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Soluble TRAP quantification as a measure of osteoclastogenesis V.1

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We use this protocol and it's working

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Abstract

The protocol details the method for quantifying the activity of secreted Tartrate-Resistant Acid Phosphatase (TRAP) via a kinetic absorbance assay. The method complements other approaches to osteoclast assessment such as microscopy and gene expression analysis and offers multiple benefits including cost-effectiveness, scalability, and, due to its lack of a requirement to fix or lyse the cells, options for longitudinal assessment of osteoclastogenesis across multiple days.

Protocol materials

- L-glutamine **Corning Catalog #**MT25005CI
- 10,000IU penicillin/10,000µg streptomycin **Corning Catalog #**MT-30-002-CI
- Minimum essential medium powder **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M0894-10X1L
- L-( ) Tartaric Acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T-6521
- Sodium Acetate Anhydrous **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S-2889
- Napthol AS-MX Phosphate Substrate mix **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**N-4875
- Ethylene Glycol Monoethyl Ether **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E-2632
- Fast Red Violet LB Salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F-3381

Before start

Prepare α-MEM

1. Combine the following: 1 jar
   Minimum essential medium powder **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M0894-10X1L , 0.88L ddH_2O, 10mL 200nM L-glutamine **Corning Catalog #**MT25005CI , 10mL 10,000IU penicillin/10,000µg streptomycin **Corning Catalog #**MT-30-002-CI , 1.98 $NaHCO_3$ (sodium bicarbonate).
2. Mix and sterile filter into a new bottle.
3. Add 100ml heat-inactivated fetal bovine serum.

Prepare Syringes

1. Working in a biosafety cabinet, add 10mL α-MEM to a syringe then attach a 28 gauge luer lock needle keeping the needle cover on. Prepare 1 syringe for each mouse to be dissected.

Disinfect Equipment

1. Sanitize scissors, forceps, push pins, and a dissecting board with 70% ethanol.

Bone marrow monocyte isolation

1 Euthanize mouse (C57BL/6 or BALB/c) by CO_2 asphyxiation followed by cervical dislocation.

2 Spray the mouse thoroughly with 70% ethanol, then pin the mouse in a supine position with limbs spread 60-90 degrees away from the body.

3 Beginning from the abdomen, carefully cut below the skin and fascia to expose the quadriceps muscles.

> Note
>
> Ensure that the peritoneum is not punctured to prevent contamination by gut microbiota.

4 Cut the patellar tendon and peel the quadriceps muscles towards the hip joint.

5 Isolate the femora by first cutting at the femoral neck then transfer each into a 60mm dish with 2mL α-MEM.

6 Cut the tibiae at the ankle joint and peel the calf muscles towards the knee joint.

7 Remove the fibulae and transfer the tibiae into the 60mm dish.

8 Working in a biosafety cabinet, flush the marrow of each bone with 2.5mL α-MEM into a 15mL conical tube for each mouse. Hold each bone with forceps while inserting the syringe needle from above.

> Note
>
> Ensure that both ends of each bone are cut to facilitate flushing. The majority of the marrow is removed when the bone's color changes from pale red to white.

9 Resuspend the cells by inverting the tube, then allow non-cellular debris to fall to the bottom of the tube for 30 seconds.

10 Transfer the cell suspension into a new 15mL conical tube then pellet by centrifugation at 350 rcf, 20°C for 5 minutes.

11 Lyse red blood cells by removing the supernatant and resuspending the cell pellet in 500µL RBC lysis buffer for 5 minutes.

12 Wash by adding 9.5mL PBS and pellet by centrifugation at 350 rcf, 20°C for 5 minutes.

13 Remove supernatant and resuspend in 10mL α-MEM, then transfer to a 100mm treated culture plate. Incubate at 37 °C for 6 to 24 hours to allow separation of suspension and adherent cell fractions.

Macrophage differentiation

14 Carefully remove the suspension fraction of the culture plate to isolate the bone marrow monocytes and transfer into a 15mL conical tube.

15 Raise volume to 10mL α-MEM and add MCSF to a final concentration of 25ng/mL.

16 Mix by inversion and transfer to a 100mm non-treated culture plate.

17 Allow macrophage differentiation by incubating at 37 °C for 36 to 48 hours.

18 Refresh the media of the macrophages by preparing 10mL of α-MEM with 25ng/mL MCSF, then aspirate and replace the media.

19 Incubate at 37 °C for an additional 24 hours.

Osteoclast differentiation

20 Lift cells by aspirating medium and adding 1mL Accutase or 2mM EDTA-PBS. Incubate at room temperature for 5-10 minutes to loosen the cells' attachment to the plate.

21 Add 1mL α-MEM, gently scrap the cells off the plate with a sterile lifter, and transfer the cells to a 15mL conical tube.

22 Invert the cells to ensure a homogenous distribution, then mix 15µL cell suspension with 15µL trypan blue in a 1.5mL tube. Proceed to count live cells.

23 Dilute the macrophages and add MCSF and RANKL as desired.

> Note
>
> For a standard differentiation, we dilute to 100,000 cells/mL to yield 10,000 cells/100µL per well of a 96-well plate and add 25ng/mL MCSF and 25ng/mL RANKL.

24 Allow osteoclast differentiation by incubating at 37 °C for 48 hours.

25 Refresh the media of the osteoclasts by preparing α-MEM with 25ng/mL MCSF and 25ng/mL RANKL, then aspirate and replace the media.

26 Incubate at 37 °C and periodically observe the cells for an additional 24-48 hours. Full differentiation of osteoclasts should be reached at 72-96 hours.

TRAP basic incubation medium preparation

27 Prepare TRAP basic incubation medium in a 2L flask by first combining 9.2 g Sodium Acetate Anhydrous **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S-2889, 11.4 g L-( ) Tartaric Acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T-6521, and 2.8 mL Glacial Acetic Acid.

28 In a separate flask, create a 5M sodium hydroxide*** solution by adding 50g of sodium hydroxide pellets to 250 mL distilled water.

29 Add drops of the 5M sodium hydroxide*** solution to the 2L Erlenmeyer flask to raise the pH to a level between 4.7 and 5.0. Add water to a final volume of 1L.

Note

This solution is typically stable at room temperature for up to 3 months.

Trap stain preparation

30 Calculate the total volume of TRAP stain needed by multiplying the number of samples by the volume of stain desired for each. Increase this volume by 20% to ensure sufficient amount of reagent.

Note

For samples in a 96-well plate, we recommend 100µL of stain for each (i.e. 15 samples with 100µL of stain each will require a minimum of 1.5mL TRAP stain).

31 Calculate the volume of

Napthol AS-MX Phosphate Substrate mix **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**N-4875

needed for a final concentration of 5µL/mL in the TRAP stain. In this volume of 2-ethoxyethanol

Ethylene Glycol Monoethyl Ether **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E-2632

weigh and dissolve napthol to a final concentration of [M] 20 mg/mL. Vortex or pipette the napthol mixture until completely dissolved.

Note

For example, a 1.7mL TRAP stain solution will require 8.5µL dissolved napthol. At a concentration of 20mg/mL, 0.17mg of napthol should be dissolved in 8.5µL 2-ethoxyethanol.

32 Calculate the mass of

Fast Red Violet LB Salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F-3381

needed for a final concentration of [M] 0.6 mg/mL in the TRAP stain, and dissolve this in TRAP basic incubation medium.

33 Add napthol AS-MX phosphate solution to Fast Red solution and mix thoroughly by vortexing.

Soluble TRAP quantification assay

34 Before preparing the osteoclast supernatant, program a plate reader to take measurements at an absorbance of 520nM every 2 minutes for 180 minutes while remaining at 37 °C .***

35 At the desired time point of osteoclast differentiation, remove 100µL of osteoclast supernatant from each well and move into a well of a new 96-well plate.

36 Add 100µL of TRAP stain to each sample and run the plate on the programmed settings.

37 Export the measurements to an excel spreadsheet.

Data analysis

38 See our annotated spreadsheet for our method of analyzing raw data.
05-05-2024 520 Abs TRAP_relativ... 379.8KB The following instructions provide an overview of our process.

39 Normalize data points by dividing each condition's measurements by the no-treatment control measurements.

40 Create a time versus condition matrix for each series of replicates.

41 Apply a polynomial function to each replicate, then retrieve the associated coefficients to calculate area under the curve (AUC).

42 Conduct pairwise or group comparisons of AUC*** by T-Test or ANOVA.