コスモ・バイオ株式会社

Product Information Sheet (M1222-009)
MarkerGene™ Fluorescent Alkaline Phosphatase Assay Kit (Product M1222)

NOTE: The following information is given as a viable methodology for use of MarkerGene™ Fluorescent Alkaline Phosphatase Assay Kit. The user may determine their own best conditions for use dependent on the specific conditions present in their experiment.

I. OVERVIEW

Phosphatases utilize a variety of phosphate and polyphosphate esters as substrates, membrane structural components, and energy resevoirs. Simple phosphatases, such as alkaline and acid phosphatase, hydrolyze phosphate monoesters to an alcohol and inorganic phosphate. Alkaline Phosphatase plays an important role in many biochemical regulatory pathways, including a possible role in cell differentiation. Alkaline Phosphatase activity can be monitored using our fluorescent substrate, Fluorescein diphosphate, tetraammonium salt (FDP) (Product No. M1222-002), contained in this kit. Alkaline Phosphatase catalyzes a two-step hydrolysis of FDP, releasing the fluorescent compound, fluorescein (Abs 488nm, EM 512nm), and activity measurements are easily obtained either *in vitro*, in cell lysate preparations, or *in vivo*. The kit contains enough substrate for as many as 100 assays and control experiments when the detailed kit protocol is used. The kit also contains reference standards and a phosphatase inhibitor cocktail, which will inhibit a variety of phosphatases. See the references below for more information and applications.

II. MATERIALS

A.) Substrate: Fluorescein diphosphate, tetraammonium salt. Dissolve using dH_2O to prepare 1mM solution for use in the assay protocol below.

B.) Reference Standard: 10mM Fluorescein in Dimethyl Sulfoxide (DMSO). Dilute to 1mM for use in the assay protocol below.

C.) Reaction Buffer: This buffer contains 100mM Diethanolamine with 1mM Magnesium Chloride, pH 7.0.

D.) Cell Lysis Buffer: 25mM Tris-Phosphate (pH 7.8) containing 10% glycerol, 1% Triton X-100, 1mg/mL BSA, 2mM EGTA, and 2mM DTT.

E.) Phosphatase Inhibitor Cocktail: This inhibitor cocktail contains 200mM imidazole, 100mM Sodium Fluoride, 115mM Sodium Molybdate, and 400mM Sodium Tartrate, and inhibits several phosphatases.

F.) Stop Buffer: This buffer contains 1.0M Sodium Carbonate. Dilute to 0.2M for use in the assay protocol below.

G.) Storage and Handling: The substrate reagent and reference standard included in this kit should be kept cold when not in use and stored at -20°C. Protect solutions of the substrate reagent and reference standard from light.

III. ENZYME ASSAY

It is recommended that a calibration curve be generated using concentrations of the Reference Standard in a range from 0 to the highest concentration of substrate reagent used for all assays.

In addition, purified enzyme assays should be performed using several enzyme concentrations in the approximate or estimated range of the enzyme concentration expected for the unknown sample. To normalize data, each enzyme reaction should be subtracted from a blank (no enzyme) sample. Adding Phosphatase Inhibitor Cocktail (Product No. 1222-006) to enzyme-containing samples may be used as an alternative or second control.

1.) Cell Lysate samples are prepared as follows: Adherent or non-adherent cells are grown to 70-80% confluency using standard tissue culture conditions. The media is removed from the cell samples by suction or mild centrifugation, and the cells washed with sterile PBS (5 mL per 100 mm plate) and the PBS removed as above. Lysis Buffer (Product No. M1222-004) is added to the cells (1 mL). Cells are incubated at 37°C for 30 mins. Lysate (1 mL) is collected by pipet and transferred to a small tube or vial. Lysate sample is placed in ice bath.

2.) Purified enzyme concentrations should be prepared fresh by diluting enzyme in reaction buffer (Product M1222-001). The concentrations of purified enzyme should be in the range of 0.01 units/mL-1.0 units/mL.

3.) To a 96-well microtiterplate (clear, flat bottom) transfer samples containing cell lysate, purified enzyme, or cell suspension. Samples are added into wells on a microtiter plate in triplicate for each concentration and sample (50 µL/well). The concentrations of purified enzyme should be in the

range of 0.001 units/mL-1.0 units/mL. Include in triplicate wells for blanks and reference standards (50 µL reaction buffer/well for blanks of known enzyme concentrations, 50 µL of lysis buffer/well for blanks of cell lysate samples). ***Optional****:* Additional wells for each purified enzyme, lysate, or cell suspension can be prepared for the addition of the phosphatase inhibitor cocktail.

4.) Add reaction buffer (100 µL) to each well prepared in step (3) above. Allow a few minutes of incubation at room temperature to ensure a homogenous reaction mixture. ***Optional:*** If enzyme-containing wells were prepared for the addition of inhibitor cocktail, add the Phosphatase Inhibitor Cocktail (10 µL/well) (Product No. M1222-006) at this point (See Note (1) below).

5.) Prepare 1mM reference standard by diluting 10mM reference standard solution (15 µL) (Product No. M1222-003) in DMSO (135 µL) (Product No. M1222-005). To wells to be used as reference standards, add 1mM reference standard (50 µL) (see note (2) below).

6.) Prepare a 1mM substrate reagent solution by dissolving substrate (2.8 mg) (Product No. M1222-002) in cold (<4°C) distilled H_2O (5 mL) (Product No. M1222-005). Add 1mM substrate reagent (50 µL/well) to all wells, except those containing reference standard.

7.) Read fluorescence (Ex/Em = 488/512 nm) in a microtiter plate reader, using appropriate filters. Use the wells containing reference standard to optimize reading conditions. It is suggested that readings be taken beginning immediately after addition of the substrate reagent, and at ~1 min. intervals and for a period of at least 20 mins. afterward.

8.) Average the readings of duplicate samples. Subtract fluorescence of control wells from that of each sample in order to normalize data.

9.) Generate a calibration curve using purified enzyme samples by plotting normalized fluorescence vs. time (log-log).

10.) Using the calibration curve generated in step 9, determine the activity (concentration) of the enzyme in the original cell/tissue suspension.

11.) ***Optional:*** If an end-point reading is desired, prepare a 0.2M Stop Buffer solution by diluting 1.0 M Na2CO3 Stop Buffer (100 µL) (Product No.

M1222-007) in distilled H_2O (900 µL). Add solution to wells (50 µL/well) to stop reaction.

Note (1.) Addition of Phosphatase Inhibitor Cocktail may not completely inhibit enzymatic cleavage of FDP by Alkaline Phosphatase (Figure 2). K_i for this inhibitor has been experimentally found to be 0.466 mM (Figure 3) Little additional inhibition occurs when more than 10 µL Phosphatase Inhibitor Cocktail is added.

Note (2.) Use of reference standard wells may be useful in calculating optimal gain with a microtiter plate reader.

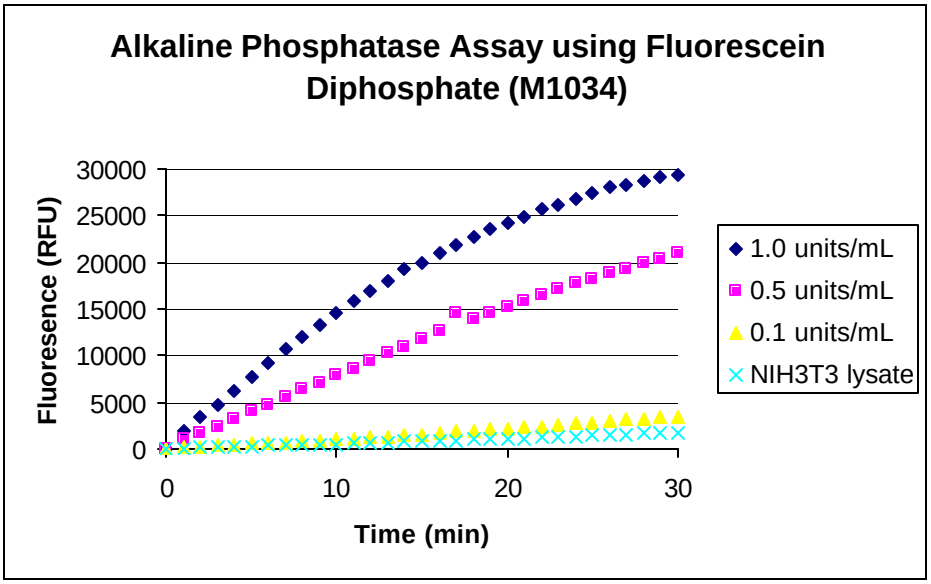

Figure 1: Three known concentrations (0.1 units/mL, 0.5 units/mL, 1.0 units/mL) of alkaline phosphatase (from bovine intestinal mucosa, SIGMA P7640) were prepared using reaction buffer as diluent. Each known concentration, as well as cell lysate obtained from NIH3T3 mouse lymphoblast tumor cells (in M1222-004 lysis buffer), was added in triplicate to wells on a 96-well microtiter plate (50 µL/well). Three wells were prepared containing reaction buffer (50 µL) and no enzyme to be used as blanks for the known enzyme concentrations, and an additional three wells were prepared containing M1222-005 lysis buffer (50 µL) to be used as a blank for the cell lysate reaction mixture. Reaction buffer (100 µL) was added to each well, followed by 1mM Fluorescein diphosphate, tetraammonium salt (50 µL) in distilled H_2O. The plate was immediately placed in a Perkin-Elmer 7000 Plus UV/FL/LUM Microtiter plate reader, and fluorescence was measured at EX/EM: 485/535 at one minute intervals for 30 minutes. The three wells for each sample/blank was averaged, and the blank values were subtracted from sample values at each time point.

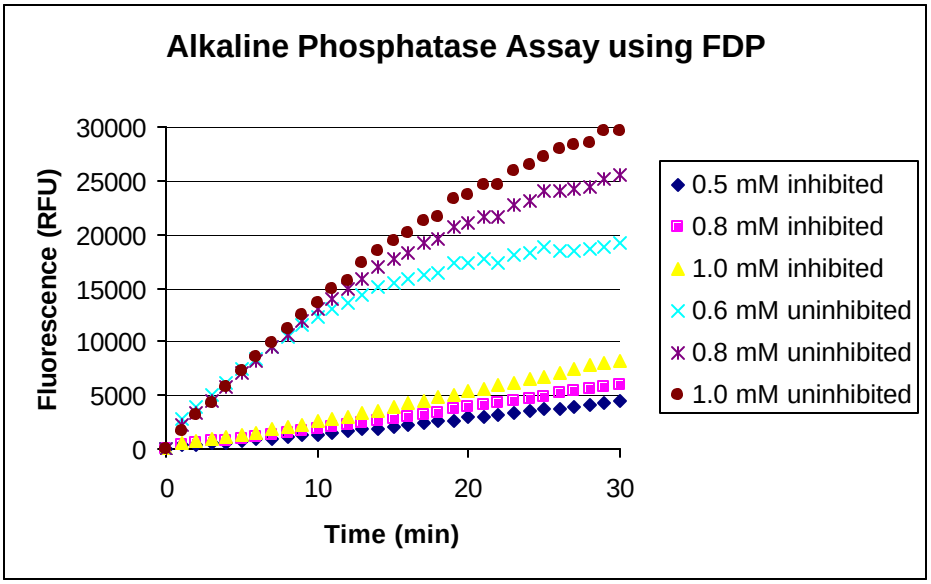

Figure 2: A dilution of alkaline phosphatase (from bovine intestinal mucosa, SIGMA P7640) with concentration 1.0 units/mL was prepared using reaction buffer as diluent. Enzyme solution was added to 18 wells on a 96-well microtiter plate (50 µL/well). Nine additional wells were prepared containing reaction buffer (50 µL) and no enzyme to be used as blanks. Reaction buffer (100 µL) was added to each well. . To nine of the enzyme-containing wells, 10 uL Phosphatase Inhibitor Cocktail was added (Product M1222-006). Three different concentrations (0.5. 0.8, 1.0 mM) of Fluorescein diphosphate, tetraammonium salt were prepared in distilled H_2O. Each concentration was added in triplicate (50 uL/well) to wells containing enzyme/no inhibitor, enzyme w/inhibitor, and no enzyme (blanks). The plate was immediately placed in a Perkin-Elmer 7000 Plus UV/FL/LUM Microtiter plate reader, and fluorescence was measured at EX/EM: 485/535 at one minute intervals for 30 minutes. The three wells for each sample/blank was averaged, and the blank values were subtracted from sample values at each time point.

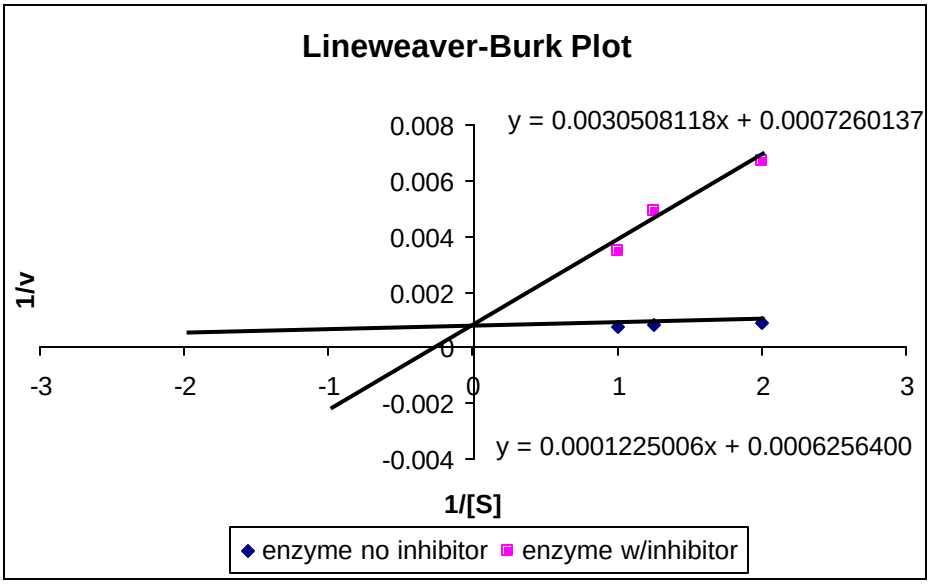

Figure 3: Lineweaver-Burk Plot constructed from data in Figure 2. Substrate concentrations are in mM. Inhibitor concentration was based on the concentration of imidazole in the Phosphatase Inhibitor Cocktail, as this is the only inhibitor of alkaline phosphatase present in the cocktail. From the data in this plot, K_i was determined to be 0.466mM.

M1222 Kit Contents

Description	Quantity	Part No.	Storage
Reagents			
Reaction Buffer	1 x 20 mL vial	1222-001	C
Substrate (Fluorescein diphosphate, tetraammonium salt)	1 x 2.8 mg	1222-002	F,R,L
Reference Standard (10mM Fluorescein in DMSO)	1x 100 µL vial	1222-003	F,R,L
Cell Lysis Buffer	1 x 10 mL vial	1222-004	C
Dimethyl Sulfoxide (DMSO)	1 x 1 mL vial	1222-005	R,FL, RT
Phosphatase Inhibitor Cocktail	1 x 500 µL vial	1222-006	R, RT
Stop Buffer (1.0 M Na2CO3 in distilled H2O)	1 x 5 mL vial	1222-007	
Documentation			
MSDS Sheets	1	1222-008	N/A
Product Information Sheet	1	1222-009	N/A

Notes: F=store at or below -20 ^{o}C; RT=store at room temperature; C=store cold (4 ^{o}C); L=light sensitive; FL=flammable; R=read protocol instructions carefully prior to use.

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