LAL Update®

ASSOCIATES OF CAPE COD INCORPORATED JUNE 1998 / VOL. 16, NO. 2

Dear LAL User,

This issue of the LAL UPDATE provides more detail on the diazo-coupling version of Pyrochrome,® ACC's chromogenic LAL product. Pyrochrome with diazo-coupling is an end-point chromogenic LAL assay. Although kinetic LAL methods may be more prevalent, the end-point LAL assay is still relied on by a large number of people due to its simplicity. The economics of the end-point assay provide its strongest recommendation. Only the simplest, inexpensive microplate reader and microplate incubator are needed to perform the test. No special computer programs are needed as the standard curve is a simple linear regression, and unlike some of the kinetic methods, the end-point assay yields a straight line, not a complex curve. Finally, the diazo-coupling modification has the added advantages of increased sensitivity and reduced sample interference.

The LAL UPDATE concludes with our regular Calendar. As we are now in full vacation season here on Cape Cod (even though we have to work), I would like to wish all our readers a very pleasant summer.

Sincerely,

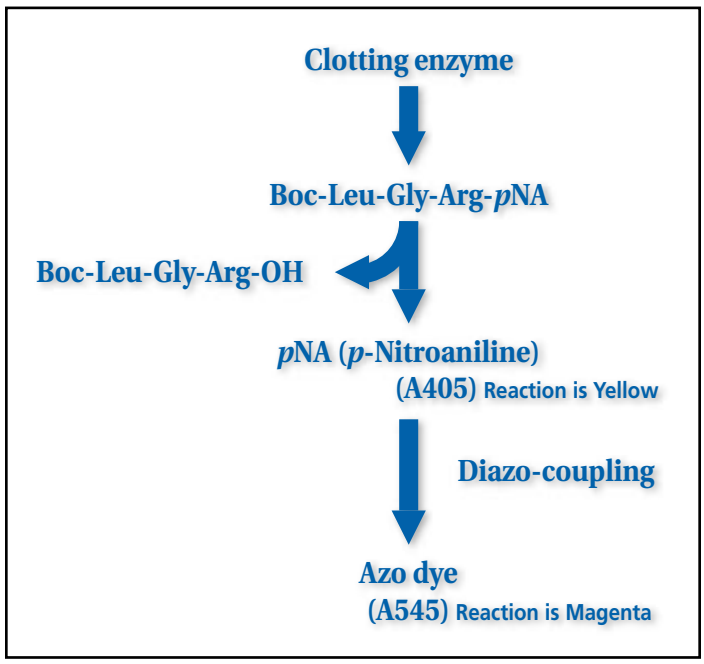
Thomas J. Novitsky, Ph.D.

Diazo-Coupling Option with Pyrochrome® Chromogenic LAL

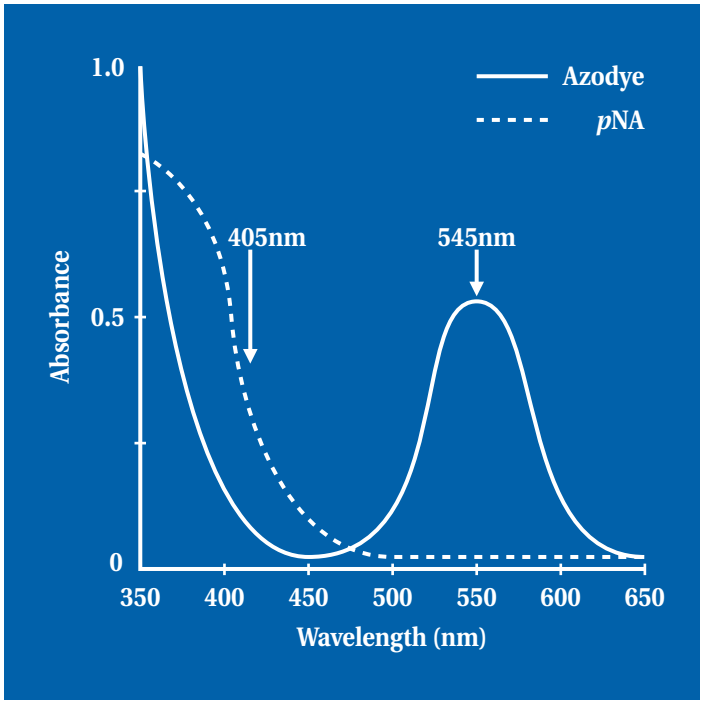
By Thomas J. Novitsky, Ph.D.

In 1993 Associates of Cape Cod, Inc. introduced Pyrochrome,® a multi-functional chromogenic LAL reagent kit (1). This reagent was the result of successful work in our laboratory to produce an LAL reagent that combined LAL and chromogenic substrate into a single reagent (1) and information gained from Seikagaku Corporation's years of experience with the chromogenic LAL assay (3). Our original intention was to produce a reagent that could be used in a kinetic assay. What we found through our collaboration with Seikagaku was that the reagent could also be used in an end-point assay, in particular an assay that employs the conversion of paranitroaniline (pNA) to the azo dye, i.e. diazo-coupling.

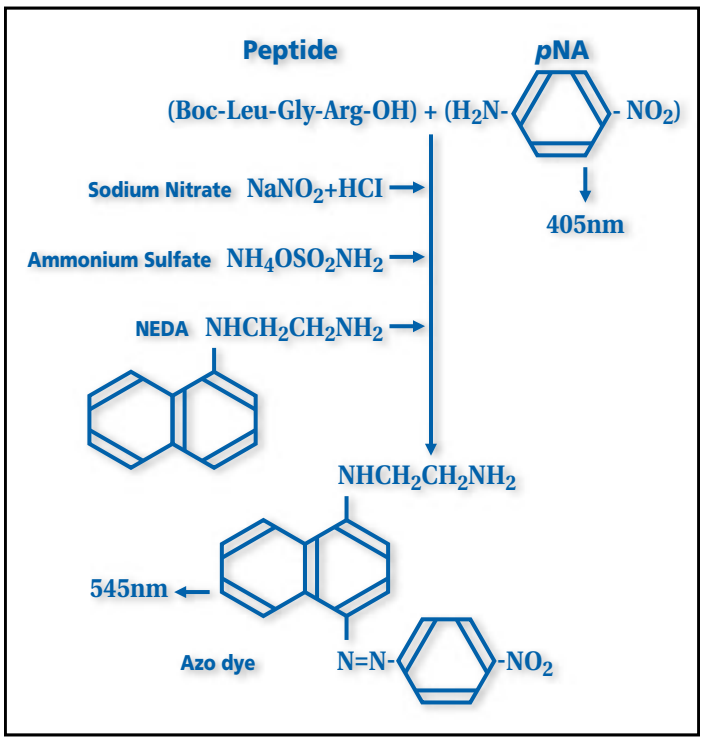
Continued on page 2



(Figure 1). Reaction of Clotting Enzyme with Chromogenic Substrate



(Figure 2). Absorption Spectra of *p*NA and Azode



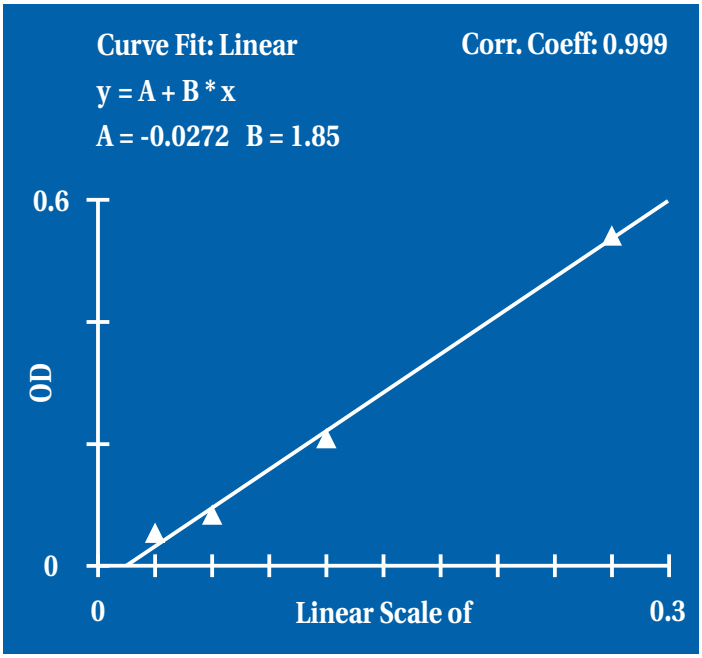
(Figure 3). Diazo-coupling Methodology Formation of the Azode from *p*NA

The reactions common to all LAL assays include the activation of an enzyme cascade that results in the activation of clotting enzyme (4). This enzyme which is a serine protease, cleaves either coagulogen which is the natural substrate (gel-clot and turbidimetric methods) or a chromogenic substrate which is a synthetic peptide containing *p*NA (chromogenic method). ACC and Seikagaku Corporation use a chromogenic substrate with the formula Boc-Leu-Gly-Arg-*p*NA. This particular substrate was selected for its high affinity to the clotting enzyme. Other commercially available chromogenic LAL kits may contain different types of chromogenic substrates, but all have *p*NA in common. When *p*NA is liberated by the action of the clotting enzyme, the yellow color characteristic of free *p*NA appears (Figure 1).

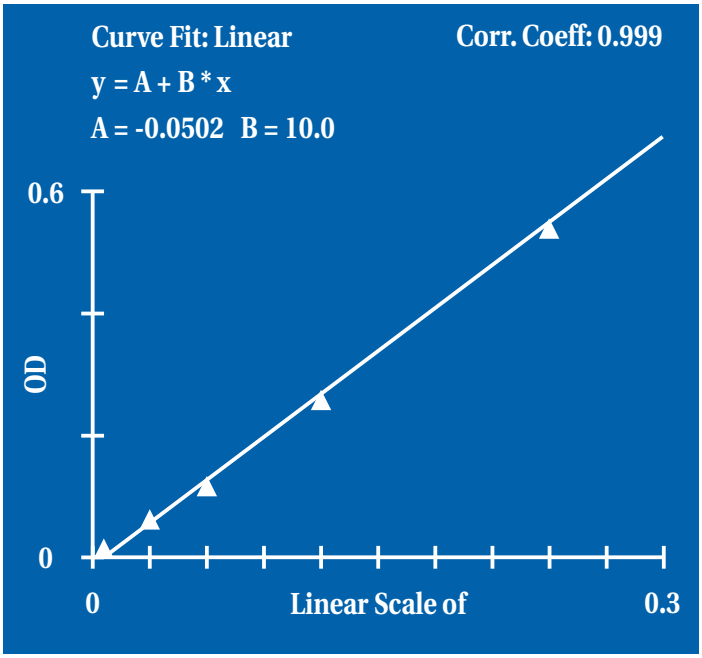
Depending on how the assay is set up, Pyrochrome can be used in either the kinetic or end-point mode. Both rely on the release of *p*NA but differ in the manner in which the *p*NA is recorded and analyzed. In the kinetic method, the continual release of *p*NA is recorded while in the end-point assay, only the amount of *p*NA released after a fixed period of time is recorded. The yellow color of *p*NA can be read in a spectrophotometer equipped with a 405 nm filter. For those of you who have seen this reaction, the yellow color does not appear very distinct to the eye. This is because the absorption maxima for *p*NA is close to the ultraviolet (UV) range. Furthermore, 405 nm is not the absorption maxima (Figure 2), but rather was selected to reduce interference from the numerous compounds that absorb in the near UV.

The diazo coupling method was developed because of the weak absorption of *p*NA and the possibility of sample interference in its absorption range. For this method, free *p*NA is derivatized to its diazo dye form. This chemistry is simple and quite well known (Figure 3). Once the LAL reaction is complete (usually 30 min), a solution of sodium nitrite/HCL is added. This not only stops the LAL reaction but also forms nitrous acid, which converts *p*NA to the p-nitrophenyldiazonium ion and nitrite. Ammonium sulfamate reagent is then added to react with the nitrite (which would otherwise interfere with the next step). The last reagent, N-(1-naphthyl) ethylene-diamine dihydrochloride (NEDA), reacts ("couples") with the diazonium ion to produce a brilliant magenta-colored derivative N-(1-naphthyl-4-diazo-4-nitrobenzene) ethylenediamine, referred to as an "azo dye". This compound has a very distinct spectra with a well-defined peak (absorption maxima of A=545) and a high extinction coefficient (ϵ_{540} = 53,300$cm^{-1}M^{-1}$ and ϵ_{545} = 53,560$cm^{-1}M^{-1}$ measured in H_2O at room temperature).

It is quite easy to compare the diazo-coupling methodology with the end-point chromogenic method using Pyrochrome,



(Figure 4a). Standard Curve of End-point (*p*NA) Pyrochrome



(Figure 4b). Standard Curve of End-point Diazo-coupling Pyrochrome

as diazo-coupling is really only an extension or enhancement of the *p*NA end-point result. Figures 4 A and B shows the standard curves produced by both methods with the same lot of Pyrochrome. As can be seen, both methods show good reproducibility (cv<10%) and linearity (r=0.999). The diazo-coupling results however indicate a steeper slope and a maximum O.D. closer to 2.5 at 0.25 EU/ml (vs. 0.45 at 0.25 EU/ml with *p*NA alone). The steeper slope and wider range of O.D. values make it easier to quantify unknowns and recover spikes while making the assay more sensitive (when compared to the same reaction time as the *p*NA end-point method, e.g. 0.007 EU/ml diazo-coupling vs. 0.03125 EU/ml *p*NA at 40 minutes incubation). In addition, reading the reaction at the higher wavelength avoids interference by yellow-colored samples, e.g. body fluids, culture media, etc. It should be pointed out, however, that the diazo-coupling method is not applicable to kinetic technology.

Pyrochrome® Chromogenic Test Kit

Each test kit contains Pyrochrome LAL reagent, Pyrochrome Reconstitution Buffer, and a 2 EU vial of Control Standard Endotoxin (CSE) with a certificate of analysis stating the potency of the CSE. A separate CSE is available for use with Pyrochrome when a greater concentration of endotoxin is required. This CSE (10 ng/vial, approx. 100 EU/vial) is useful for setting up standard curves between 50 and 0.005 EU/ml.

Pyrochrome is also offered in a diazo kit for end-point tests. The diazo reagents change the color of the chromophore form yellow to magenta making it especially useful for testing yellow samples.

Catalog#	Description
C0060	60 Test Kit
C0120	120 Test Kit
C0180	180 Test Kit
CD060	60 Test Kit (with Diazo-Coupling)

References:

1. Pyrochrome®A New Single-Step Chromogenic Limulus Amebocyte Lysate Assay. LAL Update 11 (3), June 1993.

2. Lindsay, G. K., P.F. Roslansky, and T.J. Novitsky. 1989. Single-Step, Chromogenic Limulus Amebocyte Lysate Assay for Endotoxin. J. Clin. Microbiol. 27:947–951.

3. Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai. 1985. A New Chromogenic Endotoxin-Specific Assay Using Recombined Limulus Coagulation Enzymes and its Clinical Applications. Clinica. Chimica. Acta. 149: 55–65.

4. A Wealth of Options: Choosing an LAL Test Method. LAL Update 13 (3), September 1995.

CALENDAR

JULY

July 14 – July 16
LAL Methodology and Applications Seminar and Workshop
Quality Inn
Falmouth, MA

SEPTEMBER

September 12 – September 15
International Endotoxin Society Meeting
Santa Fe, NM

September 14 – September 16
LAL Methodology and Applications Seminar and Workshop
Holiday Inn on King
Toronto, Canada

September 15 – September 17
LAL Workshop and Seminar
Woolton Redborne Hotel
Liverpool, England
Contact: Mark Childs at Associates of Cape Cod International for more details
44–151–220–3336

OCTOBER

October 7
LAL Advanced Topics Open Discussion Forum
Woolton Redborne Hotel
Contact: Mark Childs at Associates of Cape Cod International for more details
44–151–220–3336

October 14 – October 16
LAL Testing Business Seminar Presented by MMI Associates
Hamilton Park Conference Center
Florham Park, New Jersey
Session on "Choosing an LAL Test Method" will be run by Michael E. Dawson, Ph.D. Associates of Cape Cod, Inc. For more information contact Karen Zink McCullough
Tel: (908) 534–8897
Fax: (908) 534–1317
e-mail: KarenZM@aol.com

NOVEMBER

November 9 – November 11
PDA Annual Meeting
Booth 103
Washington, DC

For customer service, call (800) LAL–TEST or (508) 540–3444.

For technical service, call (800) 848–3248 or (508) 540–3444.

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