

A TD-700 Laboratory Fluorometer

Method for

Alkaline Phosphatase Fluorescence



1. INTRODUCTION

Because of their critical functions in eukaryotic cells, methods for measuring protein phosphatases were established at least as early as 1953¹. In 1965 Fernley and Walker² described the use of 4-methylumbelliferyl phosphate (MUP) as a substrate for alkaline phosphatase. Dephosphorylation of MUP yields a highly fluorescent and stable product: 4-methylumbelliferone (4MU). MUP is now widely used for phosphatase detection. In 1989 Berger³ constructed a reporter gene, secreted embryonic alkaline phosphatase (SEAP), in which alkaline phosphatase is secreted from the recombinant cell. The protein can be detected directly in the culture media with MUP. It has also been used to detect PCR amplification products in ELISAs and to identify and characterize bacteria.

We describe a method for detection of alkaline phosphatase (AP) using MUP as a substrate and the Turner Designs TD-700 Laboratory Fluorometer to measure the highly-fluorescent enzymatic product, 4MU. The TD-700 Fluorometer enables researchers to quantitate as little as 1×10^{-7} mg/ml (200 pg) 4MU and has a linear range of over 5 orders of magnitude. The sensitivity of the method described below was approximately 2 ug/ml alkaline phosphatase.

2. MATERIALS REQUIRED

- ❖ TD-700 Fluorometer with standard PMT and 10 mm × 10 mm square cuvette adaptor (P/N 7000-009).
- ❖ Near UV Lamp (P/N 10-049).
- ❖ Long Wavelength UV Filter Kit (P/N 7000-967) which includes 300-400 nm excitation filter (P/N 10-069R) and 410-600 nm emission filter (P/N 10-110R-C).
- ❖ 10 mm × 10 mm square methylacrylate disposable cuvettes (P/N 7000-959).
- ❖ 4-Methylumbelliferone, sodium salt, F.W. 198.2.
- ❖ 4-Methylumbelliferyl phosphate, free acid, MW 256.
- ❖ Alkaline Phosphatase Standard.
- ❖ Sodium Carbonate, Anhydrous Na₂CO₃, MW 106.0.
- ❖ 50mM Tris Buffer pH 8.0.
- ❖ Bovine Serum Albumin (BSA).

3. EXPERIMENT PROTOCOL

3.1 Reagent Preparation

4MU Stock Solution, 50 mM. Dissolve 99.1mg of 4MU in 10mL deionized water. Store at 4°C in the dark for up to two months.

4MU Standard Solution, 10 uM. Dilute 10 uL 4MU Stock Solution into 50 mL Tris/0.1% BSA Buffer, pH 8.0. Store at 4°C in the dark for up to two months.

Sodium Carbonate Solution, 0.2 M. Dissolve 2.12g Na₂CO₃ into 100 mL distilled water. pH is approximately 12.

MUP Substrate Stock Solution, 3.6 mM. Dissolve 9.2 mg MUP into 10 mL 50 mM Tris/0.1% BSA buffer, pH 8.0. Make up fresh daily.

NOTE: MUP spontaneously hydrolyzes in aqueous solution. It should be stored in its solid form and made up just prior to use.

MUP Substrate Working Solution.

36 μ M. Dilute 300 μ L MUP Substrate Stock Solution into 30 mL Tris/0.1% BSA Buffer, pH 8.0. Make up fresh daily.

Alkaline Phosphatase Stock Solution.

1 mg/mL. (Biozyme calf-intestine alkaline phosphatase, 15.42 mg/ml).

Dilute 100 μ L alkaline phosphatase into 1.4 mL Tris/0.1% BSA buffer, pH 8.0.

Alkaline Phosphatase Standard Solution, 500

μ g/mL. Dilute 1ml AP Stock Solution with 1mL Tris/0.1% BSA buffer.

3.2. Instrument Set-Up

3.2.1. Turn on the TD-700 Fluorometer. Allow it to warm up for 10 minutes (600 seconds).

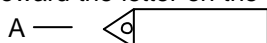
3.2.2. Ensure the lamp is installed by checking that the small window in the back panel is lit or by removing the filter cylinder and observing the lamp emission in the sample chamber.

3.2.3. Ensure that the excitation filter, P/N 10-069R, is installed in the position marked A “EX”, and the emission filter, P/N 10-110R-C, is installed in the corresponding position marked A “EM” in the filter cylinder. Install the filter cylinder so the A is next to the silver dot on the left.

3.3. Instrument Calibration

3.3.1 Prepare the 1 μ M 4MU calibration solution by adding 0.5 ml 36 μ M MUP solution into a 10x10 mm cuvette. Add 0.25ml 10 μ M 4MU Standard Solution and 1.75ml sodium carbonate solution. Cover and mix by inversion.

3.3.2. Place the cuvette in the 10x10 mm cuvette sample adapter. Place the sample adapter into the sample compartment. Be sure the pointed end of the sample adapter handle (it has a dot on it) is directed to the left and toward the letter on the filter cylinder.



3.3.3. Calibrate the instrument with the 1.0 μ M 4-MU Standard solution in the multi-optional mode according to the TD-700 Operating Manual, p. 21. Set the ‘Sample Setting’ to 900. Choose ‘9’ or ‘No’

when prompted ‘Read & Subtract Blank?’.

3.4 Alkaline Phosphatase Standard Curve

3.4.1. To generate a single-replicate, six-point standard curve from 20 μ g/mL to 1 μ g/mL, add 0.5 mL 36 μ M MUP Working Solution to each of 6 cuvettes.

3.4.2. Add an aliquot of 500 μ g/mL AP Standard Solution to a cuvette and incubate the mixture for two minutes at room temperature.

3.4.3. Add Sodium Carbonate Solution to the cuvette to make a total volume of 2.5 mL. Mix.

3.4.4. Take a fluorescence measurement immediately.

3.4.5. Repeat steps 3.4.1 through 3.4.4 with each standard (Table 1).

Volume (μ L) of 36 μ M MUP Working Soln	Volume (μ L) of 500 μ g/ml AP Std	Volume (mL) of Na_2CO_3 Soln	Final AP Concentration in Std Soln (μ g/mL)
500	100	1.90	20
500	50	1.95	10
500	20	1.98	4
500	10	1.99	2
500	5	2.00	1
500	0	2.00	0

Table 1

3.4.6. Generate a standard curve of fluorescence versus alkaline phosphatase concentration (Figure 1).

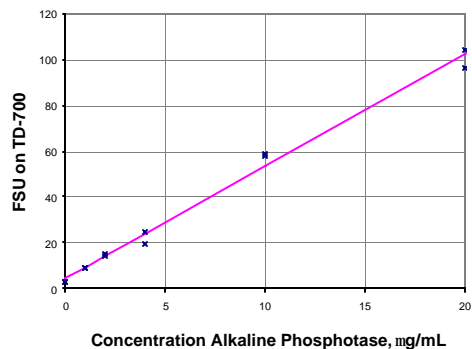


Figure 1 Fluorescence of 4MU from reaction of Alkaline Phosphatase with 36 mM Methylumbelliferone Phosphate (MUP) and quenched with 100mM NaCO₃. The standards were run in duplicate. The R² is 0.992. The fluorescence value of the reagent blank may be subtracted from that of each sample.

3.5 Alkaline Phosphatase Samples

- 3.5.1. Add 0.5 mL 36 uM MUP Working Solution to each sample cuvette.
- 3.5.2. Add 100 uL of sample to a cuvette, invert to mix.
- 3.5.3. Incubate the mixture for two minutes at room temperature.
- 3.5.4. Add 1.9 mL Sodium Carbonate Solution to the cuvette to make a total volume of 2.5 mL. Mix.
- 3.5.5. Take a fluorescence measurement immediately.
- 3.5.6. Repeat with each sample.
- 3.5.7. Calculate the amount of alkaline phosphatase from the fluorescence measurement and the linear equation determined from the AP standard concentration vs. fluorescence, step 3.4.6.

4. DISCUSSION

Alkaline phosphatase kinetics can be measured on the TD-700 using the Data Stream feature. (Press the 7 key and choose [Data Stream].) Figure 2 shows an example of the reaction data. Reagent concentrations and reaction times can be optimized using this feature.

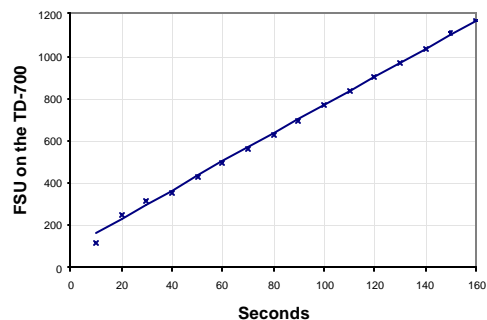


Figure 2 Real-time Fluorescence of 4MU from reaction of 1µg Alkaline Phosphatase in 36 uM Methylumbelliferone Phosphate (MUP).

The effect of pH on 4MU fluorescence is shown in Figure 3. The enzymatic reaction proceeds best at a pH of about 8; the optimum pH for 4MU fluorescence is 10 to 12.

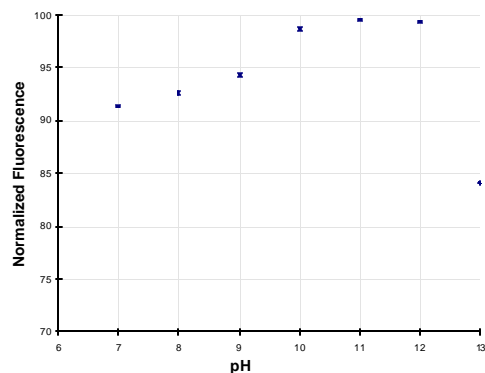


Figure 3 The Effect of pH on the Fluorescence of 4-MU in 200 mM Sodium Carbonate Solution

For quantitating enzyme, a stopped-reaction method is faster than a direct-initial reaction rate method. However, the accuracy of the method depends on precise timing and how well the reaction is quenched by the stop solution. The addition of the sodium carbonate solution slowed the reaction rate to less than 2%. Duplicate results with AP standards at two concentrations are shown in Figure 4.

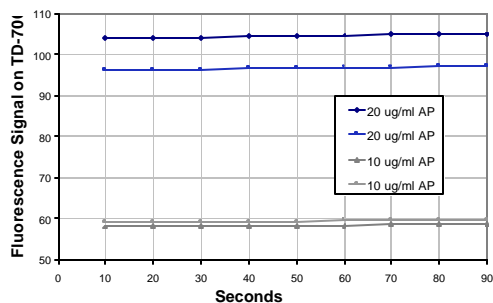


Figure 4 Stability of Fluorescence of Alkaline Phosphatase with MUP after Quenching with Sodium Carbonate, pH12.

Guilbault⁴ found that as little as 10^{-6} units/ml of alkaline phosphatase could be measured with about 1.5% accuracy by a direct initial reaction rate method using 20 mM MUP. He found that a 1.7 mM concentration of phosphate caused 50% inhibition and measured the effects of several other enzymes including β -glucosidase on the alkaline phosphatase reaction. Fernley and Walker² reported the effects of various reaction conditions. They used 0.5M K_2HPO_4 -KOH buffer, pH 10.4 to stop the reaction. They note that the addition of 5mM magnesium chloride increased the activity of the enzyme by up to 100%, but activity varied with both $MgCl_2$ and MUP concentration.

Several other enzymes can be measured using the fluorescence of 4MU derivatives. These include acid phosphatase, β -D-galactopyranoside, α -D-glucopyranoside, β -D-glucopyranoside, β -D-glucuronidase, and lipase.

5. REFERENCES

¹ Brandenberger, H., and Hanson, R., Spectrophotometric Determination of Acid and Alkaline Phosphatases, *Helv. Chim. Acta*, 36, 900, 1953.

² Fernley, H. N. and Walker, P. G., Kinetic Behaviour of Calf-Intestinal Alkaline Phosphatase with 4-Methylumbelliferyl Phosphate, *Biochem. J.*, 97, 95, 1965.

³ Berger, J., et. al., Secreted Placental Alkaline Phosphatase: a Powerful New Quantitative Indicator of Gene Expression in Eukaryotic Cells, *Gene*, 66, 1, 1988.

⁴ Guilbault, G. G. and Sadar, S. H., Umbelliferone Phosphate as a Substrate for Acid and Alkaline Phosphatase, *Analytical Letters*, 1(5), 333, 1968.

Nomenclature

4-methylumbelliferone is listed in the Merck Index as Hymecromone with the following synonyms: 7-hydroxy-4-methyl-2H-1-benzopyran-2-one, 7-hydroxy-r-methylcoumarin, 4-methylumbelliferone, β -methylumbelliferone, and 4-MU. The free acid is $C_{10}H_8O_3$, MW 176.2. Its form is off-white or yellowish crystals or powder. It is soluble in MeOH and EtOH, has blue fluorescence in alcohol and water, and is practically insoluble in cold water at neutral pH. The sodium salt, $C_{10}H_7O_3Na$, MW 198.2, is a yellow crystalline powder and is freely soluble in water. Molecular Probes lists the free acid as 7-hydroxy-4-methylumbelliferone. It is 4-methylumbelliferone (free acid or sodium salt) in the Sigma Chemical catalog.

6. ABOUT TURNER DESIGNS, INC.

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