# Substrates for Specific and Quantitative Detection of Anthrax Lethal Factor in Plasma

**N. Shine** and K. Suryadi
List Biological Laboratories
Campbell, CA USA

## Abstract

*Bacillus anthracis* is regarded as a major biological warfare threat. The inhalation form of *Bacillus anthracis* infection can rapidly lead to a blood infection and kill quickly. Antibiotic treatment can clear the bacterium from the host, but this treatment must be initiated rapidly. By the time clinical symptoms are observed, the toxin, which is rapidly produced, may already be present in lethal amounts. There is a critical need for a rapid, accurate, sensitive and simple assay to determine whether infection has occurred thereby permitting immediate treatment. The presence of one of the virulence factors, anthrax lethal factor (LF), in the blood, early in an infection, offers the opportunity for detection prior to catastrophic decline of the patient. This report describes a fast, sensitive, specific and accurate detection method to determine active infection by *Bacillus anthracis* in plasma. LF is an endopeptidase, however, the use of peptidic substrates in plasma is problematic due to the presence of other proteases and the likelihood of nonspecific cleavage of the substrate. Fluorescently labeled peptide substrates which are not cleaved by plasma proteases and thus are specific for LF are described here. The LF is enriched by capture from plasma using an LF antibody-coated microtiter plate, and the captured LF is then exposed to the fluorescent substrate. The amount of cleaved peptide substrate is determined by HPLC with fluorescence detection. Concentration of the LF using the antibody-coated plates allows for the detection of less than 5 pg LF/ml of neat plasma after 2 hours of incubation. Alternately the substrate may be added directly to diluted plasma and cleavage monitored by an increase in fluorescence as a function of time using a fluorescent microplate reader. The limit of detection by this simpler method is1 ng LF/ml of plasma after 5 hours of digestion.

## Introduction

Anthrax is caused by the gram-positive spore-producing bacterium *Bacillus anthracis*. The principal virulence factors are a γ-linked poly-D-glutamic acid (PGA) capsule and a three component exotoxin composed of protective antigen (PA), lethal factor (LF) and edema factor (EF). These proteins act in binary combinations. The complex of PA, the cell binding component, with the LF enzyme, is termed lethal toxin and can cause death. PA and the enzymatic EF together cause skin edema. Secreted PA is cleaved by membrane peptidases. This allows the 63 kDa carboxy terminal fragment to oligomerize to a heptamer or higher. Cleavage of PA is an essential step in exposing the binding sites for EF and LF. The complex enters the cell through endocytosis. PA mediates the transfer of LF and EF to the cytoplasm where these enzymes recognize and alter their targets.

The most lethal manifestation of *Bacillus anthracis* infection is by inhalation. Due to the intentional release of anthrax spores in the bioterrorism attacks of 2001, *B. anthracis* was placed at the top of the CDC list of select agents. Once symptoms are severe and diagnosis is possible, the levels of toxins are dangerously high. A quick, reliable test is needed to detect exposure early in the infection process.

There are a number of markers for inhalation anthrax infection including PA, LF and PGA, however, it has been shown that LF is present earliest in the infection (Boyer AE, et al, Infect Immun. **77**:3432-3441). The method described here takes advantage of the fact that anthrax LF is a zinc endoprotease which cleaves the N-terminus of mitogen-activated protein kinase kinases (MAPKK). Substantial signal amplification can be expected as a result of catalytic turnover of the substrate. Generally, peptide substrates based on the native sequences can be cleaved nonspecifically by other proteases in complex matrices such as plasma and serum. A series of fluorescently labeled peptide substrates based on the native substrate sequence were evaluated for specific cleavage by LF. Unnatural amino acids were substituted in the sequences to eliminate nonspecific cleavage.

This presentation focuses on one sequence subsequently named **MAPKKide® Plus***. The data presented demonstrate that **MAPKKide® Plus** is specific for LF and resistant to cleavage by nonspecific proteases found in plasma. Significantly, this newly designed substrate is highly sensitive to LF and may be used to detect early infections. Two methods for the detection of LF in plasma using this fluorogenic substrate are described: one HPLC-based and one a microplate assay.

*A patent application has been filed for this peptide substrate.

## Materials and Methods

Anthrax lethal factor (Product #169), and the chicken IgY polyclonal anti-LF antibody (Product # 769A) are products of List Biological Laboratories, Inc. The C8 Starwell Maxi Nunc-Immuno Module Plates (cat# 441653) used for LF antibody coating and the dimethyl sulfoxide (DMSO) (cat # TS-20684) were purchased from ThermoScientific. The 96-well, black, flat bottom, non binding plates used for the fluorescent plate assay were from Corning (cat # 3991). Bovine plasma (cat # 7310806) was purchased from Lampire Biological Laboratories.

**Sample Preparation:** Stock solutions of the fluorogenic substrate, **MAPKKide® Plus**, were 1.25 mM in DMSO based on the peptide content determined by elemental analysis. The substrate was diluted in assay buffer: 20 mM HEPES, pH 8.0 containing 0.1% Tween-20. For the microplate assays, the LF was dissolved in neat bovine plasma and diluted 1:10 in assay buffer. For the HPLC assays, the LF was added to neat bovine plasma and not diluted.

**LF Activity Assays:**

***Microplate reader:*** Assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices). The cleavage reaction was initiated by addition of the substrate, **MAPKKide® Plus**. The concentration was optimized to minimize background fluorescence while maintaining measureable cleavage. For all experiments the time-dependent increase in fluorescence was monitored at 37°C hourly for 5 or 6 hours followed by an additional 18 to 18.5 hr overnight incubation at ambient temperature. The excitation wavelength was set to 368 nm and emission to 452 nm with a cutoff filter at 435 nm.

***HPLC:*** The C8 Starwell Maxi Nunc-Immuno Module Plates were coated with 150 µl of a 10 µg/ml solution of a chicken affinity purified polyclonal IgY antibody to anthrax lethal factor (List Prod # 769A). Plates were incubated with the IgY overnight at 2-8°C and washed three times with 0.1M Glycine-HCl, pH 2.5. This wash was included to liberate residual LF retained after the affinity purification of the antibody and was necessary to minimize the background. After 6 washes with PBS containing 0.05% TWEEN-20 (PBST), the anti-LF coated wells were exposed to 300 µl of a series of LF concentrations in neat plasma. The plates were incubated at 22°C for 2 hours. Plates were then washed 6 times with PBST and 250 µl of 1.25 µM **MAPKKide® Plus** was added. The reaction was allowed to proceed for 2, 3.5, and 5 hours at 37°C and overnight at ambient temperature. At each time point 200 µl of the reaction mixture was removed from replicate wells and placed in HPLC sample vials.

HPLC was performed using a Zorbax Eclipse Plus C18 reverse phase column, 4.6 x 150 mm (Agilent) and a guard column containing the same resin in a Varian ProStar HPLC system (Agilent). Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The 16 minute HPLC method was as follows: 25% B for 0.75 minutes; 25 to 45% B in 4.75 minutes; 45 to 100% B in 0.75 minutes; 100% B for 3.75 minutes; 100 to 25%B in 0.67 minutes and 5.34 minute equilibration with 25% B. The column effluent was monitored using an Hitachi fluorescence detector with excitation set to 350 nm and emission at 450 nm to detect the free coumarin fluorophore cleaved from **MAPKKide® Plus**. The injection volume was 20 µl. The 7-amido-4-methylcoumarin (AMC) peak retention time was 4.8 minutes.

## Results

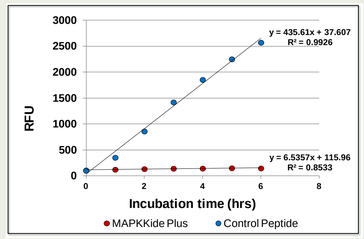
### A. MAPKKide® Plus Specificity

The change in fluorescence for 2.5 µM **MAPKKide® Plus** in the presence of 1:10 diluted plasma without LF is compared to a peptide with the same sequence but lacking the unnatural amino acids (Control Peptide). The results confirm that **MAPKKide® Plus** is specific for LF and that the peptide lacking the unnatural amino acids is non-specifically cleaved by plasma proteases (**Table 1, Figure 1**).

**Table 1:** Fluorescence observed for substrates in 1:10 diluted plasma without LF.

| Time (hrs) | Temperature ( C) | RFU | |
|---|---|---|---|
| | | MAPKKide® Plus | Control Peptide |
| 0 | 37 | 108 | 102 |
| 1 | 37 | 123 | 353 |
| 2 | 37 | 134 | 860 |
| 3 | 37 | 142 | 1420 |
| 4 | 37 | 144 | 1854 |
| 5 | 37 | 151 | 2251 |
| 6 | 37 | 147 | 2571 |
| 21 | ambient | 163 | 4074 |
| 39 | ambient | 169 | 3920 |

**Figure 1:** Fluorescence (RFU) as a function of time for **MAPKKide® Plus** (**red**) and Control Peptide (**blue**) in 1:10 plasma at 37 °C in the absence of LF.




### B. HPLC Method Using Antibody Capture to Concentrate the LF.

In order to optimize the detection of low levels of LF in plasma, the LF was enriched using an affinity purified polyclonal antibody coated on a 96-well microtiter plate. In addition to enrichment of the LF, the method allowed capture of small amounts of LF from plasma without dilution. It was found that since the antibody had been affinity purified using LF there were low levels of residual LF remaining with the antibody. Several washes with 0.1M Glycine-HCl, pH 2.5 were included to liberate residual LF retained on the antibody coated plate. This wash minimized the background observed in plasma samples without LF. After addition of the **MAPKKide® Plus**, samples were monitored using HPLC with fluorescence detection after 2, 3.5 and 5 hrs of incubation at 37°C. Representative chromatograms from the digestion of **MAPKKide® Plus** by LF in neat plasma after 2 hours at 37°C are shown in **Figure 2.** The average peak areas are given in **Table 2.**

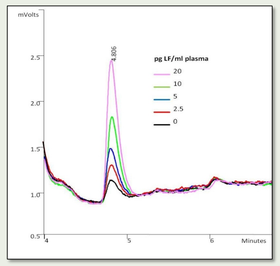



**Figure 2:** Chromatograms from the digestion of **MAPKKide® Plus** by 0 (black), 2.5 (red), 5 (blue), 10 (green), and 20 (pink) pg of LF/ml of neat plasma after 2 hours at 37°C.

**Table 2:** Detection of LF in neat plasma

| LF (pg/ml plasma) | Average Peak Area* | | | |
|---|---|---|---|---|
| | 2 hour digest | 3.5 hour digest | 5 hour digest | Overnight digest (RT) |
| 0 | 2380 | 3840 | 5033 | 7835 |
| 2.5 | 3696 | 6294 | 8788 | 14515 |
| 5 | 5459 | 8976 | 12218 | 21377 |
| 10 | 8875 | 14448 | 20487 | 34083 |
| 20 | 14311 | 25587 | 34502 | 62133 |

*The average peak areas for samples containing no LF (n=6) and for samples containing 2.5, 5, 10 and 20 pg LF/ml plasma (n=2).

A plot of the peak areas as a function of LF concentration in the plasma is shown in **Figure 3**. Analysis for each response curve is given in **Table 3**. The response at each time point is linear.

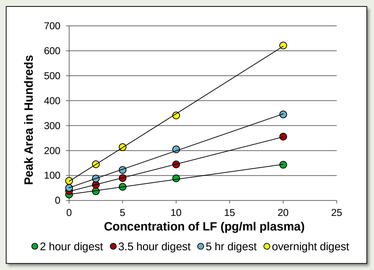



**Figure 3:** Plot of peak areas versus concentration of LF (pg/ml plasma) for 2 hour (green), 3.5 hour (red), 5 hour (blue) and overnight (yellow) digestion of MAPKKide® Plus.

**Table 3:** Slope, intercept, and correlation coefficients for each incubation time.

| Measurement | Incubation Time (hr) | | | |
|---|---|---|---|---|
| | 2 | 3.5 | 5 | Overnight (RT) |
| Slope (Peak Area/pg LF) | 604 | 1093 | 1481 | 2708 |
| Intercept (Peak area) | 2411 | 3632 | 5098 | 7676 |
| $R^2$ | 0.9971 | 0.9997 | 0.9991 | 0.9997 |
| Std deviation of the blanks | 785 | 817 | 680 | 469 |
| 3x standard deviation + the average | 4734 | 6290 | 7074 | 9242 |
| LOD (pg/ml plasma) | 3.84 | 2.43 | 1.33 | 0.58 |

The limit of detection for each incubation time was estimated from the normal distribution (3 standard deviations) of blank plasma samples (0 pg LF; n=6), calculated as pg LF/ml plasma from the standard curve generated at each incubation time. This data is shown in **Table 3**. The results indicate that the limit of detection at all time points is less than 5 pg LF/ml of neat plasma.

**Kinetic analysis of the LF activity by HPLC.** The amount of LF in an unknown sample can be determined from any single time point as shown above. However, such results might be subject to false positives due to uncertain background fluorescence. Alternately, the results can be obtained by monitoring the reaction rate, i.e. the increase in peak area as a function of time (**Figure 4**). Each concentration of LF yields a unique slope (rate) which is proportional to the concentration (**Figure 5**). The limit of detection by this method is also around 5 pg LF/ml neat plasma.

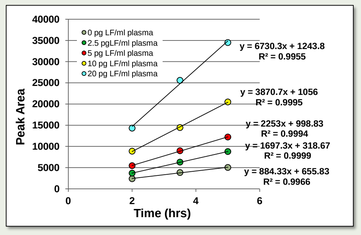



**Figure 4:** Plot of peak area versus incubation time for 0 (gray), 2.5 (green), 5 (red), 10 (yellow), and 20 (aqua) pg LF/ml plasma.

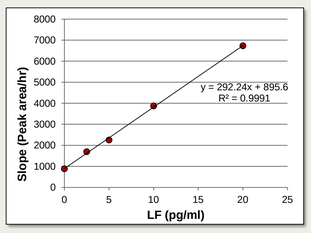



**Figure 5**: Plot of the slopes as a function of the concentration of LF (pg/ml plasma).

## Conclusion

This report describes a fast, sensitive, specific and accurate HPLC method to detect active infection by *Bacillus anthracis* in plasma at very early stages of intoxication.