyte cell line (Ref. Kato et al.)

## Additional Materials and Reagents:

- surgical instruments to dissect and mince bones: forceps, surgical scissors, and scalpels
- 25-, and/or 27- gauge needles and syringes
- sterile laminar flow hood
- 100% ethanol
- 70% ethanol
- Hank's balanced salt solution (HBSS), calcium- and magnesium-free
- α–minimal essential medium (αMEM)
- heat-inactivated fetal bovine serum (FBS)
- penicillin and streptomycin stock solution (penicillin 10,000 IU/mL and streptomycin 10,000 µg/mL)
- gentamycin (optional)
- centrifuge
- several 6-well petri dishes (non-TC treated)
- several 100mm petri dishes (non-TC treated)
- shaker in incubator
- tissue homogenizer (Medimachine (BD Biosciences, San Jose, CA) with a stainless steel mincing screen with a pore size of 50 µm)
- 1 mL syringes
- collagen coated TC treated plates for cell plating (see below)

### Collagen-Coated Plates:

* *HINT: To be completed the day before the isolation in a sterile tissue culture hood.*

Dilute sterile collagen in previously filter sterilized 0.02 M acetic acid to final concentration of 0.15 mg/mL.

* *HINT: Use a chilled pipet so the collagen doesn't stick. Generally use, 8 mL for coating a 100 mm dish. Coat plates for 1 hour at room temperature. Tilt to remove excess collagen and save. This solution can be reused approximately 6 times and should be kept at 4 °C. To use plates immediately, it is best to rinse the plate with PBS to remove residual acid; otherwise dry the plates for 1 hour (without rinsing with PBS) with the lids off before storing at 4 °C.*

## Protocol:

This protocol takes approximately 10-12 hours from the time the mice are sacrificed to the time that the bone particles are plated, this depends on the number of mice and familiarity of the researchers with the protocol.

Steps one and two can be conducted on a lab bench.
1. Aseptically dissect the long bones (femurs, tibiae, and humeri) from the mice using surgical scissors or scalpel. Be sure not to break any of the bones at this point and also try to keep the abdomen intact during the dissection to reduce contamination potential.
2. As the bones are dissected and cleaned off as much soft tissue as possible, place them in 100mm petri dishes containing αMEM with 10% penicillin and streptomycin (and gentamycin (25 μg/mL) – optional).

The following steps need to be conducted in a sterile laminar flow hood.
3. Remove any remaining muscle and connective tissue from the bones and scrape away the periosteum using a scalpel.
4. Wash the bones in sequential dishes/wells of a six-well plate filled with αMEM + 10% penicillin and streptomycin to remove any hair or other contaminants that may be present.
5. Place bones in a 100 mm petri dishes with fresh αMEM with 10% penicillin and streptomycin (and gentamycin (25 μg/mL) – optional).
6. Cut off the bone epiphyses and flush the marrow out using a needle and syringe.
7. Wash the hollowed bone pieces again in αMEM with 10% penicillin and streptomycin (and gentamycin (25 μg/mL) – optional).
8. Cut the bones in half lengthwise and then cut into 1 to 2 mm lengths using a scalpel.
9. As the bone pieces are cut, place them in HBSS for a brief wash.
10. Digest 1: Incubate the bone pieces in warmed collagenase solution for 25 min (8 mL of solution per well in a six-well plate works well for the long bones from 1-2 mice). Following the incubation, aspirate the solution and retain for cell plating (if interested in Digest 1 cells). Wash the bone pieces with HBSS three times with 5 ml each, each time adding the HBSS rinse to the aspirated solution for cell plating. Pellet, resuspend, and plate the cells on collagen-coated plates using the primary bone cell culture media.
11. Digest 2: Repeat step 10.
12. Digest 3: Repeat step 10, again.
13. Digest 4: Incubate the bone pieces in warmed EDTA solution for 25 min (8 mL of solution per well in a six-well plate works well for the long bones from 1-2 mice). Following the incubation, aspirate the solution and retain for cell plating (if interested in Digest 4 cells). Wash the bone pieces with HBSS three times with 5 mL each, each time adding the HBSS rinse to the aspirated solution for cell plating. Pellet, resuspend, and plate the cells on collagen-coated plates using the primary bone cell culture media.
14. Digest 5: Incubate the bone pieces in warmed collagenase solution for 25 min (8 mL of solution per well in a six-well plate works well for the long bones from 1-2 mice). Following the incubation, aspirate the solution and retain for cell plating (if interested in Digest 5 cells). Wash the bone pieces with HBSS three times with 5 mL each, each time adding the HBSS rinse to the aspirated solution for cell plating. Pellet, resuspend, and plate the cells on collagen-coated plates using the primary bone cell culture media. *These will be primarily osteoblastic cells.*
15. Digest 6: Incubate the bone pieces in warmed EDTA solution for 25 min (8 mL of solution per well in a six-well plate works well for the long bones from 1-2 mice). Following the incubation, aspirate the solution and retain for cell plating (if interested in Digest 6 cells). Wash the bone pieces with HBSS three times with 5 mL each, each time adding the HBSS rinse to the aspirated solution for cell plating. Pellet, resuspend, and plate the cells on collagen-coated plates using the primary bone cell culture media. *These will be primarily osteoblastic cells.*
16. Digest 7: Incubate the bone pieces in warmed collagenase solution for 25 min (8 mL of solution per well in a six-well plate works well for the long bones from 1-2 mice). Following the incubation, aspirate the solution and retain for cell plating (if interested in Digest 7 cells). Wash the bone pieces with HBSS three times with 5 mL each, each time adding the HBSS rinse to the aspirated solution for cell plating. Pellet, resuspend, and plate the cells on collagen-coated plates using the primary bone cell culture media. *These will be a mix of osteoblastic and osteocytic cells.*
17. Digest 8: Incubate the bone pieces in warmed EDTA solution for 25 min (8 mL of solution per well in a six-well plate works well for the long bones from 1-2 mice). Following the incubation, aspirate the solution and retain for cell plating (if interested in Digest 8 cells). Wash the bone pieces with HBSS three times with 5 mL each, each time adding the HBSS rinse to the aspirated solution for cell plating. Pellet, resuspend, and plate the cells on collagen-coated plates using the primary bone cell culture media. *These will be primarily osteoblastic and osteocytic cells.*
18. Digest 9: Incubate the bone pieces in warmed collagenase solution for 25 min (8 mL of solution per well in a six-well plate works well for the long bones from 1-2 mice). Following the incubation, aspirate the solution and retain for cell plating. Wash the bone pieces with HBSS three times with 5 mL each, each time adding the HBSS rinse to the aspirated solution for cell plating. Pellet, resuspend, and plate the cells on collagen-coated plates using the primary bone cell culture media. *These will be primarily osteocytic cells.*
19. Outgrowth of Cells from Bone Particles: Mince the bone pieces in αMEM utilizing a tissue homogenizer. Directly plate the resulting suspension of bone particles in αMEM on collagen-coated plates adding additional primary bone cell culture media if needed. *These will be primarily osteocytic cells.*

## Cell Plating Note

The issue of maintaining cell density is quite crucial for the cell attachment and survival of the later digests. It is recommended for an isolation using 1-2 mature mice where it is desired to plate each digest individually, one should use a 6-well plate format. If similar digests are combined together, digests 7-9 for example, one should use a 100 mm dish format. The bone particles derived from 1-2 mature mice can be split between two wells of a 6-well plate.

Cells will be immediately visible in digests 1-9 (for cell counting and trypan blue staining) and should plate in 24-48 hours. DO NOT disturb the bone particle cultures for at least 48 hours. Moving the dish will cause movement of the bone particles and therefore hinder the attachment of the osteocytes. It is recommended to leave the bone particles for as long as possible, adding additional primary bone cell media to the dishes at 72 hours, and changing to fresh media at 4 or 5 days post culture. It is recommended to use the osteocytic cultures for experimental purposes on day 7 as that is when they were characterized in the BioTechniques manuscript. Prolonged culture will otherwise lead to dedifferentiation/loss of phenotype or an overgrowth of the cultures by fibro- or osteoblasts.