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# TRACP & ALP double-stain Kit

Cat.# MK300
v.0310

TaKaRa

## I. Description

This product is a staining kit for bone-related cells, in which chromogenic substrates for alkaline phosphatase, an enzyme marker of osteoblasts, and tartrate-resistant acid phosphatase, an enzyme marker of osteoclasts, are combined with the reagent for nuclear staining that provides visualization of multinucleated osteoclasts. Both acid and alkaline phosphatase activities in the cells can be stained simultaneously for comparison. Moreover, as the substrates are provided as premixed reagents, the substrate solutions can be prepared with ease.

## II. Introduction

Phosphatase is an enzyme that acts on aliphatic and aromatic phosphate esters and hydrolyzes them to release phosphates. As phosphatases, alkaline and acid phosphatases are known, which have the optimum pHs for their activities at alkaline and acid pHs, respectively. Acid phosphatases are present in a variety of cells and tissues, such as prostate, liver, kidney, spleen, erythrocyte, platelet and ostelclast.[1,2] In 1959, Burstone[3] reported that potent acid phosphatase activity is found in the osteoclasts and alkaline phosphatase activity is found in the osteoblasts. Following this report, various search reports have been made on phosphatase activities associated with osteocytes, and acid phosphatase activity of osteoclasts was shown to be of the type that retains phosphatase activity in the presence of tartrate (tartrate-resistant acid phosphatase: TRACP). TRACP activity is now a requisite for osteoclasts. In addition to osteoclasts, hairy cells among blood cells are also known to have TRACP activity. The type of acid phosphatases that is inactivated in the presence of tartrate is called tartrate-sensitive acid phosphatase (TSACP).

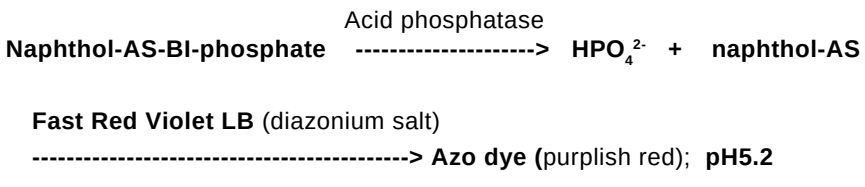
Alkaline phosphatases are membrane-bound glycoproteins and they are classified into four types, i.e. intestinal, placental, placenta-like and tissue non-specific types. Among the tissue non-specific type alkaline phosphatases, the bone-specific isozyme is called bone type alkaline phosphatase. This enzyme is bound to the membrane of osteoblasts and functions to enhance osteogenesis by degrading pyrophosphate that inhibits crystallization at the calcification site and by degrading organic phosphate esters to increase the inorganic phosphate concentration. Therefore, bone type alkaline phosphatase is particularly known as a marker of osteogenesis in the cycle of bone metabolism.

Since bone metabolism is composed of mutually balanced osteogenesis and bone resorption, simultaneous estimation with two enzyme makers is deemed useful.

## III. Principles[4]

(1) Principle for staining of acid phosphatases (tartrate-resistant and - sensitive acid phosphatases)[1,2]

For staining, cells fixed on microplate wells or slide glasses are used as samples. Two samples from the same origin are prepared, and the reaction is performed by adding the substrate solution for acid phosphatase (NABP/FRVLB) supplemented with tartrate to one of the samples and by adding the substrate solution without tartrate to the other sample. The tartrate-resistant acid phosphatase (TRACP) activity can be detected in the former sample and the total acid phosphatase activity including tartrate-resistant and-sensitive phosphatase activities can be detected in the latter. As shown below, azoic dye with purplish red color is generated in each sample in the presence of the enzyme, the object of the detection, through the reaction mediated by components contained in the substrate solution.



(2) Principle for staining of alkaline phosphatase

The substrate solution for alkaline phosphatase (BCIP/NBT) is added to cell samples fixed on microplate wells or slide glasses to carry out the reaction. As shown below, formazan dye with bluish purple color is generated in the presence of alkaline phosphatase, through the reaction mediated by components contained in the substrate solution.

**Bromo-Chloro-Indoly l phosphate** —(Alkaline phosphatase)—> **$HPO_4^{2-}$ + Br-Cl-Indol**

**Nitro Blue Tetrazolium Chloride** —> **Formazan dye** (bluish purple); **pH9.5**

## IV. Kit components

**[ Label No. ]**

[1] Fixation solution.....30 ml
Citrate buffer (pH 5.4) containing 60% acetone and 10% methanol
[2] Sodium tartrate.....4 ml
0.5 M sodium tartrate buffer (pH 5.2)
[3] Substrate for ACP (premixed).....3 x for 10 ml
NABP/FRVLB
[4] Substrate for ALP (premixed).....3 x for 10 ml
BCIP/NBT
[5] Nuclear stain.....10 ml
Methyl green (Containing acetic acid in the solvent.)

**Caution:** When handing [1] and [5], please refer to the safety cards included in the kit.

## V. Storage

-20°C
Each reagent should be stored at a suitable temperature after first use.

## VI. Preparation of reagents

This kit contains 3 x for 10 ml of premixed substrate for each enzyme. Total amount of each substrate solution is sufficient for the staining of approximately five 24-well culture plates. When performing double-staining, detection of the acid phosphatase activity has to precede the staining of alkaline phosphatase. Staining of alkaline phosphatase should be performed by replacing with the substrate for ALP after detection of acid phosphatase. Note that acid phosphatase will be partially inactivated if the staining is carried out in the inverse order.

[1] Fixation solution

This reagent should be used directly without any treatment. It can be stored at or below 4°C. Note that acetone (organic solvent) contained in the solution is inflammable. Crystal may generate during the storage. There is no problem in its quality, and it can be used directly.

[2] Sodium tartrate

This solution should be thawed at room temperature prior to use. It can be stored at 4°C or below after first use, but storage in the freezer is desirable since this solution is free of preservatives.

[3] Substrate for ACP (premixed)

The material in a vial should be dissolved in 10 ml of sterilized distilled water when used as substrate solution for the reaction of acid phosphatase. This solution looks slightly yellowish. For detection of the tartrate-resistant enzyme, 0.1 volume of Sodium tartrate [2] should be added to this solution.

NABP/FRVLB and optimized buffer are contained in this product. The substrate before dissolution and the substrate solution prepared must be stored in a frozen state at or below -20°C. The prepared substrate solution can be stored up to one month. Frozen solution may give a small quantity of precipitates. In such cases, the solution should be filtered through 0.22 µm membrane before use. The substrate solution containing tartrate also can be stored in a frozen state.

[4] Substrate for ALP (premixed)

One tablet of this component should be dissolved well in 10 ml of sterilized distilled water to be used as substrate solution for the reaction of alkaline phosphatase. Preparation of this solution should be started at least 20 minutes before use, because the dissolution will take time. The solution looks slightly yellowish. BCIP/NBT and optimized buffer are contained in this product. The substrate before dissolution and the substrate solution prepared must be stored in a frozen state at or below -20°C. The prepared substrate solution can be stored up to one month. Frozen solution may give a small quantity of precipitates. In such cases, the solution should be filtered through 0.22 µm membrane before use.

[5] Nuclear stain

This reagent should be used directly after thawing at room temperature. The thawed reagent should be stored at cool temperature (at or below 20°C). This reagent can be used for general nuclear staining or for examining whether osteoclasts are differentiated and fused into multinucleated cells.

## VII. Methods

< Cell fixation >

A. Fixation of cell samples cultured in 24-well plates (Exemplary protocol for fixation of bone marrow cells)

1. Culture the cell in the 24-well plate.
2. Remove and discard the culture supernatant and wash once with sterilized PBS.
3. Add 250 µl of Fixation solution [1] to each well, place the plate at room temperature for 5 minutes, and allow the cell to be fixed on the well.
4. Add about 2 ml of sterilized distilled water to each well to dilute the fixation solution, and then aspirate the solution. Add about 2 ml of sterilized distilled water again to wash the well, and remove and discard all the liquid from the well. Samples can be dried after this step and stored at or below -20°C for at least one week.

* Fixation solution contained in the kit is sufficient for fixation on five 24-well culture plates.

B. Fixation of cell samples cultured in 96-well plates
The procedure used for 24-well plates is employed in principle. For each well, 50 µl of Fixation solution and 250 µl of sterilized distilled water should be appropriate.

* Fixation solution contained in the kit is sufficient for fixation on five 96-well culture plates.

< Activity staining >
A. Single staining

1. Prepare the substrate solution for acid phosphatase or alkline phosphatase according to the instruction in section VI [Preparation of reagents]. For detection of tartrate-resistant enzyme, 0.1 volume of Sodium tartrate [2] should be added to the substrate solution for acid phosphatase.
2. Add the substrate solution to the well or slide glass on which the cells are fixed. Cover the plate with the lid on the slide glass with Parafilm to protect the sample from drying.

   Indication of the amount of substrate solution to be used:

| | |
|---|---|
| 24-well plate | 250 µl/well |
| 96-well plate | 50 µl/well |
| Slide glass | adequate amount |

3. Incubate at 37°C for 15 - 45 minutes for reaction.

   Note: The period for color formation will vary depending on the amount of phosphatase present in the cell.

4. Remove and discard the solution, and wash three times with sterilized distilled water to terminate the reaction.
5. Examine the sample by microscopy (Sterilized distilled water can be added for microscopic examination).

   Note: For storage of stained samples, glycerol or the like should be added to prevent dehydration.

B. Double staining

Note: When performing double-staining, detection of the acid phosphatase activity has to precede the staining of alkaline phosphatase. Staining of alkaline phosphatase should be performed by replacing with the substrate for ALP after detection of acid phosphatase. Note that acid phosphatase will be partially inactivated if the staining is carried out in the inverse order.

1. Prepare the substrate solution for acid phosphatase and perform the reaction according to the procedures 1-3 described above [A. Single staining].
2. Remove and discard the reaction solution and wash three times with sterilized distilled water.

3. Prepare the substrate solution for alkline phosphatase and perform the reaction according to the procedures 1-3 described above [A. Single staining].
4. Remove and discard the reaction solution and wash three times with sterilized distilled water.
5. Examine the sample by microscopy. (Sterilized distilled water can be added for microscopic examination.)
   Note: For storage of stained samples, glycerol or the like should be added to prevent dehydration.

< Nuclear staining >

**Note:** Staining with methyl green preceded by activity staining may hinder the visualization of activity staining. Examine by microscopy and confirm the results of activity staining prior to nuclear staining with methyl green.

1. Overlay the activity-stained well or slide glass with Nuclear stain [5].
2. Incubate the sample for staining at room temperature for 5 minutes.
3. Wash with sterilized distilled water, and examine by microscopy after adding glycerol or such to prevent dehydration.

## VIII. Application examples

**• Example 1**

Bone marrow cells collected from a 16-week old JW rabbit (male) were cultured in the presence of M-CSF and acitve vitamin $D_3$ and subjected to activity staining of tartrate-resistant acid phosphatase (TRACP) on day 6 of the culture. (Fig. 1)

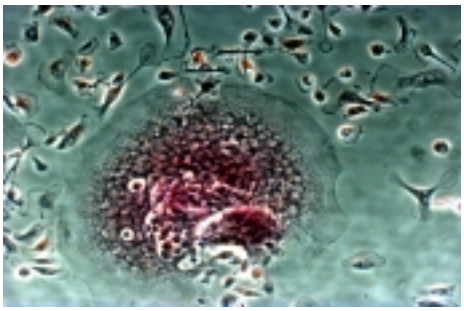


Fig. 1: Activity staining of TRACP in the cultured rabbit bone marrow cells

**• Example 2**

Bone marrow cells collected from 24-day old SD rats (female) were cultured in the presence of M-CSF and acitve vitamin $D_3$ and subjected to activity staining of alkaline phosphatase (ALP) on day 10 of the culture. (Fig. 2)

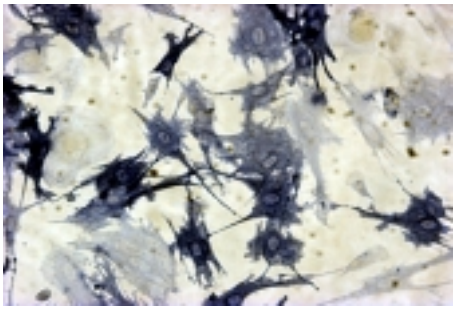


Fig. 2: Activity staining of ALP in the cultured rat bone marrow cells

• **Example 3**

Human bone marrow mononuclear cells (BIOWHITTAKER, INC.) were cultured in the presence of different additive substances, and activity staining of TRACP and ALP was carried out separately when the cells were differentiated (on day 9 of the culture) (Fig. 3).

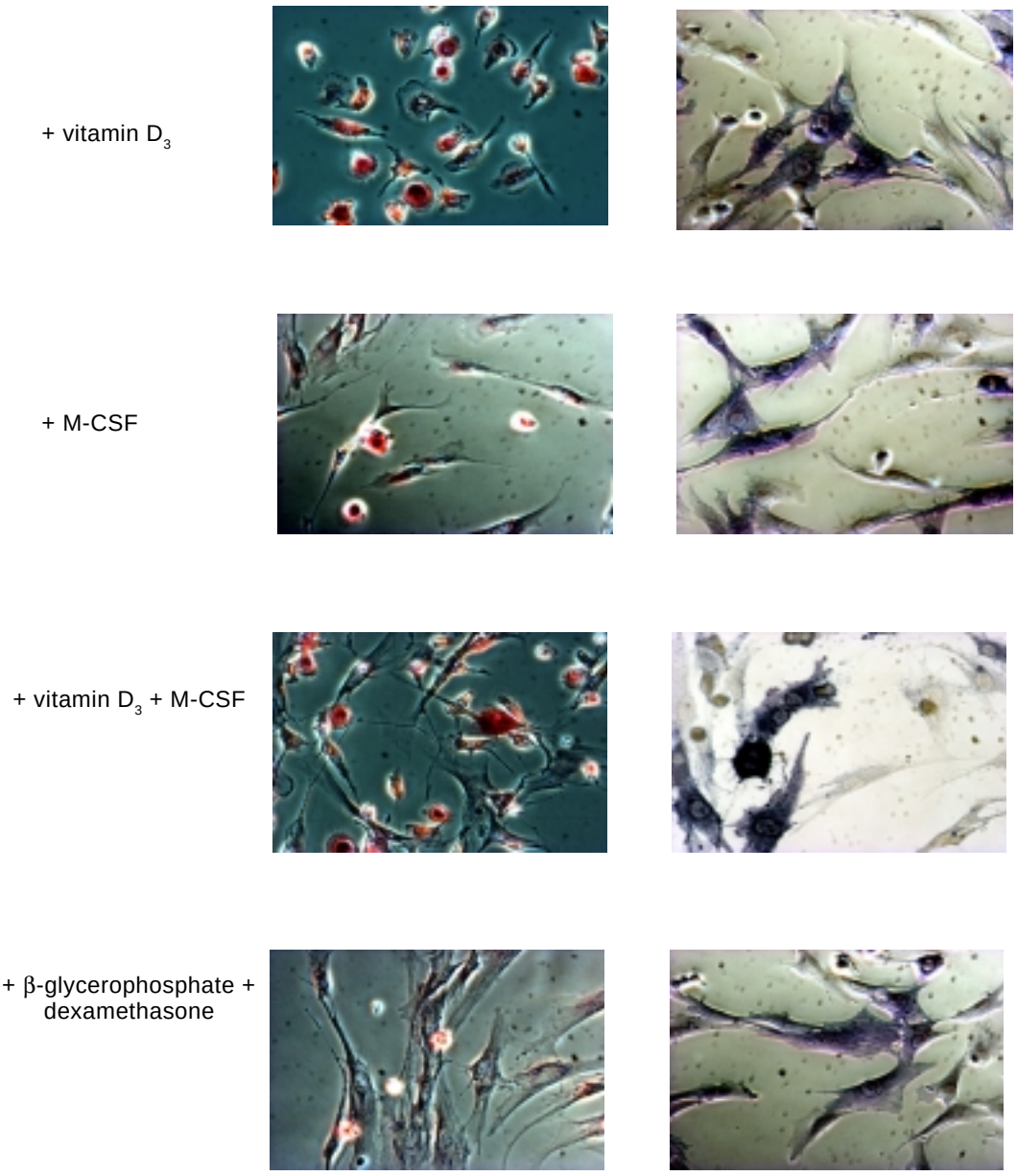



Fig 3: Activity staining of TRACP Activity staining of ALP

• **Example 4**

Rat bone marrow cells were cultured in the presence of M-CSF and active vitamin $D_3$, and differentiated. Double staining of TRACP and ALP activities was carried out on day 12 of the culture. (Fig. 4)

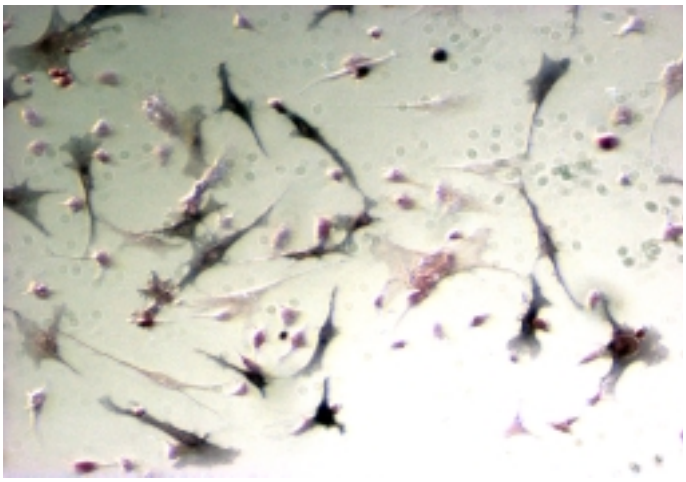


Fig. 4: Double staining of TRACP and ALP activities

## IX. Reference


1) Burstone, M. S. *et al.* (1958) *J. Natl. Cancer Inst.* **20**, 601-615.
2) Burstone, M. S. *et al.* (1958) *J. Natl. Cancer Inst.* **21**, 523-539.
3) Burstone, M. S. (1959) *J. Histochem. Cytochem.* **7**, 39-41.
4) Harlow and Lane (1988) Antibodies, A LABORATORY MANUAL, 406- 407.