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Product #532

CERTIFICATE OF ANALYSIS
MAPKKide® Plus (AMC)
Specific Substrate for Anthrax Lethal Factor
Lot #5321A1

Contents

Each vial of MAPKKide® Plus* (AMC), a substrate specific for Anthrax Lethal Factor (LF), contains 100 nmoles of lyophilized peptide. MAPKKide® Plus* (AMC) is a short peptide containing 7-amido-4-methylcoumarin (AMC). This lyophilized powder is stoppered under vacuum.

* Patent pending

Reconstitution

A small amount of peptide has been lyophilized in each vial. During lyophilization and transportation, this material may be distributed throughout the vial. Since it is common practice to reconstitute peptide in a small volume of solvent, visually locate the powder and, if necessary, shake it to the bottom of the vial prior to adding the solvent. It is recommended that initial stock solutions be made in DMSO to ensure total recovery of the lyophilized peptide. Cover the vial with foil to protect from light.

Concentration

Peptide content is obtained from nitrogen determination.

Analysis

The peptide is ≥95% pure as determined by reverse phase HPLC. The expected molecular weight was obtained by mass spectrometry.

Assay Conditions

The protocol for quantitative detection of anthrax lethal factor (LF) in plasma using MAPKKide® Plus* (AMC), Product #532 is attached. The procedure for the specific detection of active infection by *Bacillus anthracis* in plasma is described. This fluorescently labeled peptide substrate, MAPKKide® Plus* (AMC), is specific for anthrax lethal factor (LF) and resistant to cleavage by nonspecific proteases found in plasma. MAPKKide® Plus* (AMC) 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: an HPLC-based assay and a microplate assay.

The HPLC-based assay is described in "Substrates for Specific and Quantitative Detection of Anthrax Lethal Factor in Plasma" which is posted on the List Biological Laboratories, Inc. website: www.listlabs.com, under Product #532.

Handling

Good laboratory technique should be employed in the safe handling of this product. Wear appropriate laboratory attire including lab coat, gloves and safety glasses. Nitrile gloves are recommended when handling lyophilized material.

The product is intended for research purposes by qualified personnel. It is not intended for use in humans or as a diagnostic agent. List Biological Laboratories, Inc. is not liable for any damages resulting from the misuse or handling of this product.

Production: KS Date: 9/21/16 Management: NS Date: 9/21/2016 QA/QC: JC Date: 9/21/16

Made in U.S.A.

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Protocol For Quantitative Detection of Anthrax Lethal Factor (LF) in Plasma Using MAPKKide® Plus* (AMC), Prod #532

Assay Description:

This protocol describes the procedure for the specific detection of active infection by *Bacillus anthracis* in plasma. A fluorescently labeled peptide substrate, MAPKKide® Plus*, is specific for anthrax lethal factor (LF) and resistant to cleavage by nonspecific proteases found in plasma. MAPKKide® Plus* 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: an HPLC-based assay (pgs 2-5) and a microplate assay (pgs 6-9).

Briefly, anthrax lethal factor (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, MAPKKide® Plus*. 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 5, less than 2 and less than 1 pg LF/ml of neat plasma after 2, 3.5 and 5 hours of incubation, respectively.

Alternately the substrate may be added directly to diluted plasma in a microplate 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 is 1 ng LF/ml of plasma after 5 hours of digestion.

The HPLC-based assay is described in a poster entitled "Substrates for Specific and Quantitative Detection of Anthrax Lethal Factor in Plasma" which is posted on List Labs website: www.listlabs.com, under MAPKKide® Plus*, Prod #532. Data tables found in the poster demonstrate the detection of anthrax lethal factor in plasma.

* Patent pending

HPLC Method Using Antibody Capture to Concentrate LF

I. PURPOSE:

The procedure for the HPLC method using antibody capture to concentrate LF, and with detection using MAPKKide® Plus (AMC), is outlined below. Results obtained using bovine plasma are presented in the poster entitled "Substrates for Specific and Quantitative Detection of Anthrax Lethal Factor in Plasma" that can be found on List Labs website:www.listlabs.com under Prod #532.

II. MATERIALS

Material	Manufacturer	Cat #
C8 Starwell Maxi Nunc-Immuno Module Plates	ThermoScientific	441653
MAPKKide® Plus (AMC)	List	532
Anthrax Lethal Factor (LF)	List	169A
Chicken IgY polyclonal anti-LF antibody	List	769A
Bovine Plasma	Lampire	7310806
PBS, pH 7.4, 10x	Life Technologies	70011044
PBS, pH 7.4	Life Technologies	10010031
TWEEN 20	Bio-Rad	170-6531
DMSO	Pierce	TS-20684
HEPES, free acid	Sigma	H4034
HEPES, potassium salt	Sigma	H0527
Sodium Carbonate, anhydrous	Mallinckrodt	7527
Glycine	Bio-Rad	161-0718

Solution	Description
Assay Buffer	20 mM HEPES, pH 8.0 containing 0.1% TWEEN-20
Plate Coating Buffer	0.05M Sodium carbonate-bicarbonate, pH 9.6
Wash Buffer #1	0.1 M Glycine-HCl, pH 2.5
Wash Buffer #2	PBS, pH 7.4 containing 0.05% TWEEN-20
HPLC Buffer A	0.1% TFA
HPLC Buffer B	0.1% TFA in Acetonitrile

III. PROCEDURE:

A. Sample preparation:

1. MAPKKide® Plus:

A stock solution of MAPKKide® Plus was prepared at 1.25 mM in DMSO by adding 80 µl of DMSO to 1 vial of 100 nmoles MAPKKide® Plus, Product# 532. The substrate was subsequently diluted 1:5 then 1:10 and finally 1:20 to obtain the amount of 1.25 µM needed for a given assay. The diluent was 20 mM HEPES, pH 8.0 containing 0.1% Tween-20. A total of 250 µl of 1.25 µM MAPKKide® Plus is added to each well.

2. Anthrax Lethal Factor in Plasma:

One vial of 100 µg of lethal factor, Prod # 169A was dissolved in 100 µl of 20 mM HEPES, pH 8.0 containing 0.1% TWEEN-20 to make a 1 mg/ml solution. Four concentrations of LF in neat plasma; 2.5, 5, 10 and 20 pg LF/ml plasma are prepared in duplicate to generate a standard curve. A 300 µl aliquot of each amount is added to the appropriate wells. Six plasma samples without LF are included as negative controls.

Each test plasma sample will be added to the appropriately assigned wells.

3. Anthrax Lethal Factor Chicken Antibodies

The anti-LF antibody (List Prod # 769A) is diluted to 10 µg/ml in 0.05M sodium carbonate-bicarbonate, pH 9.6 buffer. Each well is coated with 150 µl of the 10 µg/ml solution for a final coating of 1.5 µg IgY/well.

B. LF Activity Assay:

1. Plate coating:

C8 Starwell Maxi Nunc-Immuno Module Plates are used in this assay to increase sensitivity. The wells of these plates have fins to increase the surface area and enhance performance. The C8 Starwell Maxi Nunc-Immuno Module Plates are coated with 150 µl of a 10 µg/ml solution of the chicken affinity purified polyclonal IgY antibody to anthrax lethal factor (List Prod # 769A) in 0.05M sodium carbonate/bicarbonate, pH 9.6 buffer. Plates are incubated with the IgY overnight at 2-8°C.

2. Washing with Wash Buffer #1:

The plate is washed three times with 0.1M Glycine-HCl, pH 2.5. This wash is included to liberate residual LF retained after the affinity purification of the antibody and is necessary to minimize the background.

3. **Washing with Wash Buffer #2.**

The anti-LF coated wells are then washed 6 times with 300 µl PBS containing 0.05% TWEEN-20 (PBST).

4. **Addition of samples, incubation, and wash.**

Three hundred microliters of each of the standard samples containing 0, 2.5, 5.0, 10 and 20 pg LF/ml plasma as well as the test samples are added to their assigned wells.

The plate is incubated at 22°C for 2 hours and then washed 6 times with PBST.

5. **Addition of MAPKKide® Plus and incubation with the captured LF.**

MAPKKide® Plus (250 µl of 1.25 µM) is added to each well. The plate is sealed and the reaction is allowed to proceed for 2, 3.5, and 5 hours at 37°C.

6. **Preparation of HPLC samples.**

At each time point; 2, 3.5 and 5 hrs, 200 µl of the reaction mixture is removed from replicate wells and placed in HPLC sample vials.

7. **HPLC**

HPLC is performed using a Zorbax Eclipse Plus C18 reverse phase column, 4.6 x 150mm (Agilent) with a guard column containing the same resin. Buffer A is 0.1% TFA in water and Buffer B is 0.1% TFA in acetonitrile. The 16 minute HPLC method is 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 is monitored using an Hitachi fluorescence detector with excitation at 350 nm and emission at 450 nm to detect the free coumarin fluorophore cleaved from MAPKKide® Plus. The injection volume is 20 µl. The 7-amido-4-methylcoumarin peak retention time is 4.78 minutes.

The HPLC-based assay is described in the poster entitled "Substrates for Specific and Quantitative Detection of Anthrax Lethal Factor in Plasma". Representative chromatograms are shown in **Figure 2**. The average peak areas for the cleaved fluorophor obtained for 2.5, 5, 10, and 20 pg LF/ml plasma are given in **Table 2.**

C. Data Analysis:

The poster includes a plot of the peak areas observed for the cleaved 7-amido-4- methyl coumarin as a function of LF concentration in plasma for each time point, 2, 3.5, 5 hours and overnight (**Figure 3**). An analysis of each response curve is given in **Table 3** including the limit of detection determined from 3 times the standard deviation plus the average of the negative controls. For all time points the limit of detection is less than 5 pg LF/ml plasma.

The amount of LF in an unknown sample can be determined from any single time point. However, such results might be subject to false positives due to uncertain background fluorescence. Alternately, a kinetic analysis of the LF activity 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. This is shown in **Figure 5**. The limit of detection by this method is also around 5 pg LF/ml neat plasma.

Detection of LF using the microplate assay method

I. PURPOSE:

MAPKKide® Plus (AMC) can be used for detection of anthrax lethal factor in a simpler microplate assay. This method is outlined below.

II. MATERIALS

Material	Manufacturer	Cat #
96-well, black, flat bottom, non binding plates	Corning	3991
MAPKKide® Plus (AMC)	List	532
Anthrax Lethal Factor (LF)	List	169A
Bovine plasma	Lampire	7310806
50% TWEEN-20	Life Technologies	00-3005
DMSO	Pierce	TS-20684
HEPES, free acid	Sigma	H4034
HEPES, potassium salt	Sigma	H0527

Solution	Description
Assay Buffer	20 mM HEPES, pH 8.0 containing 0.1% TWEEN-20

III. PROCEDURE:

A. Sample preparation:

1. MAPKKide® Plus:

A stock solution of MAPKKide® Plus is prepared at 1.25 mM in DMSO by adding 80 µl of DMSO to 1 vial of 100 nmoles MAPKKide® Plus, Product# 532. The substrate is subsequently diluted 1:10 followed by 1:10 to obtain 12.5 µM substrate in the optimized assay buffer: 20 mM HEPES, pH 8.0 containing 0.1% Tween-20. A total of 20 µl of 12.5 µM MAPKKide® Plus is added to each well containing 180 µl of diluted sample plasma. The final concentration of MAPKKide® Plus is 1.25 µM per well.

2. Anthrax Lethal Factor in Plasma:

One vial of 100 µg of lethal factor, Prod # 169A is dissolved in 100 µl of 20 mM HEPES, pH 8.0 containing 0.1% TWEEN-20 to make a 1 mg/ml solution. The plasma samples are diluted 1:10 with the assay buffer. The standard curve is generated using six serial dilutions in neat plasma from 10 ng/ml to 0.1 ng/ml. These samples are then diluted 1:10 to obtain seven samples starting with 1000 to 10 pg/ml LF. A 180 µl aliquot of each standard or test sample is added to the appropriate well.

B. LF Activity Assay:

In this simpler method the substrate is added directly to diluted plasma in replicate microplate wells and the fluorescence monitored hourly.

Assays are performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices) or an equivalent fluorescent plate reader. The LF standard solutions in 1:10 diluted plasma and the test samples also diluted 1:10 (180 µl) are added to the appropriate wells. The cleavage reaction is initiated by addition of the substrate (20 µl of a 12.5 µM MAPKKide® Plus). The substrate concentration is optimized to minimize background while maintaining measureable cleavage. The time-dependent increase in fluorescence is monitored at 37°C hourly for 5 hours followed by an additional 18 to 18.5 hr overnight incubation at ambient temperature. The excitation wavelength is set to 368 nm and emission to 452 nm with a cutoff filter at 435 nm for the 7-amido-4-methyl-coumarin containing substrate.

C. Sample Data

A plot of the cleavage of 1.25 µM MAPKKide® Plus at 5 hrs as a function of LF concentration is shown in **Figure 1**. Each RFU value for wells containing LF represents the average of 4 replicate wells and 3 reads of the plate. Each value for wells containing no LF is the average of 12 replicate wells and 3 reads of the plate. The amount of cleaved peptide is linearly proportional to the amount of LF present in the diluted plasma from 10 to1000 pg LF/ml of diluted plasma.

The limit of detection is calculated from the normal distribution (2.7 standard deviations) of blank samples (n=12) (**Table 1**). The limit of detection after 5 hrs and 24 hrs digestion is approximately 1 ng LF/ml neat plasma (**Table 1**). As can be seen in the table there is no improvement in the limit of detection after the 5 hr digestion.

Table 1. Digestion of MAPKKide® Plus by different concentrations of LF.

LF (pg/ml) in 1:10 diluted plasma	Substrate RFUs after digest	
	5 hrs	24 hrs
1000	256	354
500	190	248
200	155	186
100	141	161
50	137	152
20	137	149
10	140	147
0	129	138
Standard deviation of the blanks	7.29	7.50
LOD (pg/ml of 1:10 diluted plasma)	60	77
LOD x 10 (pg/ml) neat plasma	600	770

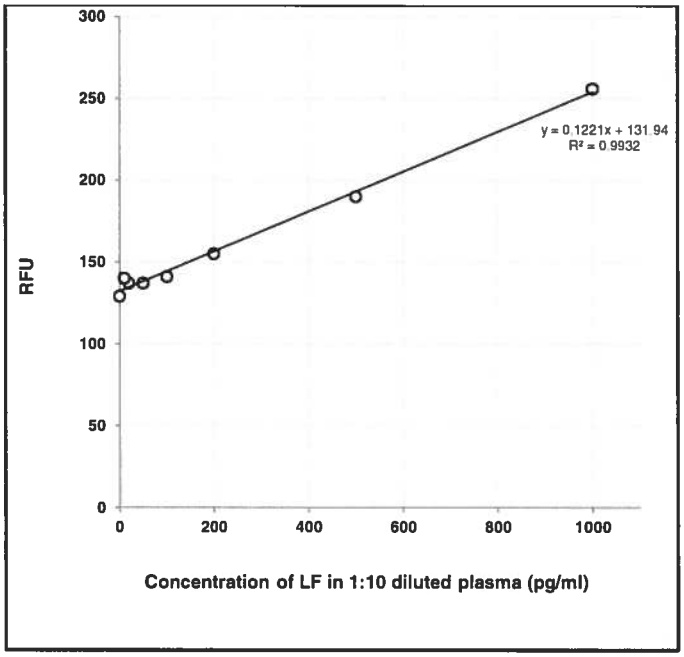

Figure 1: MAPKKide® Plus cleavage at 5 hrs as a function of LF concentration in 1:10 diluted plasma.

D. Kinetic Analysis of the LF activity using the microplate assay.

The data described above is for a single endpoint after 5 hours digestion by LF in 1:10 diluted plasma. This results in a limit of detection around 1 ng LF/ml in neat plasma MAPKKide® Plus. Subsequently, the digestion of MAPKKide® Plus by LF in the diluted plasma was repeated 6 times. A kinetic analysis of the data where the cleavage of MAPKKide® Plus as a function of time for each concentration of LF is shown in **Table 2**. The data presented are the averages of 6 data sets, each with 4 replicates for each sample which contains LF and 12 blank replicates per data set and 3 plate reads at each time point. A plot of the fluorescence (RFU) as a function of time of digestion for individual LF concentrations (pg/ml) is shown in **Figure 2**. The slopes of the curves at each concentration (see **Table 2** last row) can be plotted as a function of the concentration of LF and the resulting slope then used to determine the amount of LF present in unknown samples (**Figure 3**). The advantage of using the kinetic data is that any differences in the background observed for different sources of plasma are accommodated by this method.

Table 2. Digestion of MAPKKide® Plus by different concentrations of LF in 1:10 diluted plasma as a function of time.

Time (hours)	Substrate RFUs for LF (pg/ml)							
	1000	500	200	100	50	20	10	0
1	114	97	92	88	88	87	89	91
2	148	116	101	96	95	93	94	95
3	177	131	111	103	100	99	98	101
4	202	142	115	105	102	99	100	102
5	224	157	125	111	106	102	103	105
Slope (RFU/hr)	**27.48**	**14.72**	**8.06**	**5.46**	**4.30**	**3.61**	**3.35**	**3.39**

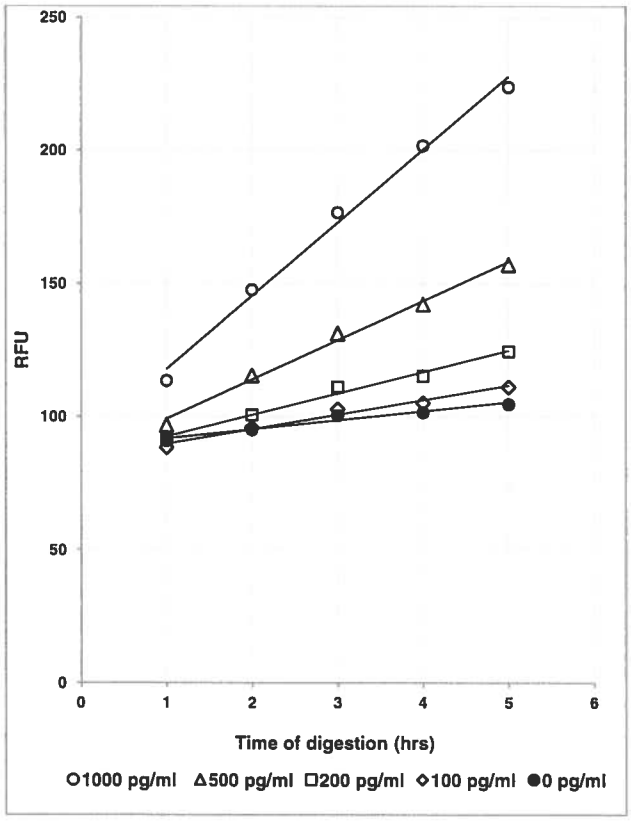

Figure 2: Cleavage MAPKKide® Plus by a series of concentrations of LF(pg/ml) in 1:10 diluted plasma as a function of time.

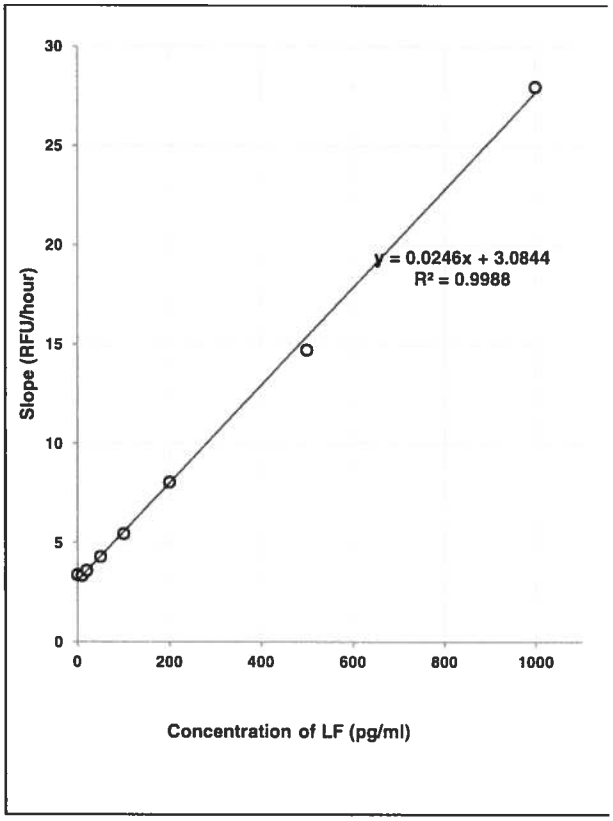

Figure 3: Slope in RFU/hour as a function of concentration of LF in 1:10 diluted plasma.

The slope is 0.0246 RFU/hr/pg/ml. The limit of detection, determined from the average standard deviation for the slopes obtained for the 6 blanks times 3, was 80 pg/ml. This is equivalent to 800 pg LF/ml in neat plasma.