Cat. # MK300

For Research Use

TaKaRa

TRACP & ALP double-stain Kit

Product Manual

From lot #AF2J200 onward, the Fixation solution in the kit is changed to Citrate buffer (pH 5.4) containing 45% acetone and 10% ethanol. There is no change to the protocol.

v201509

Table of Contents

I. Description

This product is a staining kit for bone-related cells. Chromogenic substrates for alkaline phosphatase, an enzyme marker of osteoblasts, and tartrate-resistant acid phosphatase, an enzyme marker of osteoclasts, are combined with a 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 easily prepared.

II. Introduction

Phosphatases are enzymes that act on aliphatic and aromatic phosphate esters and hydrolyze them to release phosphates. Alkaline and acid phosphatases have optimum pHs for activity 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 osteoclasts.[1, 2] In 1959, Burstone reported that potent acid phosphatase activity is found in osteoclasts and alkaline phosphatase activity is found in osteoblasts.[3] Following this report, various studies have shown phosphatase activity associated with osteocytes; the acid phosphatase activity of osteoclasts was shown to be retained in the presence of tartrate (tartrate-resistant acid phosphatase, TRACP or TRAP). TRACP activity is required for proper osteoclast function. In addition to osteoclasts, hairy cells among blood cells are also known to have TRACP activity. Acid phosphatases that are inactivated in the presence of tartrate are tartrate-sensitive acid phosphatases (TSACP or TSAP).

Alkaline phosphatases are membrane-bound glycoproteins that are classified into four types: intestinal, placental, placenta-like, and tissue non-specific. 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. Given this function, bone type alkaline phosphatase is used as a marker of osteogenesis during bone metabolism.

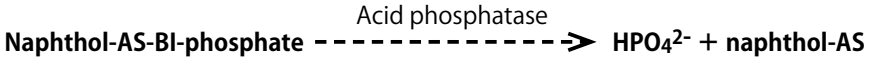
Since bone metabolism involves mutually balanced osteogenesis and bone resorption, simultaneous estimation with two enzyme makers is useful.

III. Principles [4]

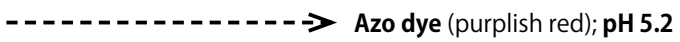
(1) Principle for staining of acid phosphatases (tartrate-resistant and-sensitive acid phosphatases) [1, 2]

For staining, cells fixed on microplate wells or slide glasses are used as the sample. 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 the substrate solution without tartrate is added to the other sample.

The tartrate-resistant acid phosphatase (TRACP) activity can be detected in the first sample and the total acid phosphatase activity including tartrate-resistant and-sensitive phosphatase activities can be detected in the second. As shown below, azoic dye with a purplish-red color is generated in each sample in the presence of the enzyme. This byproduct is detected by reaction mediated by components contained in the substrate solution.

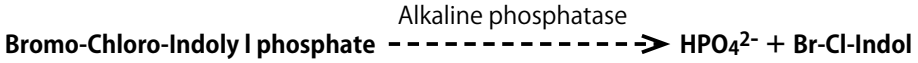


Fast Red Violet LB (diazonium salt)



(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. As shown below, formazan dye with a bluish-purple color is generated in the presence of alkaline phosphatase through a reaction mediated by components in the substrate solution.



Nitro Blue Tetrazolium Chloride

IV. Components

(1) Fixation solution Citrate buffer (pH 5.4) containing 45% acetone and 10 % ethanol	30 ml
(2) Sodium tartrate 0.5 M sodium tartrate buffer (pH 5.2)	4 ml
(3) Substrate for ACP (premixed) NABP/FRVLB	for 10 ml x 3
(4) Substrate for ALP (premixed) BCIP/NBT	for 10 ml x 3
(5) Nuclear stain Methyl green (Contains acetic acid in the solvent.)	10 ml

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

V. Storage

-20℃

Each reagent should be stored at a suitable temperature after first use.

VI. Preparation of Reagents

This kit contains for 10 ml x 3 of premixed substrate for each enzyme. The total amount of each substrate solution is sufficient for staining approximately five 24-well culture plates. When performing double-staining, detection of the acid phosphatase activity must precede the staining of alkaline phosphatase. Staining of alkaline phosphatase should be performed by replacing 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℃. Note that acetone (organic solvent) contained in the solution is inflammable.

＊ Crystals may form during storage, however the solution can be used directly.

(2) Sodium tartrate

This solution should be thawed at room temperature prior to use. It can be stored at 4℃ or below after first use, but storage in the freezer is desirable since this solution does not contain preservatives.

(3) Substrate for ACP (premixed)

The material in the vial should be dissolved in 10 ml of sterile distilled water when used as the substrate solution for the reaction of acid phosphatase. The prepared solution will have a slightly yellow color. For detection of the tartrate-resistant enzyme, 0.1 volume of Sodium tartrate (2) should be added to this solution.
NABP/FRVLB and an optimized buffer are contained in this product. Before substrate solution preparation, store frozen at or below -20℃. The prepared substrate solution can be stored up to one month. The frozen solution may form a small amount of precipitate. In such a case, the solution should be filtered through a 0.22-μm membrane before use. The substrate solution containing tartrate also can be stored in frozen.

(4) Substrate for ALP (premixed)

One tablet of this component should be dissolved well in 10 ml of sterile distilled water to be used as the substrate solution for the reaction of alkaline phosphatase. Preparation of this solution should be started at least 20 minutes before use. The prepared solution will have a slightly yellow color. BCIP/NBT and an optimized buffer are contained in this product. Before substrate solution preparation, store frozen at or below -20℃. The prepared substrate solution can be stored up to one month. The frozen solution may contain a small amount of precipitate. In such a case, the solution should be filtered through a 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 or below 20℃. 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 (example protocol for fixation of bone marrow cells)

1. Culture cells in a 24-well plate.
2. Remove and discard the culture supernatant and wash once with sterile PBS.
3. Add 250 μl of Fixation solution (1) to each well, place the plate at room temperature for 5 minutes, and allow the cells to fix in the well.
4. Add 2 ml of sterile distilled water to each well to dilute the fixation solution, and then aspirate the solution. Add 2 ml of sterile 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℃ for at least one week.

   ＊ The amount of fixation solution in the kit is sufficient for fixation of five 24-well culture plates.

B. Fixation of cell samples cultured in 96-well plates
Follow the procedure used for 24-well plates but use 50 μl of Fixation solution and 250 μl of sterile distilled water for washing.

   ＊ The amount of fixation solution in the kit is sufficient for fixation of five 96-well culture plates.

< Activity staining >

A. Single staining

1. Prepare the substrate solution for acid phosphatase or alkaline phosphatase according to the instructions in section VI [Preparation of Reagents]. For detection of tartrate-resistant enzyme, add 0.1 volume of Sodium tartrate (2) 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 or the slide with Parafilm to prevent the sample from drying.

   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℃ for 15 - 45 minutes.

   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 sterile distilled water to stop the reaction.
5. Examine the sample by microscopy. (Sterile 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 acid phosphatase activity must 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 sterile distilled water.
3. Prepare the substrate solution for alkaline 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 sterile distilled water.
5. Examine the sample by microscopy. (Sterile 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 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 at room temperature for 5 minutes.
3. Wash with sterile distilled water, and examine by microscopy after adding glycerol or the like 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 active vitamin D_3. Cells were stained for tartrate-resistant acid phosphatase (TRACP) activity on day 6 of the culture (Figure 1).

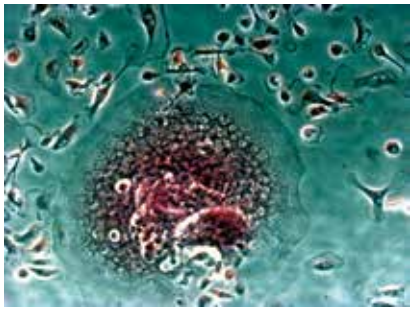


Figure 1. Activity staining of TRACP in cultured rabbit bone marrow cells.

・Example 2

Bone marrow cells collected from 24-day old SD rats (female) were cultured in the presence of M-CSF and active vitamin D_3. Cells were stained for alkaline phosphatase (ALP) activity on day 10 of culture (Figure 2).

Figure 2. Activity staining of ALP in cultured rat bone marrow cells.

・**Example 3**

Human bone marrow mononuclear cells (BIOWHITTAKER, INC.) were cultured in the presence of different additive substances. Cells were stained for TRACP and ALP activity separately when the cells were differentiated (day 9 of culture) (Figure 3).

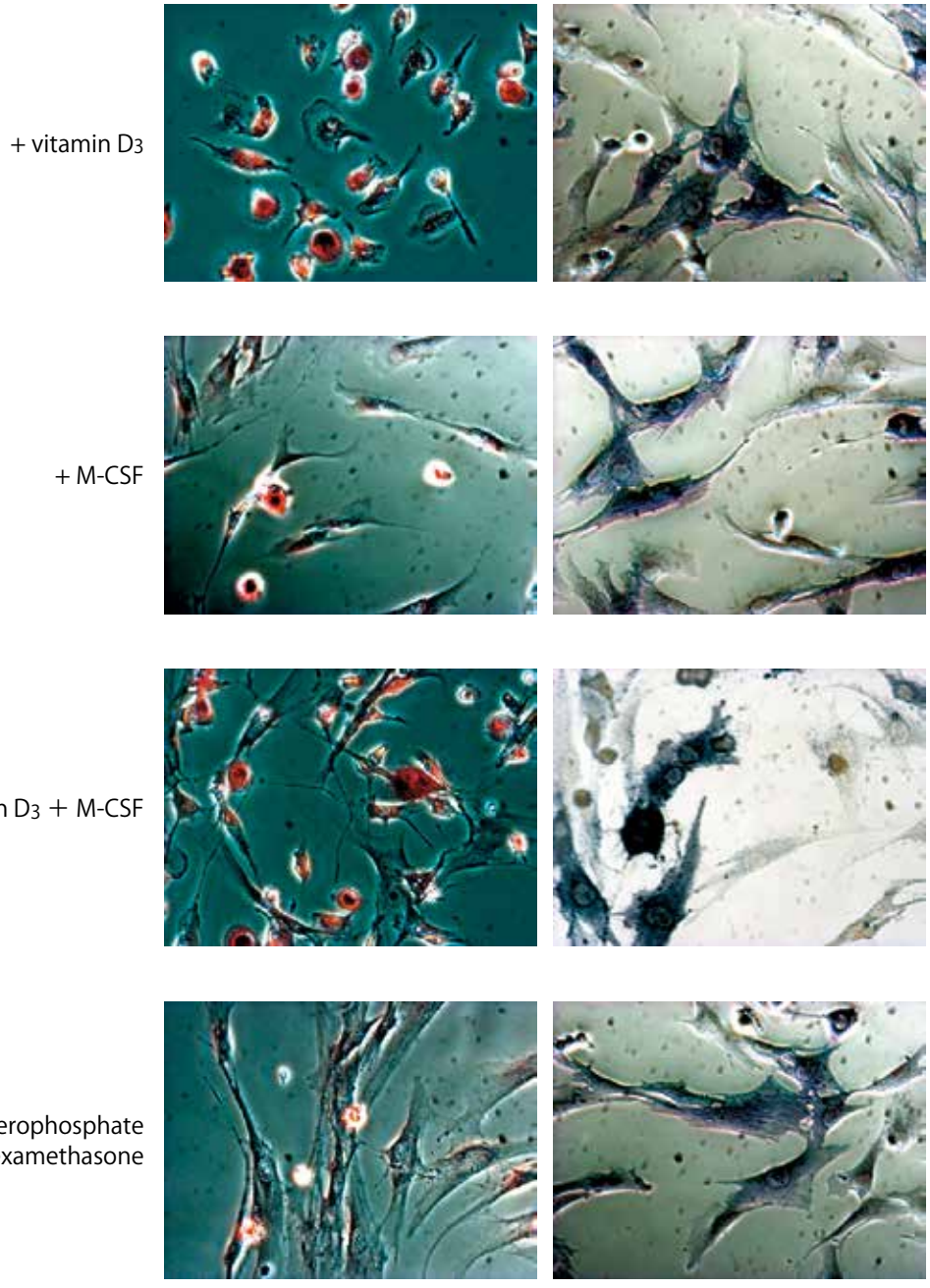


Figure 3. Activity staining of TRACP and ALP.

・**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 activity was carried out on day 12 of culture (Figure 4).

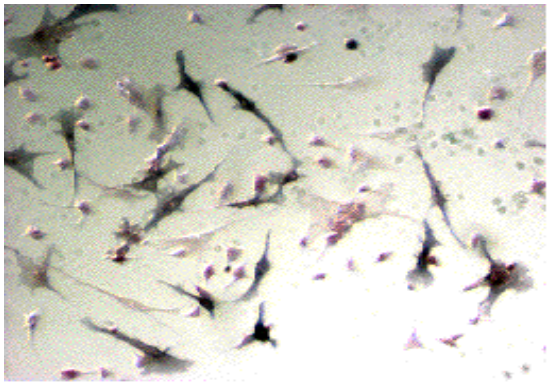


Figure 4. Double staining of TRACP and ALP activity.

・**Example 5**

The activity staining of TRACP and ALP was carried out separately using newborn mouse (1 day) frozen tissue sections. 250 μl of Fixation solution was added to 2 frozen slides, and the slides were placed at room temperature for 5 minutes for fixation. Slides were washed well with sterile distilled water and extra water was removed with a paper towel.
250 μl of substrate for AP was added on one slide and 250 μl of substrate for TRACP was added on the other slide. The slides were incubated at 37℃ for 45 minutes. The substrate solution was removed. The slides were washed with sterile distilled water and extra water was removed. Slides were observed with a microscope.
The results using frozen slides after storage for 6 months at -80℃ was almost same as the results using fresh frozen slides (Figure 5).

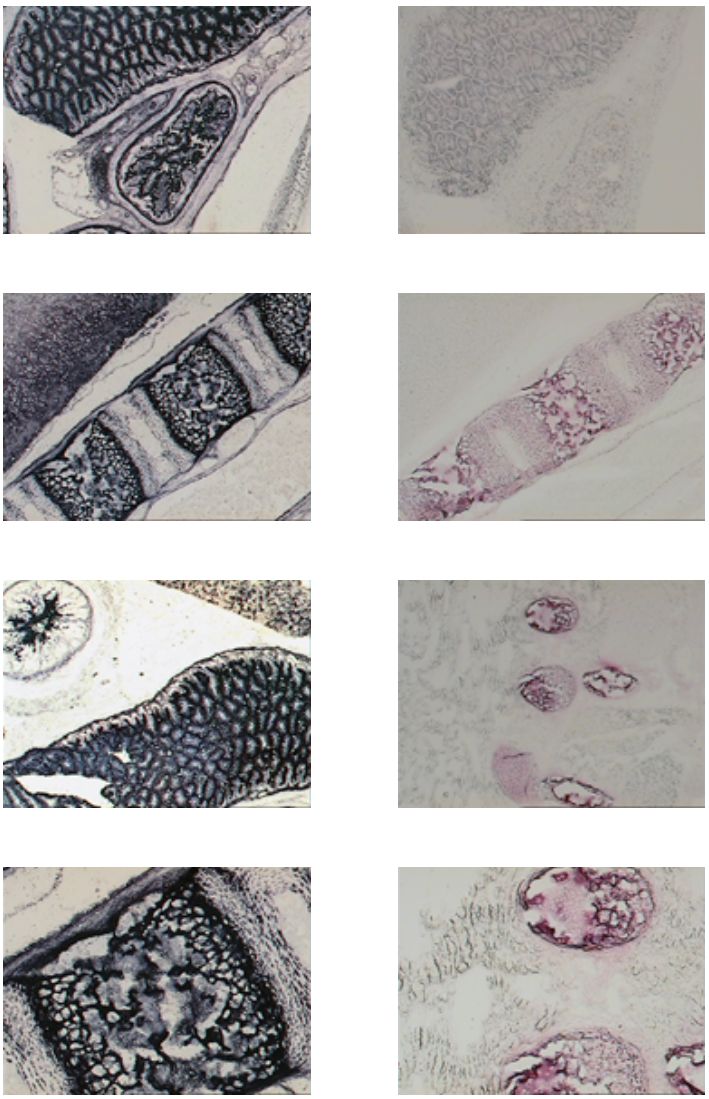


Figure 5. Activity staining of ALP & TRACP staining in the newborn mouse (1 day) frozen tissue.

IX. References

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.