

SCIENCE IMAGING SYSTEMS

Application Note No. 5

Enzyme Amplified Fluorescent Method Using AttoPhos™ and DDAO Phosphate in Southern Hybridizations

FLA-2000

Introduction

The main method used for specific detection of DNA following Southern blotting has long been the radioisotopic (RI) labeling method. In recent years, however, the increasing need for non-RI methodologies has led to the development of the enzyme amplified fluorescent method using a fluorescent substrate. (See FLA-2000 Application Note No.1.) Owing to its high stability, alkaline phosphatase (ALP) is the enzyme label of choice for use in Southern hybridization.

AttoPhos™ and DDAO Phosphate are fluorescent substrates suitable for this application. AttoPhos™ reacts enzymatically with ALP to produce a strong fluorescent product with a maximum excitation wavelength of 482nm and a maximum emission wavelength of 560nm. The reaction of DDAO Phosphate with ALP produces a strong fluorescent product with corresponding wavelengths of 647nm and 656nm.

This issue outlines the basic principles of using AttoPhos™ and DDAO Phosphate and explains the test protocol for Southern blotting.

Contents

1. Use of AttoPhos™ and DDAO Phosphate on Blotted Membranes
2. Limits of DNA Detection Using AttoPhos™ and DDAO Phosphate
3. Reagent Properties
4. References

Summary

- AttoPhos™ and DDAO Phosphate are both convenient substrates for use in Southern hybridizations by the enzyme amplified fluorescent method.
- In tests using AttoPhos™ and DDAO Phosphate, the limits of DNA detection were 5 fg and 10 fg, respectively.

1 AttoPhos™ Use on Blotted Membrane

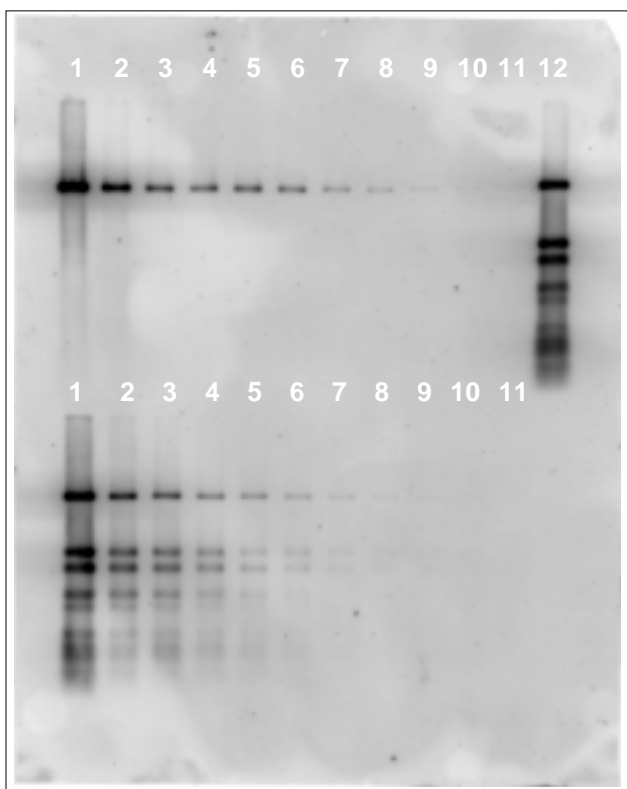


Fig. 1-1

Fig. 1-1 AttoPhos™ Reaction on Blotted Membrane

Sample :
 Upper : pBR328/*Bam* HI
 Lower : Products of digestion by pBR328, pBR322 with *Bam* HI, *Bgl* I, *Hinf* I mixed at 2:3:2

Electrophoresis was carried out on lanes 1-11 in the following amounts 1ng, 100pg, 50pg, 20pg, 10pg, 5pg, 2pg, 1pg, 500fg, 200fg and 100fg. A molecular weight marker was applied to lane 12.

The digital image was generated by scanning the membrane with the FLA-2000 under the following conditions:

Gradation : 65536(16bit)
 Resolution : 50µm
 Sensitivity : F1
 Latitude : 5
 Sample Mode : Fluor.473nm
 : Y520 Filter

Excitation/Emission Spectra of AttoPhos™

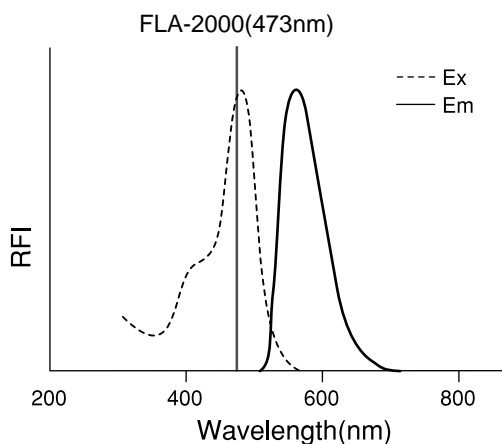


Fig. 1-2

RFI
 Relative fluorescence intensity

Fig. 1-2 Excitation/Emission Spectra of AttoPhos™ Reaction Products

The maximum excitation wavelength is 482nm and the maximum emission wavelength is 560nm.
 (Ex: Excitation spectrum, Em: Emission spectrum)

■ DDAO Phosphate Use on Blotted Membrane

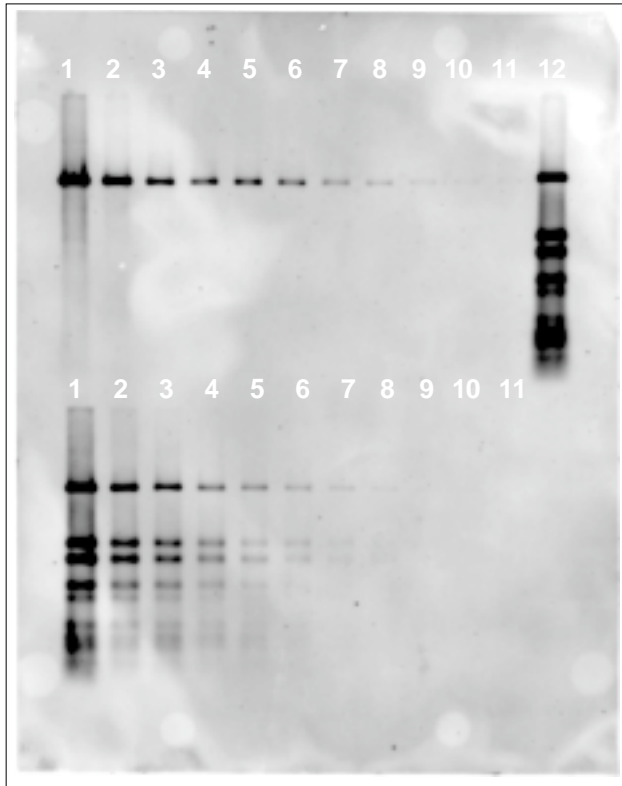


Fig. 1-3

Fig. 1-3 DDAO Phosphate Reaction on Blotted Membrane

Sample :

Upper : pBR328/*Bam* HI

Lower : Products of digestion by pBR328, pBR322 with *Bam* HI, *Bgl* I, *Hinf* I mixed at 2:3:2

Electrophoresis was carried out on lanes 1-11 in the following amounts 1ng, 100pg, 50pg, 20pg, 10pg, 5pg, 2pg, 1pg, 500fg, 200fg and 100fg. A molecular weight marker was applied to lane 12.

The digital image was generated by scanning the membrane with the FLA-2000 under the following conditions:

Gradation : 65536 (16bit)
 Resolution : 50μm
 Sensitivity : F10
 Latitude : 5
 Sample Mode : Fluor. 633nm
 : R675 Filter

■ Excitation/Emission Spectra of DDAO Phosphate

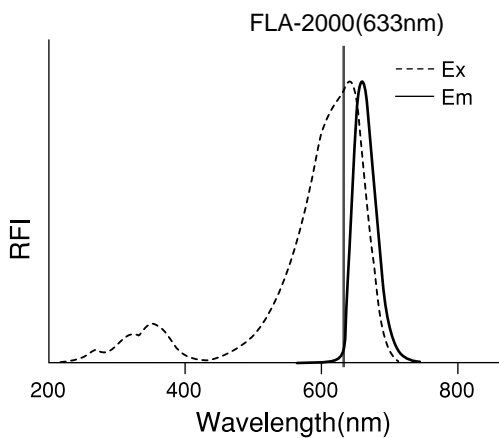


Fig. 1-4

Fig. 1-4 Excitation/Emission Spectra of DDAO Phosphate Reaction Products

The maximum excitation wavelength is 647nm and the maximum emission wavelength is 656nm. (Ex: Excitation spectrum, Em: Emission spectrum)

■ Test Protocol

Preparation of Reagents and Buffers

* Pretreatment for blotting	Denaturing solution	0.5N NaOH, 1.5M NaCl
	Neutralizing solution	1M Tris-HCl(pH8.0), 1.5M NaCl
* Blotting	Transfer buffer	20 x SSC (3M NaCl , 0.3M sodium citrate pH7.0)
* Prehybridization	Prehybridization buffer	6 x SSC (0.9M NaCl, 0.09M sodium citrate pH7.0) 5 x Denhardt's solution (0.1% Ficoll Type 400, 0.1% Polyvinylpyrrolidone, 0.1% BSA), 0.5% SDS, 100µg/ml DNA
* Hybridization	Hybridization buffer	6 x SSC (0.9M NaCl, 0.09M sodium citrate pH7.0) 0.01M EDTA pH8.0 5 x Denhardt's solution (0.1% Ficoll Type 400, 0.1% Polyvinylpyrrolidone, 0.1% BSA), 0.5% SDS, 100µg/ml DNA
	Washing buffer 1	2 x SSC, 0.1% SDS
	Washing buffer 2	0.1 x SSC, 0.1% SDS
* Blocking	Washing buffer	0.1M maleic acid, 0.15M NaCl pH7.5 0.3% Tween® 20
	Blocking buffer	Nucleic acid hybridization blocking reagent dissolved to concentration of 1% in buffer [0.1M maleic acid, 0.15M NaCl pH7.5 (+20°C)]
	Detection buffer	0.1M Tris-HCl, pH9.5, 0.1M NaCl, 50mM MgCl ₂
* Enzymatic reaction with substrate	AttoPhos™ buffer	AttoPhos™ substrate diluted with AttoPhos™ buffer (2.4M diethanolamine, 0.057mM MgCl ₂ , 0.005% NaN ₃ pH10.0)
	DDAO buffer	Diluted 1:1000 with detection buffer

Gel Sample Preparation

*Preparing agarose gel*¹

- (1) Add agarose powder to 1 x TAE buffer at 1% concentration.
- (2) Heat to 90°C with constant stirring.
- (3) Let stand until temperature falls to 70°C.
- (4) Pour 1% agarose gel solution into gel preparation tray and allow to set.*²
- (5) Wait about 1 hour.

*Sample preparation

Dilute DNA with TE buffer.

¹ Agarose produced by Boehringer Mannheim GmbH was used.

² The gel sets within approximately 30 minutes but takes about an hour to stabilize.

Electrophoresis

- (1) Pour 1 x TAE electrophoresis buffer into the buffer tray.
- (2) Place DNA samples in wells.
- (3) Electrophorese at 50V.*³
- (4) Wait about 90 minutes.

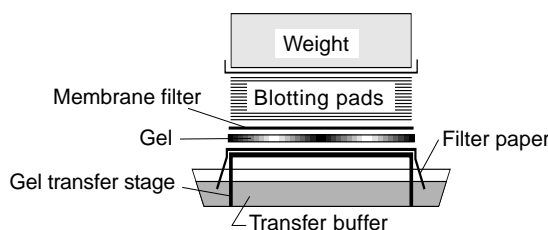
³ Gel staining before blotting makes electrophoresis easy to monitor. Quick staining and checking is possible with SYBR® Green I.

Pretreatment for Blotting

- (1) Soak gel in denaturing solution with gentle shaking.^{*4}
- (2) Wait about 30 minutes.
- (3) Discard denaturing solution.
- (4) Soak gel in neutralizing solution with shaking.
- (5) Wait about 30 minutes

Blotting

- (1) Set gel transfer stage in a container and fill it with (20 x SSC) transfer buffer.
- (2) Put filter paper on gel transfer stage, being careful not to get air in between.
- (3) Remove gel^{*5} from neutralizing solution and place it on wet filter paper.
- (4) Place membrane filter on gel.^{*6}
- (5) Put sufficient number of blotting pads on membrane filter and place a weight on top.
- (6) Wait about 3 hours.



^{*4} Denaturing solution changes double stranded DNA into single stranded DNA, which increases the blotting efficiency.

^{*5} The gel is somewhat bumpy on the top surface and should be turned upside down so the membrane filter can contact the flat side. This increases the transfer efficiency.

^{*6} Cutting off the upper right corner of the membrane makes it easy to tell how it is turned.



Cross-linking of DNA

- (1) Bake membrane at 80°C for 20 minutes.
- (2) Irradiate with UV rays at 33 mJ/cm².^{*7}

Hybridization

**Prehybridization*

- (1) Put membrane filter into hybridization bag.^{*8}
- (2) Add prehybridization buffer.
- (3) Keep at 68°C for 1 hour.

**Probe^{*9} preparation*

- (4) Keep probe at 95°C for 5 minutes.
- (5) Rapidly cool probe, then maintain as is for 5 minutes.

**Hybridization*

- (6) Discard hybridization buffer in bag.
- (7) Add probe to hybridization buffer.
- (8) Put probe solution in hybridization bag.
- (9) Keep at 68°C overnight.
- (10) Discard probe solution and wash the membrane filter twice with washing buffer 1.
- (11) Wait 5 minutes
- (12) Repeat (10) and (11).
- (13) Wash twice with washing buffer 2 at 68°C.
- (14) Wait 15 minutes.
- (15) Repeat (13) and (14).

^{*7} Highest sensitivity was obtained by baking at 80°C for 20 minutes, followed by 33 mJ/cm² UV cross-linking. Conditions on either side of the above conditions gave poorer results.

Conditions for 33 mJ/cm² UV irradiation

- UV cross-linker
Irradiate with dial set to 33mJ/cm².
- UV transilluminator at 254 nm
Illumination time (sec)
= 33 x 10⁻³/radiation intensity (W/cm²)
- UV transilluminator at 312 nm
Illumination time (sec)
= 40.5 x 10⁻³/radiation intensity (W/cm²)

^{*8} Avoid forming bubbles in the hybridization bag because they cause uneven buffer distribution.

^{*9} We used the DNA probe included in the DIG DNA labeling kit from Boehringer Mannheim GmbH.

Detection of DNA^{*10}

**Blocking*

- (1) Discard washing buffer and add fresh washing buffer.
- (2) Wait about 1 minute.
- (3) Discard washing buffer.
- (4) Add blocking buffer and shake gently.
- (5) Wait about 30 minutes.

**ALP labeling*

- (6) Dilute antidigoxigenin antibody to 1:10,000 with blocking buffer.
(ALP labeling buffer)
- (7) Discard blocking buffer.
- (8) Add ALP labeling buffer and shake gently.
- (9) Wait about 30 minutes.

**Washing*

- (10) Discard ALP labeling buffer.
- (11) Wash twice with washing buffer.
- (12) Wait 15 minutes.
- (13) Repeat (11) and (12).

**Equilibration*

- (14) Discard washing buffer.
- (15) Add detection buffer to equilibrate (pH9.5).
- (16) Wait about 5 minutes.

**Enzymatic reaction with substrate*

- (17) Discard detection buffer.
- (18) Add AttoPhos™ buffer or DDAO buffer and shake gently.
- (19) Wait about 5 minutes.
- (20) Discard AttoPhos™ buffer or DDAO buffer.
- (21) Seal hybridization bag.^{*11}
- (22) Wait 5 minutes to 1 hour.

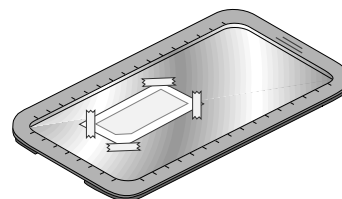
Reading

- (1) Place hybridization bag containing membrane filter on FLUOR stage.^{*12}
- (2) Bring up "Image Reader" program.
- (3) Set read-out conditions and click on "Read" button. (See page 2 or 3 for read-out conditions.)

^{*10} The buffers included in the DIG Wash and Block Buffer Set from Boehringer Mannheim GmbH were used in the DNA detection other than the enzymatic reaction.

^{*11} The reaction can be accelerated by incubation at 38°C for 20 minutes.

^{*12} Stretch and tape hybridization bag to prevent wrinkling. Diagonal taping gives best results.

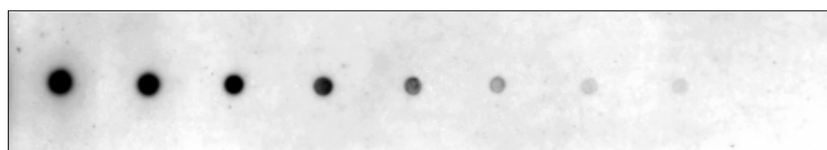


2 Limits of DNA Detection Using AttoPhos™ and DDAO Phosphate

The specific DNA detection sensitivity obtained with each of the fluorescent substrates was studied using the FLA-2000. For this, various concentrations of DIG-labeled pBR328 were dotted onto plus-charged nylon membrane. After hybridization with ALP-labeled anti-DIG-antibody, enzymatic reaction with AttoPhos™ or DDAO Phosphate as substrate was performed. The membranes were then read using the FLA-2000. The detection limit of DNA was 5 fg for AttoPhos™ and 10 fg for DDAO Phosphate.

AttoPhos™

1pg 500fg 200fg 100fg 50fg 20fg 10fg 5fg



DDAO Phosphate

10pg 1pg 500fg 200fg 100fg 50fg 20fg 10fg 5fg

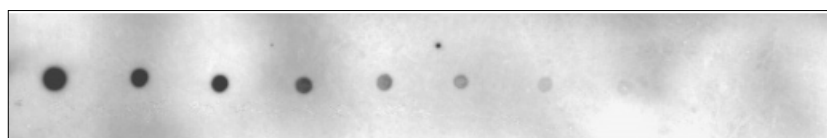


Fig. 2-1

Fig.2-1 Detection Sensitivity by Dot Blotting

Above : Detection with AttoPhos™
DNA amount : 1pg-5fg
Below : Detection with DDAO Phosphate
DNA amount : 10pg-5fg

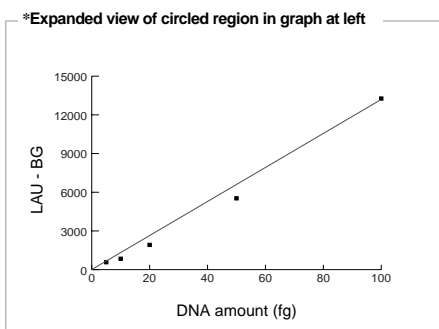
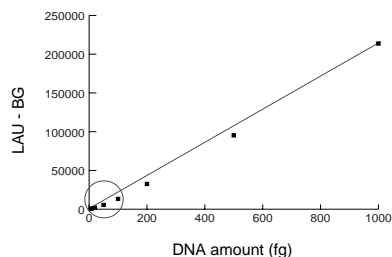
Sample was DIG-labeled pBR328 (Boehringer Mannheim GmbH)

Reading was conducted with the FLA-2000 under the following conditions.

Gradation : 65536(16bit)
Resolution : 50 μm
Sensitivity : F10
Latitude : 5
Sample Mode : Fluor.473nm : Y520
Filter (AttoPhos™) : Fluor. 633nm:R675
Filter(DDAO Phosphate)

Amount of DNA vs. Fluorescence Intensity

AttoPhos™



DDAO Phosphate

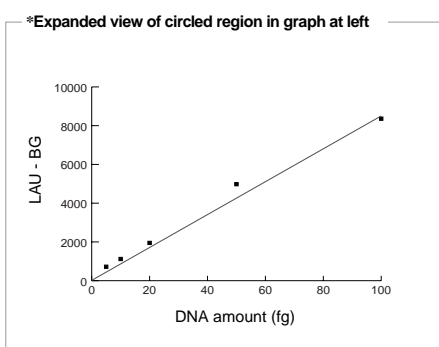
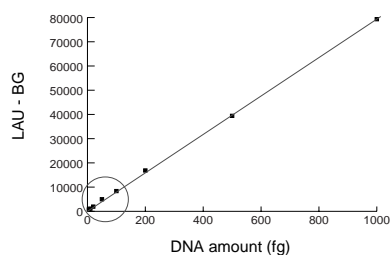


Fig. 2-2

Fig. 2-2 Amount of DNA vs. Fluorescence Intensity

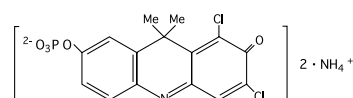
Upper left : High concentration range
Upper right : Expanded view of circled portion in graph at upper left
Lower left : High concentration range
Lower right : Expanded view of circled portion in graph at lower left

The original data were obtained using the profile analysis feature of the MacBAS software. Background was subtracted. (LAU: Linear Arbitrary Unit is the fluorescence intensity unit used in FLA-2000.)

3 Reagent Properties

Name	AttoPhos™	1,3-dichloro-9,9-dimethyl-acridine-2-one-7-yl phosphate (DDAO Phosphate)
Classification	Fluorescent enzyme substrate	
Mechanism of Action	Weak fluorescence in pH9.5 solution but strong fluorescence after enzymatic reaction with ALP.	Strong fluorescence after enzymatic reaction with ALP
Sample	DNA, protein	
Storage	Keep in dark freezer with desiccant. Shielding from light is particularly necessary when in solution.	
Caution	Wear gloves when handling.	
Manufacturer	Boehringer Mannheim GmbH (AttoPhos™ Substrate Set*)	Molecular Probes Inc.

DDAO Phosphate



* AttoPhos™ is included in the manufacturer's kit.

4 References

AttoPhos™

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2. Cano, R. J., Torres, M. J., Klem, R. E., Palomares, J. C., Casadesus, J., Detection of salmonellas by DNA hybridization with a fluorescent alkaline phosphatase substrate; *Journal of Applied Bacteriology*; 72, 393-399 (1992)
3. Kerkhof, L., A comparison of substrates for quantifying the signal from a nonradiolabeled DNA probe, *Analytical Biochemistry*; 205, 359-364 (1992)

DDAO Phosphate

4. Hisabori, T., Inoue, K., Akabane, Y., Iwakami, S., Manabe, K., Two-dimensional gel electrophoresis of the membrane-bound protein complexes, including photosystem, of thylakoid membranes in the presence of sodium oligoxyethylene alkyl ether sulfate/dimethyl dodecylamine oxide and sodium dodecyl sulfate, *Journal of Biochemical and Biophysical Methods*. 22, 253-260 (1991).

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