COSMO BIO CO., LTD.

Bone Resorption Assay Kit

Cat. No. CSR-BRA-24KIT / CSR-BRA-48KIT / CSR-BRA-48X2KIT

Last Updated: 2015/4/23

【 I 】 Background

This product is an assay kit for the measurement of bone resorption activity using a fluoresceinated calcium phosphate-coated plate. The coated calcium phosphate is first bound to fluoresceinamine-labeled chondroitin sulfate (FACS), which is released from the calcium phosphate layer into conditioned medium by osteoclastic resorption activity. Bone resorption activity is evaluated by measuring the fluorescence intensity of the conditioned medium. This assay provides a rapid evaluation system unlike that of the traditional pit assay.

【 II 】 Kit Components

	BONE RESORPTION ASSAY KIT 24	BONE RESORPTION ASSAY KIT 48	BONE RESORPTION ASSAY KIT 48x2
BONE RESORPTION ASSAY PLATE 24	1 plate	---	---
BONE RESORPTION ASSAY PLATE 48	---	1 plate	2 plates
BONE RESORPTION ASSAY FACS	1 bottle	1 bottle	2 bottles
BONE RESORPTION ASSAY BUFFER	1 bottle	1 bottle	1 bottle

Plate: Calcium phosphate (CaP)-coated, sterilized

FACS: Fluoresceinamine-labeled chondroitin sulfate (FACS) , PBS solution, sterilized, store below 4°C

Buffer: Buffer for measuring fluorescence intensity, sterilized, store below 4°C

【 III 】 Labeling FACS to CaP-coated plates

(1) Binding of FACS to the CaP-coated plate and cell culture should be performed using aseptic conditions.

(2) A: Carefully add 0.5 mL of BONE RESORPTION ASSAY FACS to each well of the BONE RESORPTION ASSAY PLATE 24.

B: Carefully add 0.25 mL of BONE RESORPTION ASSAY FACS to each well of the BONE RESORPTION ASSAY PLATE 48.

To avoid disturbing the CaP layer, do not drop the solution directly onto the CaP layer of the plate. Instead, pipet the solution slowly onto the well walls to gently flow over the CaP layer.

(3) Cover the plate with a lid and incubate at 37°C for 1 – 2 hours (in the incubator) under the light-shielded condition.

(4) A: After incubation, wash each well of the 24-well plate with 1 mL of PBS(-) twice, being careful not to touch the surface of the plate. Add 1 mL of culture medium (without phenol-red).

B: After the incubation, wash each well of the 48-well plate with 0.5 mL of PBS(-) twice, being careful not to touch the surface of the plate. Add 0.5 mL of culture medium (without phenol-red).

(5) Keep the FACS-labeled CaP-coated plate under light-shielded conditions (e.g., cover the plate with aluminum foil) during cell preparation.

【 IV 】 Cell culture and detection of the bone resorption activity

A description of a typical assay procedure for the evaluation of test substances using the murine macrophage cell line RAW264 or RAW264.7 is described below.

(1) Carefully remove the medium from the FACS-labeled CaP-coated plate and inoculate RAW264 or RAW264.7 cells into each well in culture medium (phenol red-free DMEM/F-12 or α MEM containing 10% fetal bovine serum (FBS)).

A: 24-well plate $1x10^4$ cells/mL; 1 mL/well

B: 48-well plate $5x10^3$ cells/mL; 0.5 mL/well

Add an inducer of osteoclastic differentiation, such as RANKL (Oriental Yeast Co., Ltd., Tokyo, Japan; 100 ng/mL), and the test substances to be evaluated.

(2) Culture for about 5 – 6 days without medium change. Multinuclear osteoclatic cells are observed around 4 – 6 days.

(3) On day 5 – 6, transfer 100 µL of the conditioned medium from each well into the wells of a 96-well plate (black plate for fluorescence measurement). Add 50 µL of BONE RESORPTION ASSAY BUFFER to each well and mix using a plate shaker. Measure fluorescence intensity with an excitation wavelength of 485 nm and an emission wavelength of 535 nm, identical to those used for FITC.

(4) To measure the pit area, remove the cells in the well by treating the plate with 5% sodium hypochlorite for five minutes. Wash the plates with water and dry. Using a microscope, photograph the regions in each well and measure the pit area with image analyzing software.

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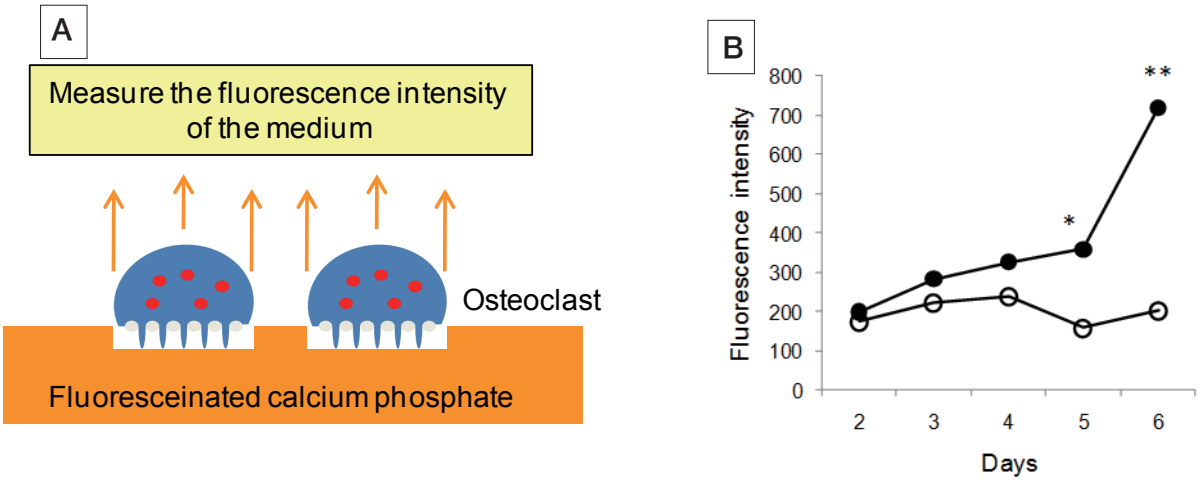
【 V 】Assay Precautions

(1) Use phenol red-free medium.
(2) The osteoclastic differentiation activity varies with cell type and culture conditions. The culture medium containing HEPES buffer influences the differentiation activity. We recommend checking of the differentiation activity of the cells using a tissue culture plate before using this kit.
(3) Because RANKL stimulation could decrease the attachment of the cells, be careful not to detach the cells when the medium change.
(4) Protect from strong light as much as possible.
(5) To stimulate cells, we recommend a RANKL concentration ≥ 100 ng/mL.
(6) Because fluorescence intensity varies with pH and is lower under acidic conditions, we recommend the use of BONE RESORPTION ASSAY BUFFER when measuring fluorescence intensity.
(7) Because FACS reduces the ability of osteoclastic differentiation of the cells to some extent when it is coated on the CaP plate, osteoclast activity may be controlled by using a chondroitin poly-sulfate (CPS)-binding protein, such as a member of the bone morphogenetic protein (BMP) or fibroblast growth factor (FGF) families.
(8) High concentration (≥ 100μM) of some kind of bisphosphonate release the FACS from CaP layer.
(9) This product is for research use only, and not for use in diagnostic or therapeutic procedures.

【 VI 】Expected Results

The data shown below are the results using RAW264 cells. (Miyazaki T., et al., Anal Biochem, 410:7-12, 2011)

Measurement Principle



A: A schematic of the mechanism for measuring bone resorption activity.
B: RAW264 cells differentiated into osteoclastic cells over days in culture.

The fluorescence intensity of the conditioned medium was increased by the addition of RANKL (100 ng/mL).
(*: p<0.05, **: p<0.001). ○ : RANKL(-), ● : RANKL(+)

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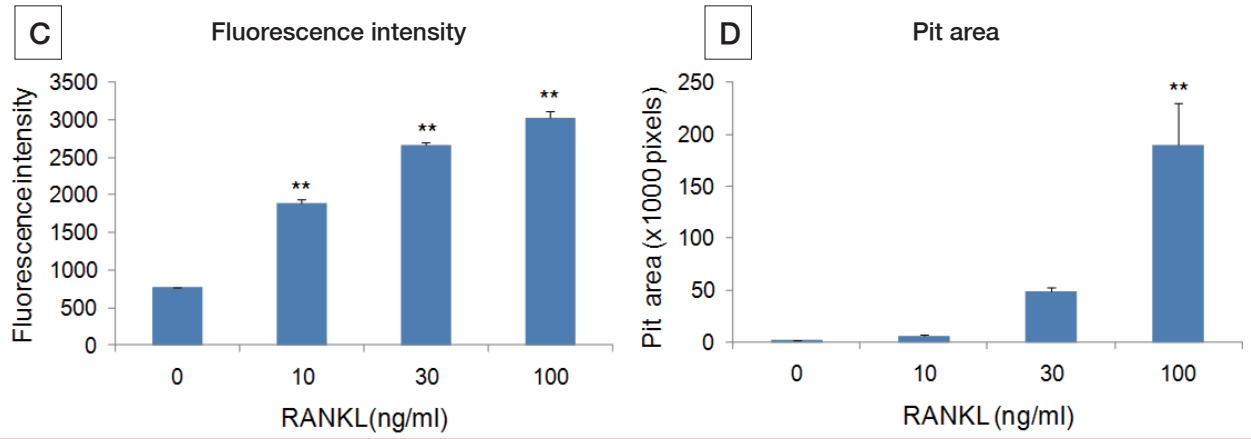
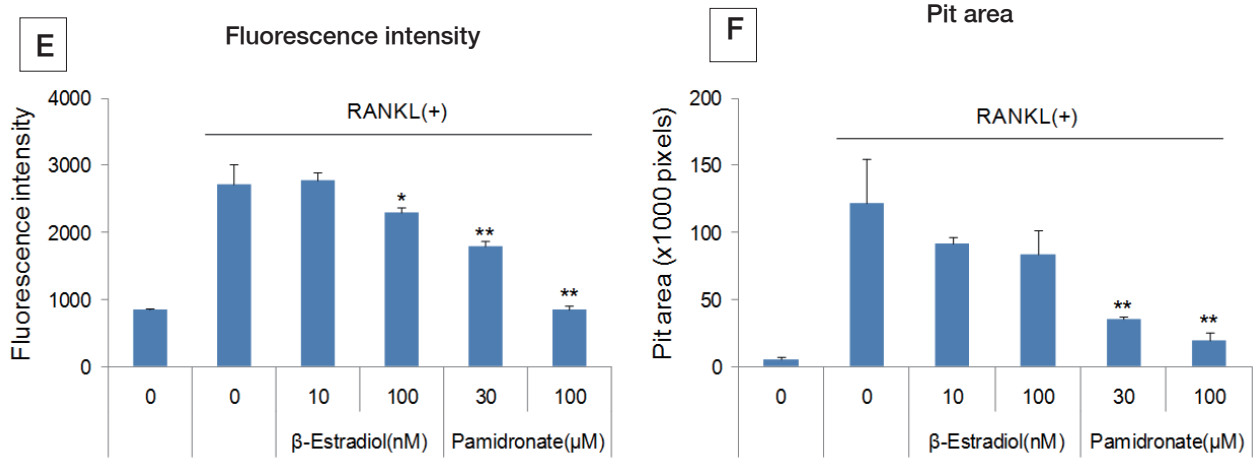
Bone resorption activity and RANKL concentration



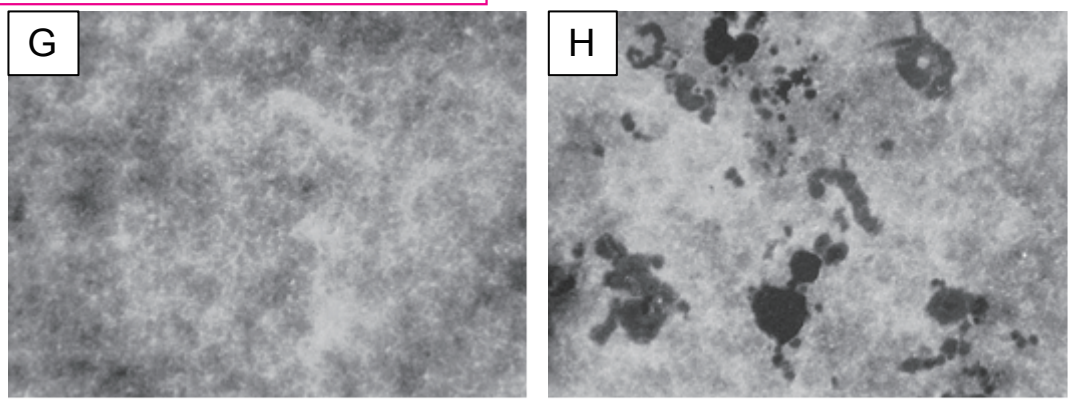
RANKL-dependent increases of the fluorescence intensity (C) and pit area (D) (mean ± S.D., n = 3, **: p<0.001).

Evaluation of drugs for treating osteoporosis



The inhibitory effects of Pamidronate and β -Estradiol on the resorption of CaP induced by RANKL (100 ng/mL) were evaluated by fluorescence intensity (E) and pit area (F) (mean ± S.D., n = 3, *:p<0.05,**: p<0.001).

Pit Formation



A microscopic photograph of a CaP-coated plate (on day 6). G: without RANKL; H: with RANKL (100 ng/mL)

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