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I. Description

TRACP&ALP Assay Kit allows for simultaneous detection of 2 enzymes which are involved in bone metabolism. TRACP which is an osteoclast enzyme marker and ALP an osteoblast enzyme marker. TRACP&ALP Assay Kit has been designed for simple and quick detection of ACP (Acid phosphatase) and ALP (Alkaline phosphatase) through the use of pNPP (*p*-nitro-phenyl phosphate) substrate. The addition of tartaric acid into the ACP assay, allows for the detection of TRACP (tartrate-resistant acid phosphatase) activity. Since this kit utilizes an aqueous substrate, it enables quick activity quantification by measuring the absorbance of the reactant. In addition to this kit, TRACP & ALP double-staining Kit (Cat.# MK300) is also available using a non-soluble substrate. The appropriate kit can be selected depending on assay interest.

II. Introduction

Phosphatase is an enzyme which hydrolyzes aliphatic and aromatic phosphate esters resulting in the release phosphates. The optimum pHs for alkaline and acid phosphatases activity are at alkaline and acid pHs, respectively. Acid phosphatases (ACP) are present in a variety of cells and tissues, such as prostate, liver, kidney, spleen, erythrocyte, platelet and ostelclast.
1,2) In 1959, Burstone3) reported that potent acid phosphatase activity is found in the osteoclasts and alkaline phosphatase activity is found in the osteoblasts. Following this report, various research reports have been made on phosphatase activities associated with osteocytes. In addition to osteoclasts, hairy cells among blood cells are also known to have TRACP activity. The 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) . The type of acid phosphatases that is inactivated in the presence of tartrate is called tartrate-sensitive acid phosphatase (TSACP) . TRACP activity is now a requisite for osteoclasts.
Alkaline phosphatases (ALP) are membrane-bound glycoproteins and 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 pyrophosphates. Pyrophosphates, inhibits crystallization at the calcification site and degrads organic phosphate esters to increase the inorganic phosphate concentration. Therefore, bone type alkaline phosphatase is known as a marker of osteogenesis in bone cycle metabolism.

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

III. Kit components (for 500 reactions)

1) pNPP (*p*-nitro-phenyl phosphate) substrate [pNPP substrate] 24 mg x 5 vials
   pNPP substrate is supplied sufficient to prepare 25 ml of substrate solution which allows 500 assays in 50 μl/well in a 96-well plate.
2) Extraction solution 11 ml x 2 vials.
   Physiological saline including 1 % NP-40
   For solubilization of suspension and adherent cells
3) Sodium tartrate solution 4 ml
   0.5 M sodium tartrate buffer, pH5.2
   TRACP : Used for the detection of osteoclast marker through the addition to the substrate solution

4) Buffer for ACP 30 ml
0.5 M Sodium acetate, pH5.2
5) Buffer for ALP 30 ml
0.2 M Tris-HCl, pH9.5, 1 mM $MgCl_2$
＊The concentration of Tris-HCl has changed for keeping pH stability from lot. 011.
6) Microplate (96-well) 1 plate
Used in sample dilution or container for reaction
The plate is reusable after soaking in 1 % sodium hypochlorite solution overnight.

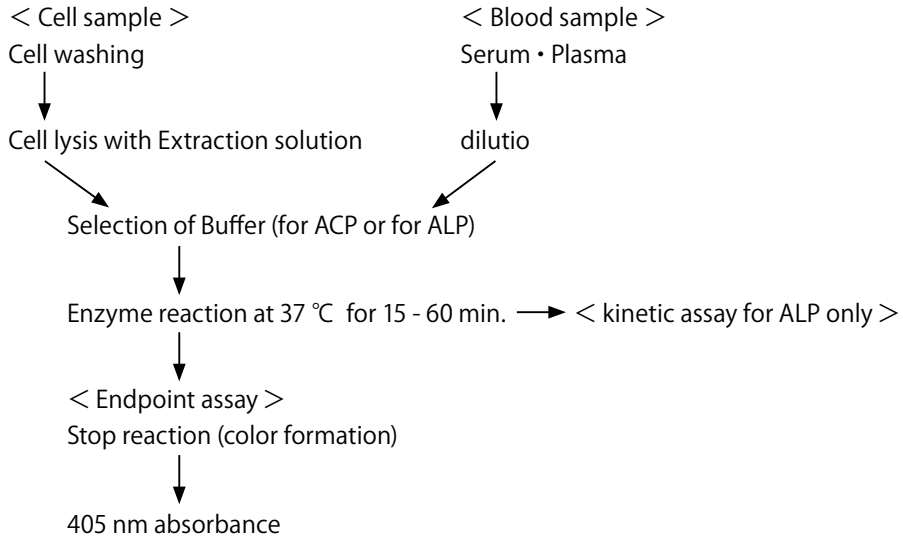
Reagent required but not supplied in the kit
Stop Solution：0.9 N NaOH ＊
Prior to starting the assay, a customer is required to prepare Stop Solution by himself.
＊：This solution is corrosive. It may cause inflammation when it contacts with skin. When it comes to contact with hands or mucous membrane, immediately wash away with large amount of water and follow instructions provided by doctors.

IV. Storage 4 ℃

V. Preparation of Reagents

1) All reagents should be brought to room temperature before use.
2) Preparation of substrate solution
Dissolve 1 vial (24 mg) of [1] pNPP (*p*-nitro-phenyl phosphate) substrate in 5 ml of the enzyme buffer to be assayed ([4] or [5]) , and use this as the substrate solution (substrate concentration：12.5 mM) . When used for tartrate-resistant acid phosphatase (TRACP) , sodium tartrate solution [3] should be added at 1/10 the volume of the substrate solution. In both cases, the prepared reagents should be stored at −20 ℃ , and used within 1 week.

VI. Outline of Procedure



VII. Procedure

(1) For adherent cells cultured in a 96-well plate

1. Remove the culture supernatant with an aspirator, etc.

2. Add 200 μl of physiological saline to each well, wash once, then discard the liquid.
   Note 1) If the cells are detached, this process should be omitted, and a blank well containing medium only should be set to correct the observed data.
   Note 2) Phosphate buffers should not be used in washing because they might inhibit the enzyme reaction.

3. Pipetting lightly, add 5 - 50 μl of Extraction solution [2] to each well.
   Note 1) The amount of Extraction solution added can be changed depending on the number of cells. Approximately, 50 μl should be used in the case of 10^4 cells.
   Note 2) Sample should be diluted with Extraction solution when the sample concentration is high. (If extraction solution is insufficient, physiological saline can be substituted.)
   Part of the diluted sample (5 - 50 μl) should be used in subsequent reactions.
   Note 3) Lysis of the cells should be confirmed by microscopic observation.

4. Add 50 μl of the substrate solution for measurement (see III. Preparation of Reagents) to each well and react at 37 ℃ for 15 - 60 minutes.
   Note 1) Reaction time can be set arbitrarily.
   Note 2) The volume ratio of the cell lysis sample (A) and substrate solution (B) should be at maximum A : B = 1 : 1. The cell lysis sample A should be set to be less than the substrate solution.
   Note 3) If high enzyme activity is expected, it is recommended to prepare a diluted cell lysis solution for measurement in the provided 96-well plate.

5. Add 50 μl of stop solution (0.9N NaOH) to each well and measure the absorbance at 405 nm after color formation.
   Note) In the case of acid phosphatase, color formation starts with the addition of stop solution.

(Reference) Cell lysis sample obtained using the provided Extraction solution [2] can be used for other measurements besides the target enzyme activity with this kit, such as protein quantification and kinase activities.

(2) For suspension cells cultured in a Petri dish

1. Collect culture medium with suspension cells into a tube and recover the cells by centrifugation.
   Wash the cells once with physiological saline and precipitate them by centrifuging again.

2. Add 50 - 500 μl * of Extraction solution [2] to each, and lyse the cells by pipetting.
   * : The amount of extraction solution added can be changed depending on the number of cells.
   Roughly, 50 μl should be used for 10^5 cells and 500 μl for 10^7 cells.

3. Dilute the cell lysate step-wise with physiological saline and add the prescribed amount (in the range of 5 - 50 μl) to each well of the provided 96-well plate.

Following this step, the procedure is the same as in (1) 4. - 5.

(3) For blood samples

1. Add 5 - 50 μl of blood samples (serum, plasma) to each well.
   Note 1) Sample should be diluted with Extraction solution [2].

Following this step, the procedure is the same as in (1) 4. - 5.

VIII. Application Examples

(1) Measurement of Alkaline Phosphatase (ALP)

This kit was used to measure ALP activity in cultured cells derived from human small intestine. At the same time, ALP staining was conducted using the fixation solution and insoluble substrate BCIP/NBT included in the TRACP & ALP Double-stain Kit (Cat. # MK300) .

＜ Method ＞

Suspension of human small intestine cells (Intestine 407) that were serially cultured in 10 % FCS/RPMI1640 medium was added to the first row of a 96-well plate. The plate was prepared up to the 11th row by 2-fold dilution (No. of cells：1 x 10^4 in row 1, 5 x 10^3 in row 2, and thereafter decreasing by half; row 12 was blank) , then further cultured for 2 days (volume：100 μl/well) . After culturing, ALP staining was conducted with insoluble substrate BCIP/NBT on columns A and B, and ALP activity was measured in columns C - F using this kit and Procedure (1) . Due to solubilization, the volume of extraction solution added to columns C - F varied from 5 to 50 μl (5 μl in column C, 10 μl in column D, 20 μl in column E, 50 μl in column F) . Enzymes reactions were conducted for 60 minutes at 37 ℃ .

＜ Results ＞

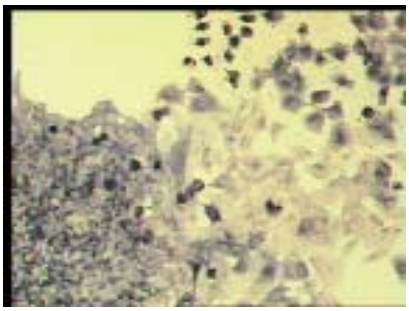
Alkaline phosphatase activity is extremely strong in the small intestine cells, and the correlation of activities and number of cells were confirmed using this kit. The detection sensitivity for human small intestine cells (Intestine 407) under the conditions of this activity measurement was 10 cells/assay. It was confirmed that even though the volume of extraction solution added varied in the range of 5 to 50 μl/well, there was little influence on the enzyme reactions. There is no problem in varying the volume of extraction solution within a fixed range if the cells are solubilized at a sufficient volume.

A405 Plate Column		Plate row	1	2	3	4	5	6
		No. of cells (cells/well)	1×10^4	5×10^3	2.5×10^3	1.25×10^3	6.25×10^2	3.13×10^2
	C	5 μl*	2.275	2.271	1.732	0.920	0.581	0.329
	D	10 μl*	2.298	2.263	1.957	1.082	0.719	0.373
	E	20 μl*	2.289	2.273	1.901	1.101	0.617	0.369
	F	50 μl*	2.236	2.214	1.964	1.287	0.744	0.341

＊：Volume of extraction solution added

7	8	9	10	11	12
1.56×10^2	7.8×10^1	3.9×10^2	20	10	0
0.239	0.180	0.160	0.158	0.154	0.145
0.252	0.200	0.176	0.154	0.158	0.148
0.228	0.192	0.168	0.162	0.152	0.145
0.234	0.175	0.161	0.152	0.157	0.145

The staining of ALP was carried out as follows.
1 x 10^4 cells / well



(2) Measurement of Tartrate-resistant Acid Phosphatase (TRACP)

＜Method＞

Bone marrow cells derived from the femur of 16-week-old rabbit were suspended in 10 % FCS/RPMI1640 medium. This cell suspension was added to the first row of a 96-well plate, and the plate was prepared up to the 11th row by 2-fold dilution of the same culture (No. of cells：7 x 10^5 in row 1, 3.5 x 10^5 in row 2, and thereafter decreasing by half in the same manner; row 12 was blank) , at a volume of 100 μl/well, and culturing was initiated. A further 100 μl of medium was added because adhesive cells had become prominent after 3 days, and culturing was continued. Eight days after seeding, TRACP activity was measured using the kit according to procedure (1) .

The degree of substrate color formation was compared when substrate solution was directly added to the adhesive cells without extraction procedure and when they were solubilated with 25 μl of extraction solution. 50 μl of substrate solution for TRACP was used, and enzyme reactions were conducted for 60 minutes at 37 ℃ .

＜Results＞
The bone marrow cell culture produces numbers of naturally differentiated osteoclast-like TRACP-positive cells, and their TRACP activity could be measured using this kit. The table below shows the numbers of cells at the start of culturing and TRACP activity as absorbency at 405 nm. Similar levels of activity were detected in the live cells to which substrate was directly added without extraction (final substrate concentration 12.5 mM) and in cells on which extraction was conducted (final substrate concentration 4.1 mM) , and results that correlated with the numbers of cells were obtained.

No. of cells (cells/well)	7×10^5	3.5×10^5	1.75×10^5	8.76×10^4	4.38×10^4	2.19×10^4
Substrate directly added	1.701	1.471	0.747	0.295	0.156	0.120
Extracted	1.754	1.880	0.781	0.246	0.150	0.125

1.1×10^3	5.5×10^2	2.25×10^2	112	56	0
0.105	0.100	0.104	0.102	0.100	0.100
0.112	0.111	0.112	0.107	0.105	0.108

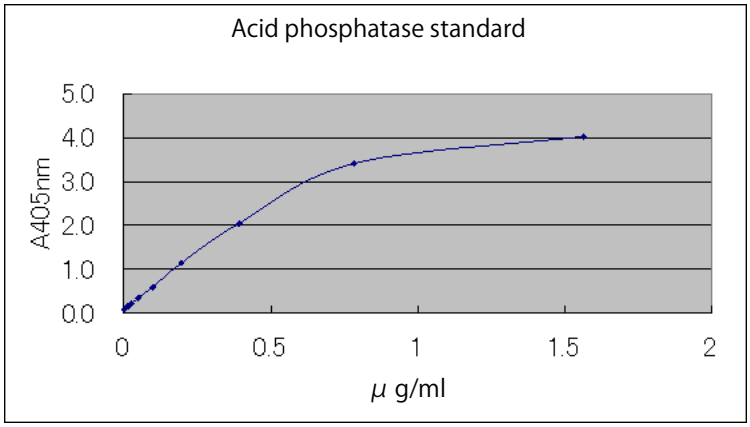
(3) Measurement with Acid Phosphatase (ACP) Standards
A standard curve was made using ACP (Code 108227, Lot 93207721) from Roche Diagnostics.
Distilled water was added to the reference standards to prepare 100 μg/ml enzyme solutions, and a 2-fold dilution series was prepared using each enzyme buffer.
Using 1 well of a 96-well plate for 1 reaction, 50 μl of enzyme and 50 μl of the substrate solution ＊ were mixed and reacted for 30 minutes at 37 ℃ . After adding 50 μl of stop solution, absorbency at 405 nm was immediately measured using a plate reader.

Note) In the case of acid phosphatase, color forms with the addition of stop solution.
＊: Tartaric acid is not added to acid phosphatase substrate.

＜Results＞
In the reaction scales produced:
For ACP, concentrations were measurable from 1.5 μg/ml or less, and actual linearity was obtained in the range up to 0.5 μg/ml.

Acid phosphatase 100 μ l/ml preparation：
$2^1 \sim 2^{15}$ stepwise dilution

μ l/ml	405 nm
50	4.014
25	3.993
12.5	3.995
6.26	4.066
3.125	4.011
1.5625	4.032
0.78125	3.411
0.390625	2.043
0.195313	1.147
0.097656	0.599
0.048828	0.356
0.024414	0.215
0.012207	0.157
0.006104	0.120
0.003052	0.108
0	0.084



(4) Measurement with Alkaline Phosphatase (ALP) as standard sample

A standard curve was made using 3 types of alkaline phosphatase, BAP (Cat.# 2120A, Lot K2601EA), CIAP (Cat. # 2250A, Lot E2301AB), or SAP (Cat.# 2660A, Lot N301CB) of TAKARA BIO INC.

The 2-fold dilution series of the enzyme solutions, which be added Buffer for ALP in the kit to each enzyme, was prepared with Extraction solution in the kit. Both 50 μ l of enzyme solution and 50 μ l of pNPP substrate dissolved in the Buffer for ALP were added and mixed in one well of 96-well plate, and incubated at 37 ℃ for 30 minutes. Immediately after stopped by adding 50 μ l of Stop solution, absorbance at 405 nm of the 96-well plate was measured using a plate reader.

＜ Result ＞

All alkaline phosphatase (BAP, CIAP, and SAP) from TAKARA BIO were available as a positive control for the kit.

(5) Comparison of Tartrate-resistant Acid Phosphate (TRACP) and Alkaline Phosphatase (ALP) by he preparation method of blood samples.

＜ Method ＞

Blood was collected from three rabbits simultaneously and the collocted blood samples were preared by three methods (citrate plasma (PPP) , serum and hemolysis serum) . 50 μ l of serial dilution samples and 50 μ l of the respective substrate solution were mixed and reacted for 30 minutes at 37 ℃ . After adding 50 μ l of stop solution, absorbance at 405 nm was measured using a plate reader. All of the samples were measured simultaneously on the day when blood samples were prepared.

＜ Results ＞

＜ TRACP activity ＞ A405 nm absorbanse

TRACP	citrate plasma （PPP）			serum			hemolysis serum		
ID No.	x 20	x 40	x 80	x 20	x 40	x 80	x 20	x 40	x 80
Rb No. 1	0.821	0.420	0.247	0.834	0.487	0.309	0.834	0.487	0.300
Rb No. 2	1.045	0.520	0.311	0.704	0.422	0.268	1.066	0.582	0.360
Rb No. 3	0.702	0.370	0.237	1.000	0.579	0.353	0.768	0.360	0.275

＜ ALP activity ＞ A405 nm absorbanse

ALP	citrate plasma （PPP）			serum			hemolysis serum		
ID No.	x 2	x 4	x 8	x 2	x 4	x 8	x 2	x 4	x 8
Rb No. 1	0.514	0.321	0.217	0.801	0.469	0.295	1.074	0.599	0.374
Rb No. 2	0.481	0.299	0.206	0.528	0.327	0.217	0.801	0.483	0.304
Rb No. 3	0.348	0.231	0.165	0.718	0.431	0.275	0.650	0.398	0.252

As the phosphate activity is different depending on the preparation method of blood sample, it is necessary to use the sample of the same preparation method.

(6) Influence of freeze-thaw cycles of blood samples on the phosphate activity.

＜ Method ＞

Serum samples were collected from three rabbits. Samples were devided into four portion and each sample was repeated various cycles of freeze-thawing (− 80 ℃ ⇔25 ℃)

For the measurement of TRACP (tartrate-resistant acid phosphate), samples were diluted by 20-, 40- and 80- fold. For the measurement of ALP (alkaline phosphate), samples were diluted by 2-, 4- and 8- fold.

50 μ l of serial dilution samples and 50 μ l of the respective substrate solution were mixed and reacted for 30 minutes at 37 ℃ . After adding 50 μ l of stop solution, absorbance at 405 nm was measured using a plate reader. All of the samples were measured simultaneously on the day when serum samples were prepared.

＜ Results ＞

＜ TRACP activity ＞ A405 nm absorbanse

TRACP	no freezing			freezing-thaw 1 cycle			freezing-thaw 2 cycles			freezing-thaw 3 cycles		
ID No.	x 20	x 40	x 80	x 20	x 40	x 80	x 20	x 40	x 80	x 20	x 40	x 80
Rb No. 1	0.826	0.477	0.295	0.832	0.438	0.284	0.778	0.452	0.292	0.736	0.432	0.277
Rb No. 2	1.087	0.577	0.342	1.078	0.573	0.343	1.021	0.580	0.353	0.980	0.565	0.344
Rb No. 3	0.743	0.409	0.252	0.754	0.429	0.269	0.749	0.434	0.279	0.710	0.424	0.275

＜ ALP activity ＞ A405 nm absorbanse

ALP	no freezing			freezing-thaw 1 cycle			freezing-thaw 2 cycles			freezing-thaw 3 cycles		
ID No.	x 2	x 4	x 8	x 2	x 4	x 8	x 2	x 4	x 8	x 2	x 4	x 8
Rb No. 1	0.616	0.338	0.281	0.602	0.328	0.212	0.592	0.335	0.214	0.588	0.330	0.221
Rb No. 2	0.577	0.327	0.206	0.600	0.338	0.214	0.562	0.324	0.209	0.577	0.334	0.213
Rb No. 3	0.400	0.243	0.164	0.423	0.255	0.170	0.440	0.261	0.175	0.415	0.248	0.173

It seemed that both of TRACP and ALP activities were not comparatively influenced by freeze-thaw cycles of blood samples, but it was preferable even twice of freeze-thaw cycles in the same condition.

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.

X. Retated Products

TRACP & ALP double-stain Kit (Cat. # MK300)

URL：http：//www.takara-bio.com
Phone：+81-77-543-7247 Fax：+81-77-543-9254