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A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro

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Abstract

Alkaline phosphatase (ALP) is the most widely recognized biochemical marker for osteoblast activity. Although its precise function is poorly understood, it is believed to play a role in skeletal mineralization. The aim of this study was to develop an assay suitable for measuring the activity of this enzyme in microtiter plate format. Using the well-characterized osteoblast-like cell line Saos-2, this paper describes an optimized biochemical assay suitable for measuring ALP activity in tissue culture samples. We have determined that a *p*-nitrophenyl phosphate substrate concentration of 9 mM provides highest enzyme activities. We have found that cell concentration, and hence enzyme concentration, affects both the kinetics and precision of the assay. We also tested several methods of enzyme solubilization and found that freeze-thawing the membrane fractions twice at −70°C/37°C or freeze-thawing once with sonication yielded highest enzyme activities. The activity of the enzyme decreased by 10% after 7 days storage. This assay provides a sensitive and reproducible method that is ideally suited for measuring ALP activity in isolated osteoblastic cells, although sample preparation and storage can influence results.

Keywords: Alkaline phosphatase; Enzyme assay; Osteoblast; Saos-2

1. Introduction

Studies on osteoblast-like cells isolated from trabecular bone, embryonic calvaria and osteosarcoma have helped to establish a set of properties associated with the os-

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teoblast phenotype [1]. These include alkaline phosphatase activity, synthesis of type I collagen, secretion of osteocalcin and production of a mineralized matrix [2]. Moreover, it has been shown that these osteoblastic characteristics are modulated by 1α,25 dihydroxyvitamin D_3, parathyroid hormone, glucocorticoids, prostanoids and various cytokine growth factors.

Of all the above, the most widely recognized biochemical marker of the osteoblast phenotype is alkaline phosphatase (ALP). ALP is a ubiquitous enzyme which catalyses the hydrolysis of phosphate esters at an alkaline pH [3]. In humans there are three isoenzymes: tissue non-specific, intestinal and placental. The tissue non-specific isoenzyme has three isoforms, namely bone, liver and kidney, which contain different carbohydrate moieties on the same polypeptide backbone [4]. Of these isoforms, bone ALP shares a common protein structure with liver ALP, however, the two can be differentiated by electrophoretic mobility, chemical inactivation, lectin reactivity and thermostability [4]. These differences have been used to distinguish bone ALP from liver ALP in serum and plasma of patients with pathological bone disorders such as osteoporosis or Paget's disease [5,6], while others have used monoclonal antibodies that react preferentially with the bone isoform [7].

There has been extensive work and publication on ALP over the years, yet the precise function of skeletal ALP in vivo is largely unknown. Evidence suggests that the enzyme is involved in skeletal mineralisation. For instance, ALP has been shown to be associated with the plasma membrane of osteoblasts, which bud out to form the matrix vesicles seen in developing bone [8–11]. In vitro studies have shown that ALP activity in foetal rat calvaria is proportional to the rate of collagen production [12]. Most conclusive evidence comes from patients suffering from hypophosphatasia, who have a defect in ALP and are characterized by abnormal skeletal mineralization [4], and from studies in which the introduction of skeletal ALP cDNA into ALP-negative cells conferred an in vitro capacity for mineralization [13]. As yet the precise role of ALP remains largely unknown.

Although ALP expression can be determined using commercially available cytochemical kits (Sigma), the most commonly used method for measuring this enzyme is based on the biochemical technique originally described by Bessey et al. [14], in which ALP at alkaline pH catalyses the following reaction:

$$\underset{\text{(Colourless in Alkali)}}{p\text{-nitrophenyl phosphate}} + H_2O \rightarrow \underset{\text{(Yellow in Alkali)}}{p\text{-nitrophenol}} + P_i$$

There have been many reports of methods for measuring ALP enzyme activity in vitro and a variety of descriptions for solubilizing the enzyme. The aim of this research was to develop a rapid, reproducible and quantitative method for measuring ALP activity using the popular 96-well microtiter plate format. Moreover, we were particularly interested in developing a protocol for quantitatively measuring ALP in isolated osteoblastic cells. Therefore, we used the well-characterized osteoblast-like cell line Saos-2 [15–18] and modified our biochemical assay from that described by Boyan et al. [19]. This paper discusses our findings.

2. Materials and methods

2.1. Materials

Saos-2 human osteosarcoma cells were obtained from European Collection of Animal Cell Cultures (Porton Down, UK). McCoy's 5a medium and foetal calf serum (FCS) were purchased from Gibco Laboratories (Paisley, UK). Antibiotics, L-glutamine, phosphate buffered saline (PBS), trypsin-EDTA, Triton X-100® and tissue culture flasks were obtained from ICN Flow Laboratories (Thame, Oxon, UK). Trypan blue (0.5%), 2-amino-2-methyl-1-propanol (AMP), *p*-nitrophenyl phosphate (*p*-NPP), *p*-nitrophenol (code 104-1) and magnesium chloride ($MgCl_2$) were all purchased from Sigma Chemicals (Poole, Dorset, UK).

2.2. Cell culture

Saos-2 cells were cultured in McCoy's 5a medium supplemented with 10% (v/v) heat inactivated FCS, 1% (v/v) penicillin (5000 IU/ml) and streptomycin (5000 mg/ml). and 1% (v/v) L-glutamine. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air.

2.3. AMP-substrate buffer

The AMP-substrate buffer was prepared by making a 0.5 M AMP solution in distilled water at pH 10. The buffer was supplemented with 2 mM of $MgCl_2$ and various concentrations of *p*-NPP to determine the optimum substrate concentration. The Michaelis constant (K_m) for ALP is 0.89 mM. In order to ensure that the concentration of ALP was the rate-limiting step of the reaction, we investigated the effect of various concentrations of *p*-NPP (1, 4.5, 9, 15 and 18 mM; i.e. 1, 5, 10, 15 and 20 × K_m, respectively) on the production of *p*-nitrophenol. The absorbance was then measured at 405 nm after 15 min incubation at 37°C.

2.4. p-Nitrophenol standards

Standard concentrations were prepared by diluting the stock *p*-nitrophenol (10 μmol/ml) in appropriate volumes of AMP-substrate buffer. The standards ranged from 0–0.5 μmol/ml and were stored in the dark at 4°C until use.

2.5. Alkaline phosphatase assay

The assay was performed by adding 100 μl of each *p*-nitrophenol standard and 50 μl of each test sample to a 96-well microtiter plate. Fifty μl of AMP-substrate buffer was then added to each of the test samples. After incubation at 37°C the absorbance was measured immediately at 405 nm using a Titertek® Multiskan spectrophotometric plate reader (ICN Flow Laboratories). A standard curve of absorbance versus *p*-nitrophenol concentration was generated and used to determine the concentration of *p*-nitrophenol in μM/ml. In the current investigation, all values are presented as absorbances determined at 405 nm.

2.6. Effect of cell concentration on p-nitrophenol production

Using various concentrations of Saos-2 cell lysates, the reaction kinetics of the

assay were investigated. The cell lysate was prepared as follows: (a) the conditioned medium was aspirated from confluent Saos-2 cell cultures, (b) the cell monolayer was washed with sterile PBS, (c) the cells were disaggregated using a trypsin-EDTA mixture and resuspended in PBS, (d) using trypan-blue exclusion, the number of viable cells was adjusted to 4×10^5 cells per ml in PBS, (e) the cell suspension was lysed by adding an equal volume of 1% (v/v) Triton X-100® in PBS. This solution, containing 2×10^5 lysed cells per ml, was then serially diluted in PBS and the ALP activity was measured at 5 min intervals for 30 min.

2.7. Effect of different methods of enzyme solubilization

As ALP is a membrane-bound enzyme, it has to be solubilized before any measurement. Previous publications have suggested the use of sonication and/or freeze-thawing prior to the biochemical measurement of ALP activity. In order to determine which method yielded the highest ALP activity, we investigated the effect of the following treatments on Saos-2 cell membrane: (a) Triton X-100® only, (b) Triton + sonication, (c) Triton + freeze-thawing once (1×), (d) Triton + freeze-thawing (1×) + sonication, (e) Triton + freeze-thaw twice (2×) and (f) Triton + freeze-thaw (2×) + sonication.

2.8. Enzyme activity during short-term storage

Saos-2 cell lysates were stored at 4°C and −70°C for 7 days. The activities of the ALP in the samples were measured after 0.5, 1, 2, 3, 5 and 7 days.

2.9. Precision

Twenty-four replicates of 3 different Saos-2 cell lysate concentrations (5×10^4, 2×10^4, 1×10^4 cells per ml) were assayed to determine the assay precision. The results were expressed as the percentage coefficients of variation for each cell lysate concentration.

2.10. Statistical methods

The data were analyzed for conformity to a Gaussian model using a normal probability plot. The results suggested that the data conformed to such a distribution. All data are presented as the mean ± 95% confidence levels of 6 replicates, except where stated otherwise. The variances of the data were studied using an F_{max} test and were found not to be significantly different. Differences between means were compared using the Student's *t*-test to determine statistical differences [21]. The results of different solubilization techniques were also analyzed using Analysis of Variance (ANOVA). Reproducibility of the measurements was expressed as the percent coefficient of variation (%CV). A probability value of less than 0.05 ($P < 0.05$) was considered significant in all cases.

3. Results

3.1. Optimization of substrate concentration

ALP enzyme activity was significantly higher ($P < 0.05$) for 4.5, 9, 15 and 18 mM

p-NPP concentrations as compared to 1 mM substrate concentration (Fig. 1). Maximal absorbances were obtained with 9, 15 and 18 mM *p*-NPP, and there were no significant differences between absorbances at these substrate concentrations ($P > 0.05$). All the subsequent experiments were therefore conducted using a substrate concentration of 9 mM (i.e. $10 \times K_m$) *p*-NPP.

3.2. Effect of cell concentration on p-nitrophenol production

We examined the ALP activity of seven different Saos-2 cell lysate concentrations every 5 min for 30 min. ALP activity was proportional to Saos-2 cell concentration ranging from 2×10^3 cells per ml to 2×10^5 cells per ml (Fig. 2). At low cell concentrations ($<2 \times 10^4$ cells per ml), there was a linear relationship over the first 15 to 25 min incubation. At higher concentrations ($>2 \times 10^4$ cells per ml), the reaction proceeded rapidly, and at cell concentrations greater than 1×10^5 cells per ml, the absorbance exceeded the reference range of the spectrophotometer within 5 min. Fig. 3 demonstrates the linear relationship between *p*-nitrophenol production and Saos-2 concentrations at various incubations. The figure illustrates that at longer incubation periods and higher cell concentrations the absorbances exceed the reference range of the spectrophotometer. After 15 min incubation, the absorbances for cell concentrations of $\leq 5 \times 10^4$ cells per ml were within the reference range, therefore this end point was used for subsequent experiments.

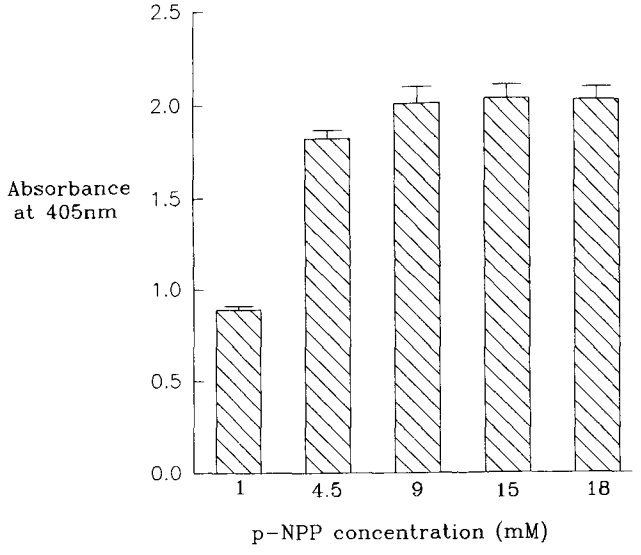

Fig. 1. Optimisation of substrate concentration. *p*-NPP concentrations of 9 mM, 15 mM, and 18 mM yielded highest ALP enzyme activities. There was no statistical difference between the activities measured between these three substrate concentrations ($P > 0.05$). The means of these substrate concentrations were statistically different from 1 mM and 4.5 mM ($P < 0.05$). Values are expressed as means of 6 replicates ± 95% confidence limits.

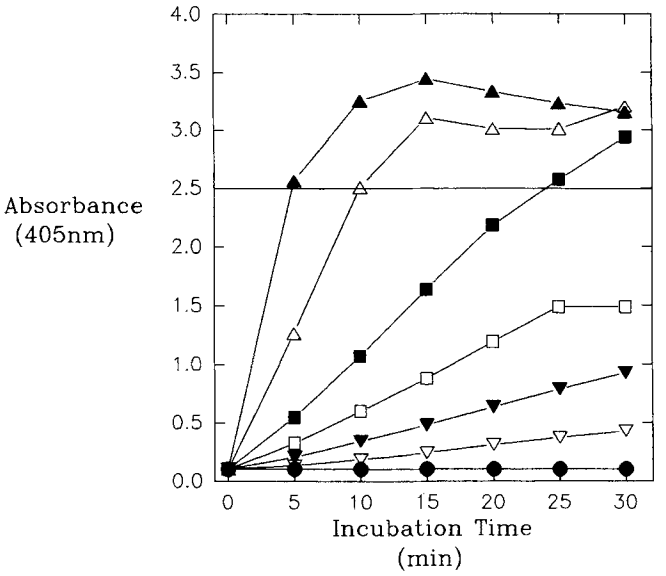

Fig. 2. Effect of cell concentration on *p*-nitrophenol production. Enzyme activity is proportional to Saos-2 cell concentrations and rapidly increases at cell concentrations greater than 2×10^4 cells per ml. Closed circles (●), 0 cells/ml; open triangles (▽), 2×10^3 cells per ml; closed triangles (▼), 5×10^3 cells per ml; open squares (□), 1×10^4 cells per ml; closed squares (■), 2×10^4 cells per ml; open triangles (△), 5×10^4 cells per ml; closed triangles (▲), 1×10^5 cells per ml. The maximal reference range of the spectrophotometer is also shown.

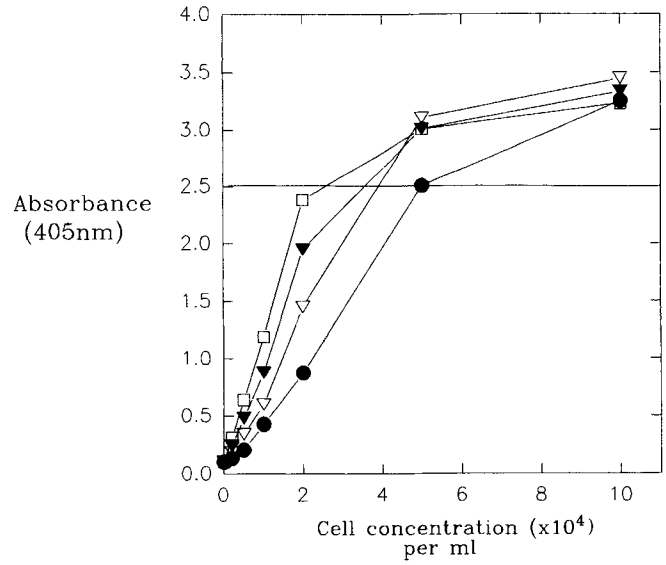

Fig. 3. Relationship between enzyme activity and cell numbers for each incubation. There is a linear relationship between the enzyme activity (expressed as absorbance at 405 nm) and increasing cell numbers, and hence, greater enzyme concentration. Closed circles (●), 10 min incubation; open triangle (▽), 15 min incubation; closed triangle (▼), 20 min incubation and open squares (□), 25 min incubation. The maximal reference range of the spectrophotometer is also given.

3.3. Effect of different methods of enzyme solubilization

Using ANOVA, the mean values of each treatment were compared and found to be significantly different ($F(5, 0.2) = 5.533$, $P < 0.001$). To determine which treatment groups were statistically different, the mean values of each set were individually compared (using Student's t-test). There was a significant difference ($P < 0.05$) between cell lysates which had Triton only and those which were (i) freeze-thawed (1×) and sonicated, (ii) freeze-thawed (2×) and (iii) freeze-thawed (2×) and sonicated. In contrast, freeze-thawing the samples once or sonicating once did not result in a higher ALP activity as compared to the Triton only group ($P > 0.05$). The highest activities were obtained from samples that were freeze-thawed once followed by sonication and freeze-thawed twice with or without sonication. There was no statistical difference ($P > 0.05$) among the three methods (Table 1).

3.4. Enzyme activity during short-term storage

The enzyme activity decreased by 10% within 2 days of storage at 4°C and −70°C (Fig. 4). Thereafter the activity remained unchanged up to 7 days at these conditions.

3.5. Precision

Coefficients of variation (%CV) of 24 replicates of three different cell concentrations are given in Table 2. Concentrations of 5×10^4 cells per ml had greater %CV values in comparison to those for lower Saos-2 cell concentrations (2×10^4 and 1×10^4 cells per ml). This indicates that at high Saos-2 cell concentrations the assay precision is reduced.

4. Discussion

Alkaline phosphatase is an important marker for osteoblast activity, and a knowledge of its function is important to our understanding of bone cell biology. The aim of this research was to develop a simple, quantitative, sensitive and reproducible

Table 1
Effect of different methods of cell lysis on ALP activity

Treatment	Mean absorbances at 405 nm	95% C.L.	Statistical comparisons vs. Triton/FT (2×)
Triton only	1.511	0.257	$P < 0.05$*
Triton/sonicate	1.648	0.099	$P < 0.05$*
Triton/FT (1×)	1.666	0.133	$P < 0.05$*
Triton/FT (1×)/sonicate	1.930	0.2	NS
Triton/FT (2×)	1.967	0.091	—
Triton/FT (2×)/sonicate	1.727	0.159	NS

After the solublisation step using 1% Triton X-100®, the sensitivity of the assay is significantly increased if the samples are subjected to freeze-thawing treatment twice (37°C and −70°C). The means of 6 replicates and 95% confidence limits (95% C.L.) are given.
*$P < 0.05$ using Student's t-test.
NS, not significantly different.

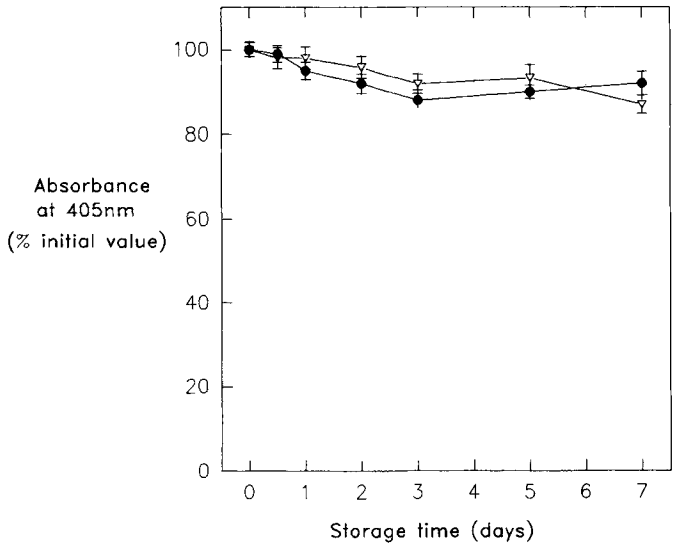

Fig. 4. Effect of short-term storage on ALP activity. Saos-2 cell lysates were stored at 4°C (●) and −70°C (▽) for 0.5, 1, 2, 3, 5 and 7 days. Each point is expressed as the mean of four replicates ± 95% confidence limits of the mean.

method for its measurement in isolated osteoblastic cells. At alkaline pH, the enzyme catalyses the conversion of *p*-NPP to the yellow coloured end-product, *p*-nitrophenol, which has a maximal absorbance at 405 nm [10,14]. AMP, an amino alcohol that is readily soluble in water and is transparent throughout the visible spectrum, has a high pK_a (9.3) which leads to good buffering in the pH range where ALP is most active [10]. $MgCl_2$ is added to the AMP-substrate buffer and acts as a co-factor for the enzyme. ALP has a Michaelis constant (K_m) of 0.89 mM, and as such a *p*-NPP concentration of 9 mM (10 × K_m) ensures that the enzyme concentration is indeed the rate-limiting step. We tested various *p*-NPP concentrations (1, 4.5, 9, 15 and 18 mM, i.e. 1, 5, 10, 15 and 20 × K_m, respectively) and found that 9 mM *p*-NPP did indeed promote the optimum enzyme activity (Fig. 1).

Table 2
Precision of alkaline phosphatase assay

Saos-2 concentration (cells per ml)	Mean	S.D.	%CV
5×10^4	2.858	0.2645	9.2
2×10^4	1.532	0.0377	2.4
1×10^4	0.841	0.023	2.7

The mean of 24 replicates of three different cell concentrations, standard deviation (SD) of the mean and % coefficient of variation (%CV) for each concentration is given. %CV was calculated using the equation %CV = (S.D./mean) × 100.

For specific samples, it is important to optimize the assay kinetically both for incubation time and enzyme concentration (reflected in cell number). As we have demonstrated in Fig. 2, it is possible to rapidly convert all the substrate to product or to exceed the reference limit of the spectrophotometer if the enzyme concentrations are too high. If necessary, the assay may be altered either by diluting samples or by changing the ratio of sample to substrate. Although other research groups have used longer incubation periods [22–24], for convenience we have optimized the incubation period for 15 min (Fig. 3).

One of the best methods for disturbing the cell membranes is the use of a non-ionic detergent, such as Triton X-100® because it preserves not only protein structure but also protein-protein interactions. In conjunction with the use of Triton, a number of authors have proposed additional steps such as freeze-thawing [19] and/or sonication [16] of the cell fraction to solubilize the membrane-bound enzyme. In the present study we investigated the effect of various methods and found that freeze-thawing the samples twice, or freeze-thawing once or twice followed by sonication yielded the highest enzyme activities (Table 1). As there were no significant differences between samples that were freeze-thawed (2×) and those which were freeze-thawed (1 or 2×) + sonication, we recommend freeze-thawing the samples twice before measurement of ALP activity.

Within 2 days of storage, the activity of the enzyme had decreased by 10% from the initial activity (Fig. 4). There was no further loss in the activity for the remainder of the experimental period. The data suggest that short-term storage of samples will affect the activity of the enzyme, and results obtained after longer periods of storage should be interpreted with caution.

At Saos-2 cell concentrations higher than 2×10^4 cells per ml, we found that ALP activity quickly exceeds the reference range of the spectrophotometer (Fig. 2). Moreover, at higher cell (and enzyme) concentrations the precision for the assay was also decreased as demonstrated by the greater %CV values given in Table 2. At lower cell concentrations, the %CV values were less than 3% indicating the excellent reproducibility of the assay.

Since the assay measures enzyme activity (rate of formation of a coloured end-product), it is necessary to divide the absorbance value by the incubation-time when expressing the results. Although the reaction can be stopped by the addition of 0.1 M sodium hydroxide [25], we found it easier to measure absorbance values immediately, thereby eliminating this additional step. While there is a small experimental delay, the 96-well format permits measurement of all samples simultaneously, and we found no evidence to suggest that this influenced our results. Using this protocol, we are able to detect ALP enzyme activities of at least 0.05 μM/min/ml, and by increasing cell concentrations have even been able to detect ALP activity in MG-63 human osteoblast-like osteosarcoma cells which express ALP at very low levels [16,24].

We feel there exists a need for a standard means of expressing ALP activity. Traditionally, ALP activity has been expressed as μmol *p*-nitrophenol per min per mg protein. Results should be comparable if the protein being measured is proportional to cell number [26]. However, this is not always the case and can lead to varied results.

We, however, found too much variability when measuring cellular protein. For example, a number of groups have measured the protein content in the lysed cell fraction containing all the cellular debris [26,27], while other authors have only measured the protein concentration in supernatants without such debris [28,29]. When dividing activity by protein content, it is easy to see how these differences could yield contrasting results and may explain why in certain osteoblastic cell lines (e.g. MG-63) some groups have seen high ALP activity [30], while others have not [24,31]. We believe that ALP activity should be normalized for cell number and expressed as μmol *p*-nitrophenol per min per cell. Results will thus be standardized and comparable which is especially useful when comparing various cell lines, or when studying the effects of agents, such as vitamin D_3, that affect both cellular differentiation and proliferation [29,32,33].

Hopefully, through a better comprehension of events occurring at the cellular level, we can come to understand the complex processes involved in bone biology. As ALP is one of the most important biochemical markers for osteoblastic activity, this is one area in which further research is certainly merited. We believe that this assay will provide an easy and precise method for measuring ALP activity in vitro.

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