**DO Bone Marrow Osteoblast and Osteoclast Differentiation Protocol**

**Red Blood Cell Lysis**

Thaw sample and re-suspend into 5mls Bone Growth Media

Spin cells at 1000rpm for 5 minutes @ 18oC

Re-suspend cell pellet into 5-10mls 0.2% NaCl. Gently pipette immediately for 20seconds followed by quick addition of an equal volume of 1.6% NaCl and mix well.

Spin cells again at 1000rpm, 5 minutes @ RT

Re-suspend cell pellet into 1ml growth media and plate into one well of a 48 well plate

Grow in 370C, 5% CO2 incubator

**Growth of Cells**

Let cells grow undisturbed until Day 3 post-plating when adherent and non-adherent cells will be separated for differentiation into osteoblasts and osteoclasts

**Osteoclast differentiation**

On Day 3, post-plating, transfer the supernatant containing non-adherent cells to a 6 well low bind tissue culture plate (Corning cat# 3471) and bring its total volume up to 2 ml. Add MCSF (R&D cat# 416-ML) to 50ng/ml.

Note that new lots of MCSF should be tested prior to use for best concentration usage.

Place samples into 370C, 5% CO2 incubator. Adherent macrophages should be evident in 48-72 hours, however maybe not with the low bind plates.

Let the cells grow undisturbed for 5 days.

On D6, re-plate the MCSF dependent macrophages by placing the plate on ice for 20 minutes. Most of the cells that may have adhered should come up. If not triterate lightly 7x and check under scope to see if they’re up. Re-triterate if not and recheck. Once cells are up, transfer to 15ml tube and spin at 1000rpm for 5 minutes at RT.

Resuspend cells into 300ul bone growth media and count and prepare for plating of pitting assay, F-actin/dapi staining and RNA collection.

**Pitting Assay**

Plate samples in triplicate @ 1.2x104 cells per well of 96 well osteo assay plates (Corning cat# 3989) in 150ul bone growth media containing 50ng/ml MCSF. Fill empty wells with DPBS to help prevent evaporation of media in sample wells. Place into in 370C, 5% CO2 incubator overnight. This is D0.

Following day (D1), cells should be adherent. Add RANK-L (R&D Systems cat# 462-TEC-010) at 50ng/ml and place back into incubator. Note that new lots of Rank-L should be tested for optimal concentration

Change ½ of the media every other day.

At D10 fix cells with 10%NBF for 10 minutes. Wash 2x with DPBS. Add 50% bleach for 10 minutes followed by 4 washes with DPBS. Scan wells on EVOS on phase contrast at 4x magnification (10x?). Count pits ?????

**F-actin and dapi staining**

Plate samples in triplicate @ 1.2x104 cells per well of normal 96 well tissue culture plates in 150ul bone growth media containing 50ng/ml MCSF. Fill empty wells with DPBS to help prevent evaporation of media in sample wells. Place into in 370C, 5% CO2 incubator overnight. This is D0.

Following day (D1), cells should be adherent. Add RANK-L (R&D Systems cat# 462-TEC-010) at 50ng/ml and place back into incubator. Note that new lots of Rank-L should be tested for optimal concentration

Change ½ of the media every other day.

At D7 stain the cells with NucBlue Live Cell Stain ReadyProbes Reagent (Invitrogen cat#R37605) according to manufacturer’s directions. Fix cells with 10% NBF for 10 minutes. Wash 2x with DPBS. Permeabilize cells in 0.5% Triton X100 in PBS for 5 min at room temperature and then incubate with 0.5 ug/ml FITC labelled Phalloidin for 30 min. The cells are then rinsed with PBS and scanned on the EVOS for both dapi and GFP channels simultaneously at 4x magnification (10x?)

**RNA Harvesting**

Plate 2.4x 104 cells per well of 48 well plate for RNA harvesting. Add MCSF @30-50ng/ml per well. Grow in 370C, 5% CO2 incubator. Cells should be adherent within 24hrs. This is D0.

Following day (D1) add RANK-L (R&D Systems cat# 462-TEC-010) @ 30-50ng/ml (new lots of RANK-L should be previously tested for best concentration).

# Harvest D7 using RNeasy Plus Micro Kit (cat# 74034) according to manufacturer’s directions. Freeze lysates at -80oC

**Osteoblast differentiation**

After removal of non-adherent cell containing supernatant, wash remaining adherent cells one time with 1ml 1x DPBS

Replace DPBS with 300ul Bone Growth Media

Repeat DPBS wash and media replacement every day through day 5 of post-plating cell growth

**Mineralization of Bone Marrow Culture**

On D6 repeat DPBS wash but replace with Bone Marrow Differentiation Media. This becomes D0 of mineralization. The cells should be 90-100% confluent ideally, however with the DO this may not be the case so scan all wells in order to record confluencies at D0 as it will affect the mineralization outcome at D10……..how to scan? In order to count cells for later normalization

Days 1,3,5,7, and 9 add 3ul of a 1:10 dilution of IRDye 680RD Bone Tag Optical Probe (Licor cat# 926-09374) to each well

Days 2,4,6, and 8 repeat the D0 wash. After washing scan the wells in the Licor Odyssey CLx . See scan procedure below.

**Licor scanning**

Wipe the platform with 70% alcohol and kimwipe as well as bottom of plate to prevent dust artifacts during scan

Place the L-shaped adaptor for plate scanning in the lower left scan. The adapter is located under the machine to the left

Place the plate in the lower left hand corner and close the lid

Login to computer and open the software

Open the workspace DO Osteoblasts in CLx

Tell the machine to scan a plate and set the focus offset at 2.87

Start the scan

**RNA harvesting**

At D10, add 350ul of Quiagen RLT Lysis buffer. Scrape well with mini-scraper, transfer lysate to 1.5ml tube and vortex 30 seconds. Store tube in -80.

**Bone Marrow Growth Media 100mls**

Alpha MEM (Gibco cat#12571-063) 88mls

FBS (previously tested for mineralization ability) 10mls

Pen/Strep (Gibco cat#15140-122) 1ml

100x Glutamax (Gibco cat# 35050-061) 1ml

**Bone Marrow Differentiation Media 100mls**

Alpha MEM (Gibco cat#12571-063) 86.9mls

FBS (previously tested for mineralization ability) 10mls

Pen/Strep (Gibco cat#15140-122) 1ml

100x Glutamax (Gibco cat# 35050-061) 1ml

50mg/ml Ascorbic Acid (in water) 100ul

1M B-glycerophosphate (in DPBS) 1ml

100uM Dexamethasone (in ethanol) 10ul