〈For Research Use Only〉

Code No. 294-67001 (for 60 tests)

Wako

(For enzyme activity staining of tartrate- resistant acid phosphatase and alkaline phosphatase)

TRAP/ALP Stain Kit

for Pathology Research

〔Introduction〕

Normal bone metabolism is based on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. When the balance is disturbed and bone resorption by osteoclasts is abnormally increased, bone mass is reduced leading to osteoporosis.

Therefore, various researches have been carried out to understand the mechanism of osteoclast and osteoblast metabolism, and to utilize this knowledge for the treatment of the diseases and for the development of effective drugs.

Today, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) are known as marker enzymes for osteoblasts and osteoclasts, respectively, and these enzymes are used as one of the markers showing the presence of osteoblasts and osteoclasts in tissue sections or cultured cells.

This kit enables you to examine the state of differentiation of bone cells and the cell distribution in bone tissues by observation of the stained images of osteoblasts and osteoclasts in the tissues and cultured cells using ALP/TRAP enzyme activities in the tissue sections and cultured cells.

〔Features〕

(1) Just mix two solutions for the preparation of the colorimetric substrate solution required for staining of TRAP activity (three solutions in case of the evaluation of TRAP).
(2) Simple steps for staining of ALP activity by using ALP substrate soln. (premixed).
(3) Double staining can be performed, reddish-purple for active region of acid phosphatase and bluish brown for alkaline phosphatase.
(4) Usable in cultured cells and bone tissue sections (GMA resin embedded section).

〔Kit Contents〕

Reagent *	Pkg. Size	Stain number	
		bone tissue sections	cell culture in 24- or 96- well multiplate
(1) Sodium tartrate soln. (× 10)	1 × 3 mL	60 pieces (0.5mL/slide)	24-well: 5 plates (120 wells) 96-well: 6 plates (576 wells)
(2) TRAP substrate soln. A **	1 × 30 mL		
(3) TRAP substrate Soln. B	1 × 0.3 mL		
(4) Nuclear stain soln. ***	1 × 10 mL	20 pieces (0.5mL/slide)	24-well: 40 wells 96-well: 192 wells
(5) ALP substrate soln. (premixed)	1 × 30 mL	60 pieces (0.5mL/slide)	24-well: 5 plates (120 wells) 96-well: 6 plates (576 wells)

(Note)

* : Please thaw each reagent at room temperature (RT). Do not leave thawed reagents at RT for a long time.

** : Repeated freeze-thaw cycles of Reagent (2) cause some precipitation in TRAP substrate soln. A. In that case, use the solution after filtration through approx. 0.2 μm filter.

*** : Since the amount of the Reanget (4) is corresponded to one-third of amount of other stain solutions, please use this solution only when it is necessary.

〔Storage〕

[**Before opening the kit**] Store at -20℃.

[**After opening the kit**]

1. Staining of ALP and TRAP enzyme activities in bone tissue section

[**Reagents and apparatuses to be prepared**]

- DDW (distilled deionized water)
- 0.1M AMPD-HCl buffer, pH 9.4 (when ALP stain is performed after TRAP stain on a section)
  AMPD: 2-Amino-2-methyl-1,3-propanediol
  (Wako Cat. No. 015-06411 (100 g))
- Optical microscope furnished with a camera
- Moist chamber
- Micropipette
- Microtubes
- Coplin-staining jars (for washing slides)
- Xylene (Wako Cat. No. 244-00086 (500 mL))
- Mounting reagent:
  Softmount® (Wako Cat. No. 199-11311 (250 mL)) or Malinol
- Coverslips
- Cover glasses

Staining example of GMA embedded thin section sample of non-decalcified bone

[**Precautions before operation**]

- In the case that double staining of TRAP stain and ALP stain is planned, first perform the TRAP stain, followed by microscopic observation and ALP stain.
- As TRAP stain and ALP stain images are possibly difficult to be observed due to nuclear stain, microscopic observation prior to nuclear stain is recommended.

[**Procedure**]

[Preparation of sample]

(1) GMA embedded thin section samples of non-decalcified bone (2 μm thick) applied to silan coated slides
(2) Wash samples with water.

[Preparation of *TRAP stain soln*.]

(3) Prepare *TRAP stain soln* in the following ratio just before use. (Do not store the solution after preparation.)

〈*TRAP stain soln.*〉

Sodium tartrate soln. (× 10)	1 mL
TRAP substrate soln. A	9 mL
TRAP substrate soln. B	0.1 mL

[TRAP stain]

(4) Apply 0.5mL of *TRAP stain soln*. on each section in a moist chamber at room temperature and allow to stand for 30 minutes at RT.
  - Colorimetric time varies according to the amount and activity of TRAP in the samples. Stop the reaction in the appropriate state while observation is carried out microscopically. Note that long reaction time may cause precipitation of the reactant and nonspecific reactions to various cells besides osteoclasts.

(5) Add a sufficient amount of distilled water to soak the sections in 3 Coplin-staining jars and wash the sections in these jars for 1 min. each.
(6) Add a sufficient amount of 0.1M AMPD-HCl buffer (pH 9.4) to soak the sections in each Coplin-staining jar, soak the sections and allow to stand for 10 minutes.
(7) Remove excess moisture on the slides.

[ALP stain]

(8) Apply 0.5mL of *ALP substrate soln. (premixed)* on each section and allow to stand for 30 minutes in a moist chamber at room temperature.
* Colorimetric time varies according to the amount and activity of alkaline phosphatase in the samples.
Stop the reaction in the appropriate state while observation is carried out microscopically.
(9) Add a sufficient amount of distilled water to soak the sections in 3 Coplin-staining jars and wash the sections in these jars for 1 min. each.

[Nuclear stain]

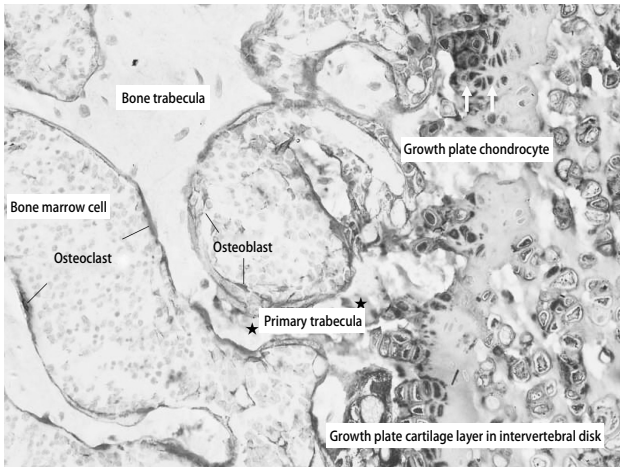
(10) Add a sufficient amount of distilled water to soak the sections in a Coplin-staining jar and apply 0.5 mL of *Nuclear stain soln.* on the sections.
After 4~5 seconds, immediately wash one section by moving them up and down in distilled water.
(When more than one section is stained, it is recommended to repeat the staining and washing steps one by one as immediate washing after the application of *Nuclear stain solution* is necessary for the procedures for nuclear stain.)
(11) Add a sufficient amount of distilled water to soak the sections in each Coplin-staining jar and wash the sections.

[Observation]

(12) Dry the sections on a heater plate at 37℃.
(13) Add a sufficient amount of xylene to soak the sections in each Coplin-staining jar and soak the sections.
(14) Mount the sections using mounting agents such as Softmount and Malinol and perform observation.

Stained images and staining method are provided by Dr. Hajime Kawahara.

Non-decalcified mouse spinal bone GMA resin embedded 2μm-section



2. Staining of ALP and TRAP enzyme activities in cultured cell

[**Reagents and apparatuses to be prepared**]

- DDW (distilled deionized water)
- Wash buffer (Dulbecco's Phosphate buffered saline: D-PBS (-) (Wako Cat. No. 045-29795 (500 mL))
- Fixative*: Dilute 37% Formaldehyde soln. to 1/10 with cold PBS at 2~10℃ and place on ice. (*: Prepare the fixative just before use.) Formaldehyde soln. (Wako Cat. No. 061-00416 (500 mL))
- Permeate [Ethanol / Acetone (50 : 50 v/v)] Ethanol (99.5) (Wako Cat. No. 057-00456 (500 mL)), Acetone (Wako Cat. No. 016-00346 (500 mL))
- 37℃ Incubator
- Optical microscope furnished with a camera
- Micropipette
- Microtubes

Staining example of cells cultured in 24-well plate

[**Precautions before operation**]

- In the case that double staining of TRAP stain and ALP stain is planned, first perform the TRAP stain, followed by microscopic observation and ALP stain.
- As TRAP stain and ALP stain images are possibly difficult to be observed due to nuclear stain, microscopic observation prior to nuclear stain is recommended.
- The methods for cell fixation and permeation are not limited to these procedures.
  If done properly, other fixation and permeation techniques suitable for the samples used can be employed.

[**Procedure**]

Culture cells in a 24-well plate.

[Fixation of cell]

(1) Immediately after removal of the culture media, add 3 mL of PBS and rinse the cells gently.
(2) Remove the PBS added, slowly add 500μL of pre-cold fixative so as not to remove the cells and allow to stand on ice for 10 minutes. (Carry out the following steps at room temperature.)
(3) Dilute the fixative by adding 2 mL of PBS to the wells containing the fixative.
(4) Remove the solution in the wells and add 2 mL of PBS. Repeat this step 2 additional times.

[Permeation]

(5) Remove PBS, add 500μL of Ethanol/Acetone (50 : 50 v/v) and incubate for 1 minute at -30 ~ -20℃.
(6) Gently remove the solution in the wells and add 2 mL of PBS. Repeat this step 2 additional times.

[Preparation of *TRAP stain soln.*]

Just before use, mix each reagent in the following ratio according to the number of samples. (Do not store the solution after preparation.)

〈*TRAP stain soln.*〉

Sodium tartrate soln.（× 10）	100 μL
TRAP substrate soln. A	900 μL
TRAP substrate soln. B	10 μL

24-well multiplate: 250 μL/well
96-well multiplate: 50 μL/well
Slide: 500 μL/piece

［TRAP stain］

(7) Add 250 μL of prepared *TRAP stain soln.* in each well, cover the plate to prevent them from drying and allow them to react at 37℃ in an incubator for 15~45 minutes.
- Colorimetric time varies according to the amount and activity of tartrate-resistant acid phosphatase in the samples. Stop the reaction in the appropriate state while observation is carried out microscopically.
  Note that long reaction time may cause precipitation of the reactant and nonspecific reactions to various cells besides osteoclasts.

(8) Dilute the reaction solution by adding 2 mL of DDW in the wells.
(9) Remove the solution in the wells and add 2 mL of DDW. Repeat this step 2 additional times.
(10) When necessary, remove excess moisture in the wells and perform ALP stain or nuclear stain.

［ALP stain］

(11) Add 250 μL of *ALP substrate soln.（premixed）* in each well, cover the plate to prevent them from drying and allow them to react at 37℃ in an incubator for 15~45 minutes.
- Colorimetric time varies according to the amount and activity of alkaline phosphatase in the samples. Stop the reaction in the appropriate state while observation is carried out microscopically.

(12) Dilute the reaction solution by adding 2 mL of DDW in the wells.
(13) Remove the solution in the wells and add again 2 mL of DDW. Repeat this step 3 additional times.
(14) When necessary, remove excess moisture in the wells and perform nuclear stain.

［Nuclear stain］

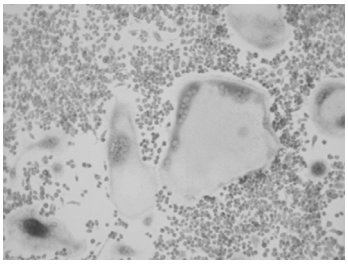
(15) Add 250 μL of *Nuclear stain soln.* in the wells and stain for 5~15 minutes at room temperature. (The time for staining is provided only as a guide. Employ the time suitable for the sample used.)
(16) Add 2 mL of DDW to dilute *Nuclear stain soln.* in the wells.
(17) Remove the solution in the wells and add again 2 mL of DDW. Repeat this step 2 additional times.
Repeat this step until the DDW added to the wells becomes clear.

［Observation］

(18) If the samples get dry, drop some DDW to them and perform observation.

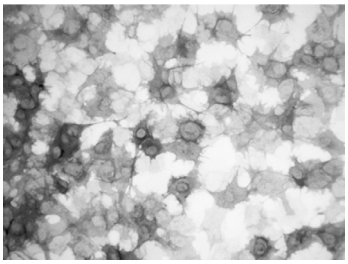
［**Example of staining results**］

Figure 1. Enzyme activity staining of tartrate-resistant acid phosphatase (TRAP) in RAW264 cells



RAW264 cells (cell line derived from Mouse leukemic monocyte, differentiate into osteoclast-like cells) were cultured in the presence of sRANKL. On day 6 of culture, the cells were fixed in neutral formalin and treated with Ethanol/Acetone (50 : 50 v/v) for permeation, and then TRAP stain was performed.
TRAP-positive, multinuclear osteoclast-like cells were observed.

Figure 2. Enzyme activity staining of alkaline phosphatase (ALP) in MC3T3-E1 cells



MC3T3-E1 cells (cell line derived from Mouse calvaria, differentiate into osteoblasts) were cultured in the presence of BMP-2. On day 7 of culture, the cells were treated with Ethanol/Acetone (50 : 50 v/v) for permeation and then ALP stain was performed.

〔**Expiration Date**〕

24 months after the manufacturing (Indicated on each label as "使用期限")