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PI: [redacted]	Title: Antigen Detection assay for the Diagnosis of Melioidosis	
Received: 12/05/2013	FOA: PA10-124	Council: 05/2014
Competition ID: ADOBE-FORMS-B1	FOA Title: NIAID ADVANCED TECHNOLOGY STTR (NIAID-AT-STTR [R41/R42])	
2 R42 AI102482-03	Dual:	Accession Number: 3650491
IPF: 3966401	Organization: INBIOS INTERNATIONAL, INC.	
Former Number:	Department:	
IRG/SRG: ZRG1 IDM-V (12)B	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 3: [redacted] Year 4: [redacted] Year 5: [redacted]	Animals: N Humans: Y Clinical Trial: N Current HS Code: E4 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

Additions for Review

Accepted Publication

Accepted manuscript news

Post-submission supplemental material. Information about manuscript accepted for publication.

This sample is a multi-page PDF document.

Continue scrolling to see the remainder of the application, navigate using the bookmarks in your PDF reader of choice, or skip to page 4 for the Table of Contents.

If you have any questions, contact deaweb@niaid.nih.gov .

OMB Number: 4040-0001
Expiration Date: 06/30/2011

APPLICATION FOR FEDERAL ASSISTANCE

SF 424 (R&R)

3. DATE RECEIVED BY STATE

State Application Identifier

1. * TYPE OF SUBMISSION

☐ Pre-application ☐ Application ☒ Changed/Corrected Application

4. a. Federal Identifier [redacted]

b. Agency Routing Identifier

2. DATE SUBMITTED 12/04/2013

Applicant Identifier

5. APPLICANT INFORMATION

*** Organizational DUNS:** [redacted]

* Legal Name: InBios International Inc

Department:

Division:

* Street1: 562 Ist Avenue S., Suite 600

Street2:

* City: Seattle

County / Parish: King

* State: WA: Washington

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 98104-3829

Person to be contacted on matters involving this application

Prefix: [redacted]

* First Name: [redacted]

Middle Name:

* Last Name: [redacted]

Suffix:

* Phone Number: [redacted]

Fax Number: [redacted]

Email: [redacted]

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN):* [redacted]

7. * TYPE OF APPLICANT: R: Small Business

Other (Specify):

Small Business Organization Type ☒ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:

☒ New ☐ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY: National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:

TITLE:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

Antigen Detection assay for the Diagnosis of Melioidosis

12. PROPOSED PROJECT:

* Start Date: 07/01/2014

* Ending Date: 06/30/2017

*** 13. CONGRESSIONAL DISTRICT OF APPLICANT** WA 007

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: [redacted]

* First Name: [redacted]

Middle Name:

* Last Name: [redacted]

Suffix: [redacted]

Position/Title: [redacted]

* Organization Name: InBios International Inc

Department:

Division:

* Street1: 562 Ist Avenue S., Suite 600

Street2:

* City: Seattle

County / Parish: King

* State: WA: Washington

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 98104-3829

* Phone Number: [redacted]

Fax Number: [redacted]

* Email: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	[redacted]
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	[redacted]
d. Estimated Program Income	0.00

16. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 18, Section 1001)

☒ *** I agree**

**** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.***

18. SFLLL or other Explanatory Documentation

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: * First Name: [redacted] Middle Name:

* Last Name: [redacted] Suffix:

* Position/Title: [redacted]

* Organization: InBios International Inc

Department: Division:

* Street1: 562 Ist Avenue S., Suite 600

Street2:

* City: Seattle County / Parish: King

* State: WA: Washington Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 98104-3829

* Phone Number: [redacted] Fax Number: [redacted]

* Email: [redacted]

*** Signature of Authorized Representative** [redacted]

*** Date Signed** 12/05/2013

20. Pre-application Add Attachment | Delete Attachment | View Attachment

424 R&R and PHS-398 Specific
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OMB Number: 4040-0010
Expiration Date: 08/31/2011

Project/Performance Site Location(s)

Project/Performance Site Primary Location

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: InBios International

DUNS Number: [redacted]

* Street1: 562 1st Avenue S., Suite 600

Street2:

* City: Seattle

County: King

* State: WA: Washington

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 98104-3829

* Project/ Performance Site Congressional District: WA 007

Project/Performance Site Location 1

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: University of Nevada, Reno

DUNS Number: [redacted]

* Street1: 1664 North Virginia Street

Street2:

* City: Reno

County: Washoe

* State: NV: Nevada

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 89557-0240

* Project/ Performance Site Congressional District: NV 002

Project/Performance Site Location 2

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: Menzies School of Health Research-Royal Darwin Hospital

DUNS Number:

* Street1: PO Box 41096

Street2:

* City: Casuarina

County:

* State:

Province: Northern Territory

* Country: AUS: AUSTRALIA

* ZIP / Postal Code: 0811

* Project/ Performance Site Congressional District:

Performance Sites

Project/Performance Site Location(s)

Project/Performance Site Location 3

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: Mahidol University

DUNS Number:

* Street1: 420/6 Ratchawithi Road

Street2:

* City: Bangkok

County:

* State:

Province:

* Country: THA: THAILAND

* ZIP / Postal Code: 10400

* Project/ Performance Site Congressional District:

Project/Performance Site Location 4

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: Health Protection Agency

DUNS Number:

* Street1: Research Department

Street2: Porton Down

* City: Salisbury

County: Wiltshire

* State:

Province:

* Country: GBR: UNITED KINGDOM

* ZIP / Postal Code: SP4 0JG

* Project/ Performance Site Congressional District:

Project/Performance Site Location 5

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: Menzies School of Health Research-Royal Darwin Hospital

DUNS Number:

* Street1: PO Box 41096

Street2:

* City: Casuarina

County:

* State:

Province: Northern Territories

* Country: AUS: AUSTRALIA

* ZIP / Postal Code: 0811

* Project/ Performance Site Congressional District:

Project/Performance Site Location(s)

Additional Location(s) Add Attachment Delete Attachment View Attachment

[redacted]

RESEARCH & RELATED Other Project Information

OMB Number: 4040-0001
Expiration Date: 6/30/2016

1. Are Human Subjects Involved? ☒ Yes ☐ No

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☒ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☒ 4 ☐ 5 ☐ 6

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Human Subject Assurance Number: [redacted]

2. Are Vertebrate Animals Used? ☐ Yes ☒ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☐ Yes ☐ No

IACUC Approval Date:

Animal Welfare Assurance Number:

3. Is proprietary/privileged information included in the application? ☒ Yes ☐ No

4.a. Does this Project Have an Actual or Potential Impact - positive or negative - on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. Does this project involve activities outside of the United States or partnerships with international collaborators? ☒ Yes ☐ No

6.a. If yes, identify countries: Australia, Thailand, UK

6.b. Optional Explanation: Non STTR funds will be used for foreign sites

7. Project Summary/Abstract 1241-Phase II Abstract.pdf Add Attachment Delete Attachment View Attachment

8. Project Narrative 1242-Phase II Project Narrative.pdf Add Attachment Delete Attachment View Attachment

9. Bibliography & References Cited 1243-Bp Phase II REferences.pdf Add Attachment Delete Attachment View Attachment

10. Facilities & Other Resources 1244-Burk_FACILITIES_Nov_2013.pdf Add Attachment Delete Attachment View Attachment

11. Equipment 1245-Burk_Equipment_UNR_Nov_2013.pdf Add Attachment Delete Attachment View Attachment

12. Other Attachments Add Attachments Delete Attachments View Attachments

Abstract

Burkholderia pseudomallei is a Gram-negative bacterium that is the causative agent of melioidosis. The bacterium causes significant morbidity and mortality in tropical regions and endemic areas are expanding. Melioidosis is difficult to treat and diagnose due to the fact that *B. pseudomallei* is resistant to common antibiotics and symptoms are non-specific. Mortality rates reach 45% and 20% in Thailand and Australia respectively, even with antibiotic treatment. Currently, there is no validated diagnostic product for the diagnosis of melioidosis. Culturing of patient samples is the "gold standard" for diagnosis, however this can take 3-7 days. Our goal is to develop a rapid point-of-care lateral flow immunoassay (LFI) for the detection of the *B. pseudomallei* capsular antigen (CPS) directly from patient samples. Phase I STTR Specific Aims focused on producing a library of anti-CPS monoclonal antibodies (mAbs), optimizing the LFI components and sample preparation, and performing a feasibility study on archived melioidosis patient samples. The objectives of this Phase II proposal is advanced development and validation of the prototype AMD LFI that incorporates a higher affinity CPS mAb. By completing these goals we hope to produce an optimized LFI that possesses sensitivity equal to or greater than culturing of patient samples. To achieve these goals we have established an experienced team. A strong partnership exists between Dr. AuCoin's laboratory and InBios International that has lead to the development of an encouraging prototype LFI. In addition, both groups will work closely with experts in endemic areas (Thailand and Australia) who will be conducting a large preclinical evaluation of the LFI with different patient sample types.

Project Narrative

Melioidosis is a rapidly progressing and frequently fatal disease if left untreated. *Burkholderia pseudomallei* is the causative agent of melioidosis; this soil dwelling bacterium has been listed as a Tier I Select Agent by the CDC. In the endemic areas of Thailand and northern Australia melioidosis is a major public health concern. Rapid diagnosis of melioidosis is needed in order to properly administer effective treatment with specific antibiotics. At present, the diagnostic “gold standard” is culturing of patient samples, which takes 3-7 days and significantly delays administration of proper treatment. In this proposal, we offer to develop a point-of-care, cost-effective, and rapid diagnostic for melioidosis that can deliver results within minutes. Such a rapid diagnostic test will have the capability to detect acute *B. pseudomallei* infections in endemic areas as well as following a bioterrorist attack.

FACILITIES

InBios International

InBios International Inc. is a ten-year old biotechnology company focusing on developing novel diagnostic products and reagents based on proprietary antigens and technologies. The company's objective is to become a leader in the development and manufacturing of novel diagnostic tests for infectious diseases. The company specializes in development of diagnostic products for diagnosis of less frequent infectious diseases that may be overlooked due to a perceived small market size. The company's founders and employees have extensive experience in developing, manufacturing and marketing diagnostic tests worldwide. In-house expertise includes various aspects of chemistry and immunology including immunochemistry, protein expression/purification, and their manipulation. InBios is also specialized in particle and conjugation chemistry that includes our confidential gold-based technology. The company manufacturing, quality control and regulatory staff are experienced in producing large-scale lots of rapid and ELISA based assay reproducibly. The company is FDA registered and GMP compliant.

Laboratory: InBios occupies approximately 9,000 sq ft of laboratory space on three floors of a seven story secure building in downtown Seattle, WA. The core facility is designed for molecular, immunological, and biochemical research in support of developing and manufacturing ELISA and lateral flow based immunoassay diagnostics. A minimum of 7 separate benches exists for technician and scientist work. There exists a standard laboratory fully equipped with a BSL-2 biosafety hood for working with human biological fluids, as well as all equipment necessary for assay development. A dedicated dry room that is humidity (<15% RH) and temperature controlled, and everything needed for analyte conjugation is housed at InBios. A separate molecular biology laboratory and cold room exist for all protein expression/purification work. InBios also has a tissue culture room for culturing monoclonal antibody cell lines and a dedicated Quality Control room/staff.

Computer: All scientific staff have dedicated computers. All computers are on a company server providing regular backups and access to various peripheral devices. They are supported by DNA sequence analysis and other scientific, data and word processing software and also provide internet and email access.

Office: In addition to lab space, approximately 3,000 sq. ft. of office spaces exists at InBios. The facility contains office space for all scientists, administrative staff (including project manager, administrative assistant, accountant, and document control specialist) as well as general use areas including conference rooms; library space and staff lounge. All office supplies are kept on-hand, in addition to copiers, projector, scanner, fax, and telephone capabilities.

Other: There is full-service media prep; glass wash and autoclave area located within InBios laboratories. InBios also has access to photography, microscopy, machine, electronic shops, and other facilities and services at local research companies in the area on an as needed and fee-for use basis.

University of Nevada, Reno

Studies at UNR will be done in the Laboratory for Diagnostics Discovery and Development (LDDD) located in the newly constructed (October, 2010) Center for Molecular Medicine. The LDDD houses the research programs of Dr. David AuCoin and Dr. Thomas Kozel. LDDD is dedicated to identification of targets for diagnosis of infectious diseases and the production of reagents for immunoassay construction. LDDD consists of 2,850 sq. ft. of bench laboratory space that includes 1,300 sq. ft. of general laboratory space and 1,550 sq. ft. of specialty laboratory space.

Specialty laboratories within the LDDD include i) a hybridoma production laboratory, ii) a tissue culture laboratory dedicated to large-scale production of engineered antibodies, iii) a serology laboratory for analysis of human and non-human primate samples under BSL-2 conditions, iv) a BSL-2 laboratory dedicated to growth of bacteria and fungi, and v) a separate laboratory that is capable of functioning under GLP.

Capabilities of the LDDD in routine use at this time include i) immunization of mice, fusions and screening for production of mAbs; ii) subclass switching of mAbs to optimize antibody performance; iii) large-scale (gram

amounts) production of mAbs; iv) antibody purification, fragmentation and labeling (enzyme, colloidal gold and fluorophore); v) molecular characterization (sequencing of variable region genes) and engineering of mAbs; vi) affinity enhancement via phage display; vii) production of engineered antibodies in CHO cells; viii) antibody characterization, including evaluation with Biacore; ix) immunoassay construction and evaluation, including ELISA and lateral flow immunochromatography; x) production and purification of microbial antigens; and ix) identification of microbial protein antigens by 2D gel immunoblotting and sequencing of peptides via mass spectrometry at the UNR Proteomics Core Laboratory.

The Center for Molecular Medicine includes a state-of-art vivariam that is suitable for immunization of mice for mAb production. Also included in the CMM is a BSL-3 suite that meets the security and safety requirements of the Patriot Act. Designed through consultation with Ted Tram (World BioHazTec Corp.), commissioning and CDC certification of the facility are scheduled for January 2012.

Mahidol-Oxford Tropical Medicine Research Unit, Mahidol University, Bangkok Thailand

Laboratory: Dr. Limmathurotsakul and collogues have full access to a new 5,400 sq. ft. microbiology laboratory which includes a 1216 sq. ft. BSL3 laboratory. The laboratory is located on the second floor of the building. The BSL3 laboratory can accommodate up to eight people at the same time, and is equipped with eight -80°C freezers, one -20°C freezer and six Class II biosafety cabinets. Two biosafety cabinets are dedicated to *B. pseudomallei* work. One -80 freezer is dedicated to select agents including *B. pseudomallei*. This freezer is locked and access is given to authorized personnel only. Freezerworks, the sample tracking system complying with international conference on harmonization-good clinical practice (ICH-GCP), is used for storage and tracking all specimens. Mahidol-Oxford Tropical Medicine Research Unit is enrolled in a Select Agent Program developed by CDC/NIH.

Office: Dr Direk Limmathurotsakul has a 150 sq. ft. office on the third floor of the building for his use, and there is another shared office next to the microbiology laboratory for the use of lab technicians. Mahidol-Oxford Tropical Medicine Research Unit has a variety of support personnel, including purchasing and account agents.

Computer: The laboratory is equipped with Windows and Mac desktops and laptop computers, local-area network and servers, which are sufficient for the basic support for this project. Internet access is secured by a well-established firewall. The server is well secured and maintained to collect data for clinical studies.

Scientific Environment: The laboratory is within the Faculty of Tropical Medicine at Mahidol University. There are large communities of microbiologists, molecular microbiologists, epidemiologists, statisticians and health economists in the unit. Dr Direk Limmathurotsakul has been leading a study team researching bacterial infectious diseases, particularly melioidosis. Dr Ben Cooper is assisting with the studies involving modeling of infectious disease and development of preventive guidelines for melioidosis. Prof Nicholas Day is assisting with the design and analysis of the studies.

Sappasithiprasong Hospital, Ubon Ratchathani, northeast of Thailand

Laboratory: Dr. Limmathurotsakul and collogues have an office within Sappasithiprasong Hospital, Ubon Ratchathani. The hospital laboratory is very familiar with *B. pseudomallei* as it is one of the most common bacteria isolated from clinical specimens, and safety measures of this laboratory are at the highest standard required by the Ministry of Public Health, Thailand. *B. pseudomallei,* which is isolated from clinical specimens from patients, will be transferred to the BSL3 laboratory of Mahidol-Oxford Tropical Medicine Research Unit under appropriate containment using a high level biosafety standards. Freezerworks, the sample tracking system, has been set up at Sappasithiprasong Hospital and will be used to track the records of specimen transportation.

Office: Dr. Limmathurotsakul has an 800 sq. ft. office for use by his study team. The office at Sappasithiprasong Hospital has secretarial support for the projects and accounting.

Computer: The office is equipped with Windows and Mac desktops and laptop computers, local-area network and servers. Internet access is secured by a well-established firewall. The server is well secured and maintained to collect data from clinical studies.

Clinical: Dr. Limmathurotsakul and his study team have a strong collaboration with physicians from all departments, including pediatricians and director of the Sappasithiprasong hospital.

Scientific Environment: The laboratory and the office are within Sappasithiprasong Hospital. The collaboration between Mahidol-Oxford Tropical Medicine Research Unit and Sappasithiprasong Hospital has been established for more than 30 years. Dr. Limmathurotsakul has been working with Sappasithiprasong Hospital for more than 5 years, performing clinical, laboratory and environmental studies.

Menzies School of Health Research - Royal Darwin Hospital (Menzies)

Menzies School of Health Research is an independent research organization that has research programs focused on Indigenous, tropical, and remote health, and is the focal point for medical research in northern Australia. The mission of Menzies is to improve the health of people living in northern and central Australia, and regions to the near north, through multidisciplinary research and education.

Menzies has a Melioidosis Research program within its Tropical and Emerging Infectious Diseases Division. Menzies is based in Darwin, on the grounds of the Royal Darwin Hospital. The Royal Darwin Hospital is the primary acute care facility for the Australian Northern Territory, providing a wide range of medical services. Royal Darwin Hospital has been accredited under the Australian Council of Health Care Standards since 1997. These team members are responsible for the Darwin Prospective Melioidosis Study. This study has a clinical-research arm, which over the last 22 years has established state-of-the-art therapy for melioidosis, and a laboratory-research arm, which maintains the most extensive collection of patient clinical sample DNA in the world, as well as a meticulously curated collection of over 3000 *B. pseudomallei* isolates from humans, animals, and the environment.

Dr. Bart Currie is the Head of the Tropical and Emerging Infectious Diseases Division at Menzies and is an internationally recognized expert in melioidosis and *B. pseudomallei.* Menzies, in association with Royal Darwin Hospital, where Dr. Currie is also Head of Infectious Diseases, has generated diverse *B. pseudomallei* isolates and clinical specimens for assay validation. These isolates are available to the AuCoin laboratory and InBios for evaluation of assay specificity. Menzies has a CDC-certified PC3 laboratory for handling of *B. pseudomallei* cultures and also designated space at PC2 level for handling patient samples. Full IT support is also available.

EQUIPMENT

InBios International

InBios is fully equipped for the development and production of both ELISA and dipstick diagnostic tests. All equipment is housed in BSL-2 level facilities and secured. Equipment includes: a BSL-2 Biosafety hood, refrigerated centrifuges (low and high speed), microfuges, water baths, both analytical and conventional balances, mixers/stirrers, UV/vis spectrophotometer, pH meter, -20°C freezer, -70°C freezer, LN2 tank, Baxter Cryofridge, environmental chamber with recorder, microplate ELISA readers and plate washers. Accessories required for a full range of protein purification are available which include peristaltic pump, UV monitor and fraction collector. Fully equipped strip production facility with Biodot Quanti 3000, laminator, strip cutter, heat sealer and automatic cassette closure are also available. A PCR thermal cycler machine and equipment for agarose and polyacrylamide gel electrophoresis. The company also has a Perkin Elmer microarray reader and devices for making hand made arrays as well as a Biorad Odyssey calligrapher for machine made arrays. Also available is a 3D printer for making cassette designs prior to sending to manufacturer for scale up.

University of Nevada, Reno

Work for this proposal at UNR will be done in the Laboratory for Diagnostics Discovery and Development (LDDD) suite. Please see the Facilities section for a description of the LDDD. Relevant equipment in the LDDD includes standard laboratory and tissue culture equipment such as biosafety cabinets (4), HERAcell 150i CO_2 incubators (8), Isco low pressure chromatography systems (three complete systems), refrigerated low speed benchtop centrifuges (3), a large capacity liquid nitrogen system for preservation of hybridoma cell lines, -80°C freezers (2), a Sorvall RC6 high speed centrifuge, a New Brunswick Excella E25 incubator shaker, a Wave 2/10 EH bioreactor system for large scale growth of hybridoma and CHO cells, multiple electrophoresis rigs, a BioTek EL808 plate reader, and a BioTek ELx405 plate washer.

Large equipment within the LDDD includes a Biacore X100 for assessment of mAb binding. The Biacore X100 has recently been upgraded to a Biacore X100 Plus with software for extended control of reaction conditions and determination of analyte concentrations. Additional large equipment includes a complete system for fabrication of lateral flow strips that includes a Biodot XYZ 3050 dispensing system, a Biodot guillotine strip cutter and a Biodot laminator; a Zeiss LSM 700 laser scanning microscope; a BioRad ChemiDoc XRS+ imaging system; and a complete BioRad high pressure liquid chromatography system.

Finally, the LDDD has licensed the OptiCHO Antibody Express system from Invitrogen. This system is used for cloning and expression of recombinant antibodies in dihydrofolate reductase (DHFR)-deficient, Chinese hamster ovary (CHO)-derived DG44 cells in suspension culture. The system includes the pOptiVEC-TOPO TA cloning system, the pcDNA 3.3-TOPO TA cloning system and DG44 cells. This system is currently being used for production of chimeric mouse/human antibodies and for production of antibodies that have been affinity-enhanced via phage display. Optimization of cell lines for large scale antibody production with the Wave bioreactor system is currently underway.

[redacted]

OMB Number: 4040-0001
Expiration Date: 06/30/2011

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: [redacted] * First Name: [redacted] Middle Name:
* Last Name: [redacted] Suffix: [redacted]
Position/Title: [redacted] Department:
Organization Name: InBios International Inc Division:
* Street1: 562 Ist Avenue S., Suite 600
Street2:
* City: Seattle County/ Parish: King
* State: WA: Washington Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 98104-3829
* Phone Number: [redacted] Fax Number: [redacted]
* E-Mail: [redacted]

Credential, e.g., agency login: [redacted]

*** Project Role:** PD/PI **Other Project Role Category:**

Degree Type: Ph.D.
Degree Year: 1973

***Attach Biographical Sketch** 1235-[redacted] Biosketch Nov 2 Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 1

Prefix: [redacted] * First Name: [redacted] Middle Name:
* Last Name: [redacted] Suffix: [redacted]
Position/Title: [redacted] Department:
Organization Name: University of Nevada, Reno School of Medicine Division:
* Street1: Department of Microbiology and Immunology/320
Street2: 1664 North Virginia Street
* City: Reno County/ Parish: Washoe
* State: NV: Nevada Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 89557-0000
* Phone Number: [redacted] Fax Number:
* E-Mail: [redacted]

Credential, e.g., agency login: [redacted]

*** Project Role:** PD/PI **Other Project Role Category:**

Degree Type: Ph.D.
Degree Year: 2002

***Attach Biographical Sketch** 1236-aucoin biosketch.pdf Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

[redacted]

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 2

Prefix: [redacted] * First Name: [redacted] Middle Name:

* Last Name: [redacted] Suffix: [redacted]

Position/Title: [redacted] Department: Research

Organization Name: InBios International Inc Division:

* Street1: 562 1st Avenue South

Street2: Suite 600

* City: Seattle County/ Parish: King

* State: WA: Washington Province:

* Country: USA: UNITED STATES * Zip / Postal Code: 98104-3829

* Phone Number: [redacted] Fax Number: [redacted]

* E-Mail: [redacted]

Credential, e.g., agency login: [redacted]

*** Project Role:** Co-Investigator **Other Project Role Category:**

Degree Type: Ph.D.

Degree Year: 1981

***Attach Biographical Sketch**	1237-BIOGRAPHICAL SKETCH SRay	Add Attachment	Delete Attachment	View Attachment
Attach Current & Pending Support	1238-Other support - July 201	Add Attachment	Delete Attachment	View Attachment

PROFILE - Senior/Key Person 3

Prefix: [redacted] * First Name: [redacted] Middle Name:

* Last Name: [redacted] Suffix:

Position/Title: [redacted] Department:

Organization Name: Mahidol University Division:

* Street1: 420/6 Ratchawithi Road

Street2: 24 Medicine Research Unit Rajvithee 25 Rd

* City: Bangkok County/ Parish:

* State: Province:

* Country: THA: THAILAND * Zip / Postal Code: 10400

* Phone Number: [redacted] Fax Number:

* E-Mail: [redacted]

Credential, e.g., agency login:

*** Project Role:** Other Professional **Other Project Role Category:** Collaborator

Degree Type: MD

Degree Year: 2000

***Attach Biographical Sketch**	1239-Limmathurotsakul_BIO.pdf	Add Attachment	Delete Attachment	View Attachment
Attach Current & Pending Support		Add Attachment	Delete Attachment	View Attachment

[redacted]

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 4

Prefix: [redacted] * First Name: [redacted] Middle Name:

* Last Name: [redacted] Suffix:

Position/Title: Department:

Organization Name: Menzies School of Health Royal Darwin Hospital Division:

* Street1: PO Box 41096

Street2:

* City: Casuarina County/ Parish:

* State: Province: Northern Territories

* Country: AUS: AUSTRALIA * Zip / Postal Code:

* Phone Number: [redacted] Fax Number: [redacted]

* E-Mail: [redacted]

Credential, e.g., agency login:

*** Project Role:** Other Professional **Other Project Role Category:** Collaborator

Degree Type: MD

Degree Year: 1978

***Attach Biographical Sketch**	1240-Currie_NIH_Biosketch_Pha	Add Attachment	Delete Attachment	View Attachment
Attach Current & Pending Support		Add Attachment	Delete Attachment	View Attachment

Travel
Domestic travel: $[redacted]/year for trips to UNR and scientific meetings on Melioidosis and Biodefense.

Materials and Supplies
All supply costs are estimates, based on current usage for similar projects.
Annual costs include:

- General laboratory reagents
- Disposable Plasticware
- Tissue culture media (Mab development)
- Strip materials
- Membranes, pads and backing materials
- Cassettes (as needed)
- Colloidal gold and conjugate development
- Gel electrophoresis materials for evaluating purity of purified antibodies
- Reagents for internal control e.g. goat anti mouse IgG or chicken IgY/anti IgY
- ELISA plates
- HRP antibody conjugation materials

Baseline costs: $[redacted]/year for years 1 through 3 ($[redacted]/month).

Subawards
As part of this STTR there is a sub contract between InBios and the University of Nevada and the budget for this is attached. Total is $[redacted] for a total of three years

Shipping costs
$2000/year for three years will cover the estimated costs of shipping all analytes and prototype tests.

Fee at 7% (Including Subaward): A total of $[redacted] is requested.

Fringe Benefit and overhead rates:
Fringe benefit rate: 33%, overhead rate: 38%
Determined by Andrew Sandberg, Procurement Analyst with the Office of Acquisitions, Management, Contracts & Grants (AMCG), HHS, at meeting at InBios on 3/29/11.

Budget Justification UNR

Fringe Benefit Rate Justification: The fringe benefits percentages used are average rates by employee type that are approved by the University for use in budgeting fringe benefits in proposals. However, awards are charged the actual fringe benefit cost for each employee as they are incurred. Fringe benefits cover Worker's Compensation, Medicare taxes, retirement and health insurance.

Materials and Supplies Budget Period 1:

Disposable plastics (microtiter plates, tissue culture supplies, pipettes, pipette tips, etc.)	$
Immunochemicals, reagents, ELISA supplies	$
Tissue culture media	$
Integra bioreactors ($ /bioreactor x 10)	$
Columns, gels, reagents for mAb and CPS purification and characterization	$
BSL-3 PPE (tyvek suites, shrouds, gloves, scrubs, etc.)	$
Biacore NTA sensor chips (3 pack)	$
Total	$
Funds for 2 trips per year to InBios and a yearly melioidosis meeting (AuCoin)	$
Publication costs	$
Biacore service contract (50% of total contract ($)	$

Materials and Supplies Budget Period 2:

Disposable plastics (microtiter plates, tissue culture supplies, pipettes, pipette tips, etc)	$
Immunochemicals, reagents, ELISA supplies	$
Tissue culture media	$
Integra tissue culture flasks for large scale mAb production	$
Columns, gels, reagents for mAb and CPS purification and characterization	$
BSL-3 PPE (tyvek suites, shrouds, gloves, scrubs, etc.)	$

[redacted]

Total	$[redacted]
Funds for 2 trips per year to InBios and a yearly melioidosis meeting (AuCoin)	$[redacted]
Publication costs	$[redacted]
Biacore service contract (50% of total contract ($[redacted])	$[redacted]

Materials and Supplies Budget Period 3:

Disposable plastics (microtiter plates, tissue culture supplies, pipettes, pipette tips, etc)	$[redacted]
Immunochemicals, reagents, ELISA supplies	$[redacted]
Tissue culture media	$[redacted]
Integra bioreactors ($[redacted]/bioreactor x 5)	$[redacted]
Integra tissue culture flasks for large scale mAb production	$[redacted]
Columns, gels, reagents for mAb purification and characterization	$[redacted]
BSL-3 PPE (tyvek suites, shrouds, gloves, scrubs, etc.)	$[redacted]
Biacore NTA sensor chips (3 pack)	$[redacted]
Total	$[redacted]
Funds for 2 trips per year to InBios and a yearly melioidosis meeting (AuCoin)	$[redacted]
Publication costs	$[redacted]
Biacore service contract (50% of total contract ($[redacted])	$[redacted]

UNR Indirect Cost Rate:

Indirect costs are included at the University's federally negotiated rate of 43.5%.
This rate is based on modified total direct costs for on–campus research.

SBIR/STTR Information

OMB Number: 4040-0001
Expiration Date: 6/30/2016

*** Program Type (select only one)**

☐ SBIR ☒ STTR

☐ Both *(See agency-specific instructions to determine whether a particular agency allows a single submission for both SBIR and STTR)*

*** SBIR/STTR Type (select only one)**

☐ Phase I ☒ Phase II

☐ Fast-Track *(See agency-specific instructions to determine whether a particular agency participates in Fast-Track)*

Questions 1-7 must be completed by all SBIR and STTR Applicants:

☒ Yes ☐ No	* 1a. Do you certify that at the time of award your organization will meet the eligibility criteria for a small business as defined in the funding opportunity announcement?
	* 1b. Anticipated Number of personnel to be employed at your organization at the time of award. 30
☐ Yes ☒ No	* 2. Does this application include subcontracts with Federal laboratories or any other Federal Government agencies? * If yes, insert the names of the Federal laboratories/agencies:
☐ Yes ☒ No	* 3. Are you located in a HUBZone? To find out if your business is in a HUBZone, use the mapping utility provided by the Small Business Administration at its web site: http://www.sba.gov
☐ Yes ☒ No	* 4. Will all research and development on the project be performed in its entirety in the United States? If no, provide an explanation in an attached file. * Explanation: 1257-SBIR__STTR_4_Information M [Add Attachment] [Delete Attachment] [View Attachment]
☐ Yes ☒ No	* 5. Has the applicant and/or Program Director/Principal Investigator submitted proposals for essentially equivalent work under other Federal program solicitations or received other Federal awards for essentially equivalent work? * If yes, insert the names of the other Federal agencies:
☒ Yes ☐ No	* 6. Disclosure Permission Statement: If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?
	* 7. Commercialization Plan: If you are submitting a Phase II or Phase I/Phase II Fast-Track Application, include a Commercialization Plan in accordance with the agency announcement and/or agency-specific instructions. * Attach File: 1258-MELIOIDOSIS commercializat [Add Attachment] [Delete Attachment] [View Attachment]

SBIR/STTR Information

SBIR-Specific Questions:

Questions 8 and 9 apply only to SBIR applications. If you are submitting ONLY an STTR application, leave questions 8 and 9 blank and proceed to question 10.

☐ Yes ☐ No	* 8. Have you received SBIR Phase II awards from the Federal Government? If yes, provide a company commercialization history in accordance with agency-specific instructions using this attachment. * Attach File: [ ] Add Attachment Delete Attachment View Attachment
☐ Yes ☐ No	* 9. Will the Project Director/Principal Investigator have his/her primary employment with the small business at the time of award?

STTR-Specific Questions:

Questions 10 and 11 apply only to STTR applications. If you are submitting ONLY an SBIR application, leave questions 10 and 11 blank.

☒ Yes ☐ No	* 10. Please indicate whether the answer to BOTH of the following questions is TRUE: (1) Does the Project Director/Principal Investigator have a formal appointment or commitment either with the small business directly (as an employee or a contractor) OR as an employee of the Research Institution, which in turn has made a commitment to the small business through the STTR application process; AND (2) Will the Project Director/Principal Investigator devote at least 10% effort to the proposed project?
☒ Yes ☐ No	* 11. In the joint research and development proposed in this project, does the small business perform at least 40% of the work and the research institution named in the application perform at least 30% of the work?

SBIR/STTR INFORMATION

Melioidosis is caused by the Gram-negative bacterium *Burkholderia pseudomallei* transmitted through contaminated water and soil, and prevalent in tropical regions of the world. At present, only non-indigenous cases of melioidosis have been reported in the United States. The worldwide distribution is as yet unknown due to lack of rapid field tests. In the studies proposed we plan to further evaluate a rapid test with the view to eventual FDA clearance.

In order to conduct an appropriate, statistically valid evaluation of our tests, it is necessary to perform the field studies with a significant number of samples (good sample size with enough power for significance calculation). Considering that this disease is non-endemic to the US; is difficult to treat; and the causative agent of this disease is listed as a bioterrorism agent we propose to collaborate by providing tests to experts in the endemic regions where this disease has a public health concern.

Such centers will include Mahidol University, THAILAND and Menzies School of Health Research-Royal Darwin Hospital, AUSTRALIA. These sites have appropriate facilities to conduct the testing and sufficient number of archived melioidosis positive patient samples for the evaluation. We have also recently been asked to participate in the Detection of Melioidosis in Africa Working Group (DMAWG).

In addition, we plan to test the LFI and quantitate via ELISA the CPS from *B. pseudomallei* and *B. mallei* in archived blood and urine samples from non-human primates. Collecting time course samples after exposure will provide supportive data on kinetics of CPS production and the ability of the LFI to detect *B. mallei* (Glanders). Only non-STTR funds will be used to compensate these foreign sites.

We also plan to evaluate a Deki reader with data capture, software and cloud computing capabilities to facilitate data analysis in the field.

[redacted]

Active Melioidosis Detect (AMD) Commercialization Plan

Value of the STTR Project, Expected Outcomes and Impact.
Intended use of the product: The Active Melioidosis Detect™ (AMD) is a point of care diagnostic test for active melioidosis. The AMD is an in vitro immunochromatographic device for visual and rapid qualitative detection of the *Burkholderia pseudomallei* capsular polysaccharide (CPS) directly from patient samples or cultured samples (Figure 1). The disease can present as local to very generalized and disseminated infections. The assay will accept multiple sample matrices that are known to accumulate CPS during melioidosis. These matrices include blood, urine, pus, and respiratory secretions. The assay is also applicable for cultured clinical samples from enrichment broth. Particularly hemoculture broth, and bacterial colonies from cultured samples on solid medium.

Figure 1. Active Melioidosis Detect kit

The final test is intended for use in diagnosis of *B. pseudomallei* infection in subjects with early non-specific signs and symptoms and/or individuals with confirmed, presumed or likely exposure to *B. pseudomallei*. Tests are for use by healthcare professionals and medical personnel, including emergency personnel who have been trained. AMD positive results may be confirmed by blood culture (the current gold standard). The test could also have the potential for detecting presence of *B. pseudomallei* in (a) soil samples where the organism resides (b) contaminated water as well as (c) monitoring for infection in laboratory animals such as non-human primates.

Currently, there is no FDA cleared (point-of-care) POC test for the diagnosis of melioidosis. A rapid diagnosis of melioidosis is essential to the survival of patients infected with *B. pseudomallei.* The bacterium is resistant to commonly prescribed antibiotics; therefore, timely diagnosis is needed for administration of effective antibiotics. Diagnosis in endemic and non-endemic regions is extremely challenging due to a lack of experience of identifying *B. pseudomallei* and a lack of effective diagnostic tools.. Isolation of *B. pseudomallei* from cultures of patient samples remains the "gold standard" for the diagnosis of melioidosis. However, accurate diagnosis from culturing of patient samples can take 3-7 days for bacterial isolation and another 1-2 days for bacterial identification. Use of the Active Melioidosis Detect Assay will reduce the time to diagnosis to 10-15 minutes from clinical specimens, in addition the assay can also be used to rapidly identify *B. pseudomallei* bacterial colonies with high accuracy.

Serious problems exist with current rapid diagnostics such as the indirect hemagglutination assay (high background levels of seropositive individuals) and PCR (reduced sensitivity), immunofluorescence assay (requires fluorescent microscope and skilled personnel) and latex agglutination (not applicable to clinical samples.). As a consequence there is not an FDA cleared diagnostic available for the diagnosis of melioidosis. Therefore, there is a critical need for a rapid, effective and low cost point-of-care (POC) diagnostic test for melioidosis and an improved algorithm for early diagnosis. A field test is also needed to fully assess the incidence of melioidosis worldwide.

The global incidence of melioidosis. To date there have been no good estimates of the global incidence of melioidosis. However our collaborator in Thailand (Dr. Direk Limmathurotsakul) has put significant time and effort into generating a risk map for melioidosis based on epidemiological data and geospatial statistical modeling (Fig. 2). Importantly, it can be used to predict areas at risk of acquiring human melioidosis worldwide, including areas where melioidosis might be endemic but under diagnosed or undiagnosed. [redacted] Also, Table 2 is an estimate of the mortality of the countries that are within known and predicted melioidosis endemic regions.

Established methods and models were used to generate a risk map [1]. Briefly, data from recent reviews of melioidosis and environmental *B. pseudomallei* studies [2,3] along with a systematic literature review were used to obtain updated evidence of clinical melioidosis worldwide. This included peer reviewed journals (PubMed, Google Scholar, ISI Web of Science), disease surveillance networks (ProMED archives, Eurosurveillance archives), Genbank database and health websites of low and middle-income countries.

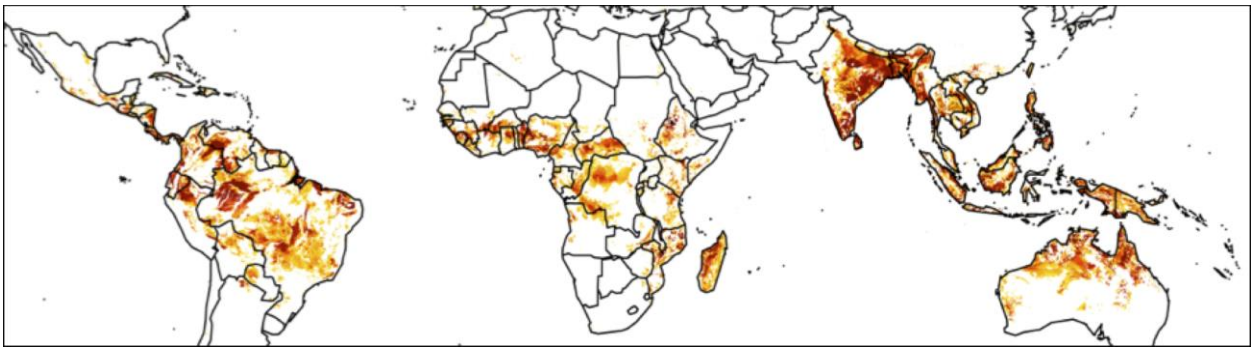

Figure 2. Risk map for populations in predicted and known melioidosis endemic regions. Colored areas indicate regions predicted areas of incidence of melioidosis. Dark red areas indicate areas at highest risk.

Table 1. Predicted global incidence of melioidosis as compared to other infectious diseases

Disease	Predicted incidence	Predicted mortality	Predicted case fatality rate	Source of data
Tuberculosis	8,600,000	1,300,000	15%	WHO website
Malaria	219,000,000	660,000	0.3%	WHO website
2009 H1N1	N/A	284,000	N/A	LID 2012, 12(9)687-695
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Severe Dengue	500,000	125,000	2.5%	WHO website
Leptospirosis	1,000,000	60,000	6%	ILS 2013

Table 2. Predicted incidence of melioidosis by country

Country	Predicted mortality
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

The above studies and data analysis would indicate the incidence of melioidosis is under reported. By providing prototype tests to potential collaborators we expect to better understand the prevalence of melioidosis. This will then lead into larger market size for finalized tests. To this end Dr. AuCoin and his collaborator in Thailand (Dr. Limmathurotsakul) created an initiative to "Explore the Presence of Melioidosis in Africa". Please see initiative below that has been posted to "groups.google.com/group/melioidosis".

Exploring the Presence of Melioidosis in Africa

Goal: To determine if *Burkholderia pseudomallei*, the causative agent of melioidosis, is an underreported cause of infection in Africa. Hospital/microbiological facilities in Africa will be offered the use of the prototype Active Melioidosis Detect™ Lateral Flow Immunoassay (AMD LFI) developed by the University of Nevada and InBios International to rapidly identify *B. pseudomallei* isolate.

Organizers: Detection of Melioidosis in Africa Working Group (DMAWG)

Significance: *Burkholderia pseudomallei* is an environmental Gram-negative bacillus and the cause of melioidosis. Melioidosis is highly endemic to northeast Thailand and northern Australia, but more recent evidence indicates a much wider distribution that includes the Indian subcontinent and southern China, Hong

Kong, and Taiwan [4]. There are also an increasing number of reports of sporadic and possibly endemic disease in the Caribbean, Central and South America and East and West Africa [4-8]. The extent of melioidosis in Africa remains uncertain. Melioidosis may pass unrecognized because diagnostic confirmation relies on microbiological culture, which is often unavailable in resource-restricted regions of the Africa. Even with such facilities, *B. pseudomallei* may be dismissed as a culture contaminant, or be misidentified as *Pseudomonas spp.* or other organisms by standard identification methods including API 20NE and automated bacterial identification systems.

Assay: The AMD LFI is a point of care diagnostic test, based on the detection of capsular polysaccharide (CPS) produced by *Burkholderia pseudomallei* (Fig. 1). The assay is an immunochromatographic strip that is similar in design to the rapid Strep test or home pregnancy test. The assay is designed to accept all routinely collected clinical samples from patients with suspected melioidosis (blood, urine, pus, respiratory secretions, etc.) and to identify the organism isolated from culture. Preliminary sensitivity and specificity of AMD LFI to identify a *B. pseudomallei* isolate is 98.7% and 97.2%, respectively. **For the current initiative, the AMD LFI is to be used for testing all Gram-negative, oxidase positive bacilli that are isolated from blood culture and that cannot be simply identified as *Pseudomonas aeruginosa*.**

Participants: Hospital/microbiological facilities in geographic areas of Africa

This is an open call for hospital/microbiological facilities in Africa to receive free AMD LFI kits.

Work plan:

1) This initiative is for hospital/microbiology facilities that perform blood culture for either routine practice or research, and have capacities to perform Gram strain and oxidase tests in Africa.
2) The University of Nevada and InBios International will provide a set of LFI tests (e.g. 20-50 strips) to be used with **all Gram-negative oxidase-positive bacilli that are isolated from blood culture**.
3) Isolates that are positive by the AMD LFI test should be suspected to be *B. pseudomallei* and should be further evaluated by the participating sites using SOPs for identification that will be provided by the DMAWG.
4) Alternatively, the Centers for Disease Control and Prevention has agreed to offer confirmation for any positive isolates and genotyping them as well (See CDC contacts below).
5) There is usually no need for Ethical Clearance for work of this nature. However, if the participating site considers that it is required, it is the responsibility of the participating site to obtain EC clearance prior to the participation.
6) The overall result that the DMAWG would like to receive back is, i) how many tested were used?, ii) how many were positive? and iii) what is the final identification of those positive isolates? The DMAWG will release the results only as an overall result.
7) The participating site can write up the case report with full clinical details. If needed, collaboration for writing case report can be sought from the DMAWG.
8) Clinical and public health advice from experts in the field will be made available through the network to any site where melioidosis is discovered.
9) If you are interested to participate to this initiative, please email Dr David P Aucoin

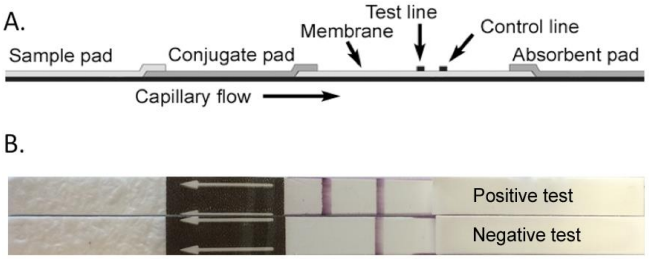

Figure 1. Prototype Active Melioidosis Detect (AMD) LFI. (A) Schematic of AMD LFI components. (B) *B. pseudomallei* colony grown on an agar plate was picked and suspended in 2 drops of lysis buffer. The lysate was added to the sample pad followed by three drops of LFI chase buffer (top LFI). The LFI was imaged following a 15 min run time. The same test condition were used with a colony of *E. coli* (bottom LFI).

[redacted]

Assay Contact: [redacted]

Clinical contact: [redacted]

WHO contact: [redacted]

CDC contact: [redacted]

Isolate analysis by CDC: [redacted]

Current members of DMAWG

[redacted]

The DMAWG was formed following the 7th World Melioidosis Congress 2013. This was in response to increased reports of melioidosis in Africa. Following release of this initiative we have requests for the 4C4 AMD LFI from around the world. The Africa initiative will be modified to include any region predicted to be endemic for melioidosis. In just one month, we have received requests from 13 laboratories from around the globe. Some examples are Papua New Guinea, Singapore, Brazil, Nairobi, Nepal, and Seychelles. Importantly we have formed a collaboration with [redacted] **We anticipate by spearheading these initiatives and collaborations we will expand the market significantly for the AMD LFI.**

In Phase I we identified a "lead candidate" antigen (*B. pseudomallei* CPS) and a "lead candidate" monoclonal antibody (mAb 4C4) for the diagnosis of melioidosis. A prototype Active Melioidosis Detect Assay kit was initially developed by InBios and UNR based on mAb 3C5 was tested for feasibility in Thailand and Australia (Figure 1) with various sample types. With the isolation of IgG1 mAb 4C4 the assay achieves improved analytical sensitivity This latest generation of test is available and is being scaled up for more extensive testing. Therefore, the goal of the project is assay optimization (improved sensitivity and specificity), improved process development, and validation of the diagnostic assay in a variety of patient samples. A prototype antigen-capture ELISA and a prototype lateral flow immunoassay for the detection of *B. pseudomallei* CPS based on 4C4 are two intermediate products that have been developed and will be improved upon during the Phase II award period. Both of these diagnostic products possess encouraging limit of detection values for CPS, however improvements can surely be made. Both tests will also be made available for testing in archived samples from non-human primates. Tests will also be used to understand prevalence levels of melioidosis outside of Thailand and Australia.

Following the completion of the product development plan the goal is submission of a 510(k) application (by the end of Project Year 3) and FDA clearance. The assay would be approved for use with a variety of patient samples that are relevant to a melioidosis infection (blood, urine, pus, respiratory secretions, and pleural fluid).

Data that support the selection of the candidate product for further development, including and assessment of the present capacity of the diagnostic to meet the performance specifications:

- InMAD (In vivo Microbial Antigen Discovery) has identified CPS as a secreted antigen that is shed into samples from animal models of melioidosis and humans infected with *B. pseudomallei*.
- The qCPS ELISA and AMD LFI identified shed CPS in filtered urine and serum samples from melioidosis patients.

- AMD LFI is able to identify CPS in a variety of melioidosis patient samples (blood, urine, pus, sputum, and pleural fluid)
- Preliminary studies have determined that the CPS is highly conserved.
- CPS is a well-established virulence factor and its expression is necessary for disease in animal models of melioidosis.
- The LFI is ideal for the point-of-care and resource poor setting (rapid, sensitive, specific, easy to use, adaptable, allows for random access and cost effective).
- Clinicians in Thailand (Dr. Direk Limmathurotsakul) and Australia (Dr. Bart Currie) are quite encouraged by the performance of the prototype CPS LFI (see letters of support).
- Both the Department of Homeland Security and the Defense Threat Reduction Agency are very interested in the AMD LFI and are potential end users (see letters of support).
- Clinicians in Laos, India, Singapore and Africa as well as WHO requested evaluation of the AMD LFI (see letters of support)

Discussions with the FDA, which are relevant to development for the candidate product:
A major goal of the proposal is to submit a 510(k) to the FDA. InBios International has a well-established history of product development with five approved medical diagnostics (Table 4). InBios and UNR will take the lead in drafting the pre-submission to FDA (Project Year 2) to seek advice and requirements for prospective studies and review of in house performance studies. Estela Raychaudhuri at InBios who is experienced in FDA submissions will provide regulatory assistance. It is anticipated that Dr Steven Hawes, a consultant to InBios, will perform Biostatistics.

Regulatory items to be covered by the InBios team:
- Development of pre-market submission strategies
- Premarket submission preparation (510(k)s, PMAs, IDEs, reclassification petitions, etc.) and reviews
- FDA correspondence
- Procedure and user manual development
- Assistance with FDA compliance matters, GMP/QSR reviews, development and auditing (including SOP development and review)
- Good clinical practice auditing
- Clinical protocol development
- Statistical support services
- FDA staff meeting preparation and participation
- FDA Advisory Panel preparation and participation
- Technology and regulatory due diligence assessments

Compliance with GLP defined by 21 CFR (58) and cGMP defined by 21 CRF (211):
Prototype LFIs will be produced under GLP (Project Years 1-2) and the optimized LFI will be produced under GMP (Project Years 2-3). A Research Associate, who has recently completed and FDA GLP training course for pre-clinical testing, will oversee all GLP requirements in the AuCoin laboratory. InBios is a FDA registered and an ISO (International Organization for Standardization) certified company who has established a set of general manufacturing principles in compliance with Good Manufacturing Practice (GMP) ensuring that products are consistently produced and controlled according to quality standards. InBios manufacturing processes are clearly defined, controlled and documented. All critical processes are validated to ensure consistency and compliance with design specifications. All operators are trained to carry out and document procedures. Deviations are investigated and documented. Records of manufactured goods that enable the complete history of a batch to be traced are retained in a comprehensible and accessible form.

The entire staff at InBios manufacturing is trained for GMP practices. James Needham who is also responsible for final development and transition efforts to manufacturing leads the InBios manufacturing team. The manufacturing team includes an engineer, wet chemistry application specialists and a team of assemblers.
A design plan will be written to establish the overall design goals, specifications, input and output objectives, project schedule, studies to be performed, and when formal design reviews shall be held. As required by CFR 820.30, The Contractor shall maintain a design history file that contains or references the design of this assay. It will include the design plan, design specifications, protocols for the studies to be conducted, study results and associated data, a record of project meetings minutes and design review minutes, and copies of all communications with collaborators and FDA. At the beginning of this project, InBios shall establish a design

review body consisting of scientists, clinicians, and experts in regulatory affairs, quality control and manufacturing. The team will meet on a regular basis and assess the developmental work and shall recommend future efforts. All input discussion shall be documented and design output will be followed for the product's continued development. In Table 3 we have described our preliminary product specification for Active Melioidosis *Detect*™ assay. This will be considered as input of the AMD assay system and will be constantly monitored and modified depending on the feedback and results.

Table 3. PRODUCT SPECIFICATION

• ***Product Identification:***	Active Melioidosis *Detect*™, device not yet classified by FDA
• ***Device:***	Active Melioidosis *Detect*™ (AMD; Lateral flow immunoassay (LFI))
• ***Intended Use:***	In vitro diagnosis of active melioidosis for visual and rapid qualitative detection of the *Burkholderia pseudomallei* capsular polysaccharide (CPS) antigen
• ***Analyte:***	Whole blood (venous and/or capillary blood), blood, urine, pus, and respiratory secretions
• ***Antibodies to be used:***	Proprietary antibodies to the *B. pseudomallei* CPS
• ***Detection agent:***	Selected antibody(s) to CPS conjugated with gold
• ***Buffer system:***	Chase buffer that should not require cold storage
• ***Instrument size/weight:***	No additional instrumentation required.

Performance input:

• ***Clinical Sensitivity***	Pus > 98% Sputum > 98% Urine > 95% Blood > 90%
• ***Clinical Specificity:***	≥95%
• ***Analytical sensitivity:***	Limit of detection ≤ 1 ng/ml CPS
• ***Analytical Specificity:***	Reactivity with *B. pseudomallei* and *B. mallei* strains but not other *Burkholderia* spp.
• ***Cross Reactivity:***	No reactivity with other pathogenic bacteria e.g. *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumonia* etc.
• ***Comparative Methods***	PCR, Immunofluorescence, Culture
• ***Sample preparation time:***	None (depending on the matrix to be used)
• ***Reading time (time to result):***	5 to 20 minutes *(TBD)*
• ***Sample throughput rate:***	Unlimited. Many samples can be run in parallel on separate strips.
• ***General assay characteristics:***	Clear test and control lines
• ***Shelf life:***	24 months at room temperature (≤30°C)

Company Background: InBios is a 12-year old FDA-registered ISO-certified biotechnology company focusing on developing novel immunologic diagnostic products and reagents based on proprietary antigens/antibodies and technologies. The company's objective is to become a leader in the development and manufacturing of novel diagnostic tests for infectious diseases. The company's primary focus has been on: 1) infectious diseases that plague developing countries and have a great potential to impact the United States due to immigration; and on 2) emerging infectious diseases such as Chagas (*T. cruzi*), West Nile Virus, Dengue fever, and Japanese Encephalitis 3) biodefense e.g. anthrax and melioidosis. The company believes that major diagnostic firms are not focused on emerging infectious disease diagnostic markets or third world diseases because of their perceived small market size and/or profit margin. InBios believes that this niche market needs to be filled and is committed to developing high quality, cost-effective diagnostics tests. To that end InBios has developed the following diagnostic assay services:

- Twenty plus years of experience in assay development, both lateral flow and ELISA based
- Expertise in production of conjugates, including various gold sized particles
- Feasibility studies
- Prototype and beta test development
- Full FDA compliance-design plan and design history files prepared
- Laboratory studies- accelerated stability, cross reactivity, interference and precision

- Analytical sensitivity and specificity studies
- Clinical trial management
- FDA product submissions (510(k) & PMA)
- Small and large scale manufacturing of rapid and ELISA based immunoassays

The company's founders and employees have extensive experience in developing, manufacturing, and marketing diagnostic tests worldwide. In-house expertise includes various aspects of chemistry and immunology including immunochemistry, protein expression and purification, and protein manipulation. InBios also specializes in particle and conjugation chemistry that includes our proprietary gold-based technology for assay read-out. Regulatory approval and commercialization of a new diagnostic test is confronted with many significant scientific and regulatory hurdles. InBios has an excellent track record of obtaining funds to develop diagnostic tests/products. Over 75% of InBios' externally-funded projects have successfully obtained competitive renewal, indicating that InBios performs excellent science and achieves significant progress. Recently, InBios released an FDA-cleared Dengue virus IgM detection diagnostic test which is the first and only FDA-cleared dengue test available in the market. Here is the NIAID webpage link to the dengue diagnostic test: http://www.niaid.nih.gov/topics/DengueFever/Research/Diagnosis/Pages/default.aspx

Please note we outcompeted a number of big players in our race to gain FDA clearance of our dengue IgM ELISA product. The competition included companies like Alere (Inverness), Panbio, Standard Diagnostics, etc. to name a few. We have a number of advanced levels of products that are progressing towards the FDA submission process. Our typical FDA submission process ranges between 3-5 years. A brief overview of Inbios selected product portfolio is shown in Table 1 below.

The company has successfully applied for and received SBIR funding for the development of its TB test (Phase I) and for its *T. cruzi,* Dengue and Leishmania assays (Phase I and II). Its test for visceral leishmaniasis received FDA 510(k) clearance May 2003; commercialization of this product is well underway and sales are being realized. InBios has also received competitive funding from the Department of Defense for development of multi-pathogen dipstick rapid tests for enteric diseases, febrile illnesses and sand fly fever viruses as well as cutaneous leishmaniasis. Development of diagnostics to meet a growing need in the military and abroad presents unique commercialization opportunities for a variety of the company's product portfolio.

The company has significant regulatory experience. It is registered with the Food and Drug Administration and several of its products have gained FDA clearance (see Table 4 below). Establishment licensure with the USDA has been received and several tests with veterinary applications will be marketed in the coming year. The company adheres to the Quality Systems regulations of the FDA from product inception by following Design Controls mandated by CFR 820 to the validation of its equipment and processes. FDA inspection of its facility has not yielded any complaints (483s) regarding its quality system. InBios became ISO 13485:2003 certified in 2011 which has increased its ability to market its products internationally. Several products are also CE marked and can be sold in Europe.

The vision of InBios is simple: develop and market high quality diagnostic assays with superb performance at an affordable price. The founders' of InBios have maintained that vision since the company's inception. To accomplish that goal, the company has invested the majority of the private investment dollars it has received as well as product sales revenue into research and development. Overhead at InBios is kept low and the "burn rate" is below average for a company of its size. The company believes that its new products, such as the West Nile Virus and Dengue ELISA will result in significant sales and revenue in years to come. The company understands that as it grows, changes in management will take place. The company already plans to add positions in management for sales and marketing. At present, the company relies on consultants and focuses its employees on its core competencies – research and development as well as in-house manufacturing and regulatory affairs.

Market, Customer, and Competition: We anticipate that Active Melioidosis *Detect* ™ (AMD) marketing will be analogous to our FDA cleared Kalazar *Detect* ™ test. Kala-azar or Visceral leishmaniasis (VL) is a severe disease of children and women. There is an effective therapy if an accurate diagnosis is made. Many of the symptoms are similar to malaria, dengue and other tropical diseases. We developed a rapid test for early diagnosis of VL that changed VL disease management completely. We started with an export use only product, obtained FDA approval and created this market when the only diagnosis was splenic and/or bone marrow aspirate, microscopy and culture. At this time we are the leader in this field and our customers include

US and UK Army who are/were engaged in Gulf and Afghanistan conflict. We are selling hundreds of thousands of this test every year. However, the price ranges between 1$ US and 10$ US, depending on the markets. Similar to the VL target the meliodosis target is proprietary and IP protected.

Table 4. InBios selected product portfolio

Product	Test type	510(k) No.	Status
Generic Blood Glucose	***Dipstick***	***K001092***	***Cleared; de-listed***
Kalazar Detect (a test for Visceral Leishmaniasis; Class I)- NIH funded	***Dipstick***	***K023483***	***Cleared***
West Nile Virus (Class III)- CDC collaboration	***ELISA***	***K041817***	***Cleared***
Dengue IgM (Class II)- NIH funded	***ELISA***	***K100534***	***Cleared***
Cutaneous Leishmaniasis (class I) – DOD funded	Dipstick	-	FDA submission 1st Qtr 2014
Dengue NS1 – NIH funded	ELISA, dipstick	-	FDA submission of ELISA product is imminent
Chagas (T. cruzi) – NIH funded	Dipstick	-	Clinical studies have begun
Scrub typhus – DOD funded	Dipstick	-	Pre-IDE communication stage
Japanese Encephalitis (class III) – CDC collaboration	ELISA	-	In clinical
AMD	Rapid		In development
AAD	Rapid		In development

Melioidosis will follow a very similar development plan. *B. pseudomallei* is found all over the world, but *Melioidosis* is most frequently reported in Southeast Asia and Northern Australia (Fig. 2). It also occurs in South Pacific, Africa, India, and the Middle East. People acquire the disease by inhaling dust contaminated by the bacteria and when the contaminated soil comes in contact with an open wound of the skin. Infection most commonly occurs during the rainy season. Melioidosis symptoms most commonly stem from lung disease where the infection can form a cavity of pus (abscess). The effects can range from mild bronchitis to severe pneumonia. As a result, patients also may experience fever, headache, loss of appetite, cough, chest pain, septicemia and general muscle soreness. The symptoms are very much like many other diseases like dengue, leptospirosis, and rickettsial diseases. The septicemia caused by *B. pseudomallei* infection is clinically similar to that caused by other major pathogens. Subacute and chronic disease may mimic tuberculosis clinically. Considering the rate of mortality of this disease, acute and early diagnosis is critical.

If untreated, melioidosis is fatal. When treated with antibiotics, severe forms of the illness have a 50% chance of recovery, but overall the mortality rate is 40% in resource poor settings. Early detection is critical. In the United States, confirmed cases range from 0 to 5 each year and occur among travelers and immigrants, according to the U.S. Centers for Disease Control and Prevention (CDC). However, in the US, the melioidosis is further complicated because it has the potential to be developed as a biological weapon. It is classified by the US Centers for Disease Control (CDC) Tier 1 select agent. The Department of Defense and Homeland Security is very interested in the diagnosis and control of melioidosis (see support letters).

Current diagnosis is limited to specialized laboratories and the number of competitors is also limited. We believe that number of cases is much higher than reported. Many cases are not reported because of a lack of diagnosis. Recently, more and more cases are reported because of awareness of the disease from the level of Government agencies (CDC, Army, local universities, etc.). We also believe that the testing will be performed on a greater number of people with unrelated febrile symptoms, just to rule out the possibility of melioidosis. The diagnostic assay InBios is developing for melioidosis is very robust, field deployable, and simple to use and does not require any special equipment or any trained personnel.

Intellectual Property (IP) Protection:

Patent Title: Method of Diagnosing and Treating Melioidosis
PCT Patent Application No. PCT/US2011/032155, filed on April 12, 2011
This application claims the benefit of U.S. Provisional Application:
U.S. Provisional Patent Application No. 61/323,236, filed on April 12, 2010
U.S. Provisional Patent Application No. 61/452,388, filed on March 14, 2011
Inventors: David P. AuCoin; Thomas R. Kozel (University of Nevada School of Medicine)

Abstract: Non-invasive methods are provided herein for the diagnosis of melioidosis with specific antibodies capable of detecting molecules associated with melioidosis in a biological fluid, such as urine or serum. These molecules can be identified using proteomic methods, including but not limited to antibody based methods, such as an enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), or a lateral flow immunoassay. Methods of inducing an immune response to melioidosis are also disclosed. The methods include the use of the immunogenic melioidosis polypeptides, nucleic acids encoding these polypeptides, and/or viral vectors encoding an immunogenic melioidosis polypeptide, alone or in conjunction with other agents, such as traditional melioidosis therapies. Also disclosed are methods for treating a subject having melioidosis. These methods include inducing an immune response to melioidosis and/or using an inhibitory nucleic acid, such as a siRNA or antisense molecule, to decrease a melioidosis associated molecule expression in order to treat melioidosis. InBios' innovation in Melioidosis diagnostics is protected by intellectual properties through the University and Inbios know how and trade secrets.

Finance Plan: The maturity of AMD candidate is to a point where achieving FDA clearance within 3-4 years is likely. While challenging, FDA clearance is critical to provide dependable products manufactured under GMP. For melioidosis where there is no huge market at this time (besides the military use), the FDA clearance is hindered by the limited availability of well-defined samples in the US. The cost of organizing clinical studies in the endemic areas (primarily international sites) is also cost prohibitive unless strong financial support is in place. We anticipate seeking help from our existing collaborators with pre-clinical and clinical studies. Some of the current funding resources will support in house performance studies and enhance our documentation capability, GMP process development, GMP manufacturing and overall QC manpower and capability of performing in house studies. All these are needed for FDA submission and continued GMP manufacturing of the AMD product. As needed we will seek additional funds through Phase III as well funds from sales of AMD research use only (RUO) and analyte specific reagent (ASR).

Production and Marketing Plan: InBios has the facility and manufacturing experience to produce LFI and ELISA tests on a large scale. It has automatic, quantitative reagent dispensing equipment, automated strip cutter machine, high speed labeling and package sealer as well validated manufacturing processes to produce all rapid tests. The company is currently producing several lots a week of its Visceral Leishmania test and are making small lots of prototype melioidosis tests. The manufacturing process for a rapid melioidosis test will be similar to the process currently used for its Visceral Leishmaniasis test and other rapid tests in development. However, we will gear towards more automation in the near future. Therefore, in-house manufacturing of this product will follow under a routine process. The work that will be required to manufacture this AMD assay will be finalization of documentation of the manufacturing and quality control as well as validation of the design and process. The company is confident its manufacturing processes are under quality control as it has been audited recently by the FDA and did not receive any complaints during the inspection.

InBios, in collaboration with UNR, has already created awareness regarding availability of the rapid melioidosis diagnostic market by introducing the first rapid test which is being evaluated by a number of clinicians and has received considerable interest from government agencies. InBios also has distributors in India, Pakistan, Vietnam, Singapore, Thailand, South America, and the U.S. Once developed, we will approach all the distribution channels and reference laboratories. In the U.S., we are connected with all major State Health and reference laboratories such as ARUP and the Mayo clinic. However, for marketing in the US we will need FDA approval. We shall market the test in the US as a research use only (RUO). We shall definitely market outside US as an export use only product. We have many years of experience with a number of products for "export use only" category. In the context of RUO we have already established a contract with the Navy (DTRA) to provide a cassette version of rapid test that has combined capability of detecting Dengue NS1 as well as CPS (See letter of support). This test is designed only for using large volumes of whole blood or serum and is not adapted or intended for the multi sample type described in this application.

We strongly believe that having a FDA cleared AMD test will be needed for creating and penetrating markets not familiar with this novel test. Our experience confirms that end users are looking for quality and affordability. FDA clearance will provide a stamp of approval, consistency and quality**.** In addition, solid publications from the opinion makers will definitely help to bring in public awareness about this disease and expand markets for AMD. FDA clearance will also be important for marketing to government agencies e.g. DoD, DTRA and Department of Homeland Security.

To maintain our competitive advantage and thwart competition we shall employ several strategies: (a) pursue FDA clearance and CE marking for our products to demonstrate product quality; (b) consider manufacturing

cost-reduction strategies so that we could maintain a competitive and affordable price for the AMD; (c) re-enforce alliance with existing distributors and bring in additional well-known international distributors, if warranted, who can further provide a reputable presentation for our products and saturate the competitive market; (d) use customer feedback to continuously improve the product quality and our services.

Revenue Stream: InBios is a privately-held, for-profit company with operation resources from contract manufacturing, product sales, and federal-funded Small Business programs. Commercialization costs for these products will be funded by the company's revenue and potential future Government funding. InBios forecasts its service and sales revenue for fiscal year 2013 to be close to $2MM, not including grant funding. Our 2012 sales have reached close to $1.5MM, not including grant revenues. Table 5 shows the selective revenues generated and forecast by major product/service type. Our dengue IgM and NS1 ELISA business is growing faster and therefore will generate increased revenues essential for additional hiring and new product development. Please note we do not have a dedicated in house marketing and sales team. The revenues are all generated through our web site and via references. We anticipate recruiting sales and marketing once sales reach $5MM. The sales of AMD will move slowly (similar to Kalazar Detect). Once the incidence of melioidosis worldwide is better understood we expect sales to increase significantly. We are looking for a steady growth year after year once the technology is proven and markets are aware of the test. We are hoping to sell close to 10 to 20K tests in the first year once a final prototype and selection of matrix or matrices are established. Overall pricing structure, government requirement and insurance reimbursement, if allowed will dictate the final pricing.

Table 5.: Selected Product/Service Revenue of 2012 and Forecast 2013

Product description	*Revenue earned 2012,*	*Forecast for revenue for 2013*
Kalazar Detect ™ (FDA-cleared)	$ [redacted]	$ [redacted]
West Nile Detect (FDA-cleared)	$ [redacted] (burst of sales compared to last year due to outbreak)	$ [redacted] *(not anticipating any outbreak this year)*
Dengue ELISA (FDA-cleared)	$ [redacted]	$ [redacted]
Dengue NS1 Detect (ELISA and RAPID) (FDA studies have begun)	$ [redacted]	$ [redacted]
T cruzi Human (FDA studies underway)	$ [redacted]	$ [redacted]

InBios has also received USDA approval to begin selling its Equine West Nile test, which will generate additional revenue.

Future Developments: Product development to be completed following the award period: Following FDA clearance of the Melioidosis Antigen Detect Assay "multiplexing" will be evaluated. Dr. Thomas Kozel (University of Nevada, Reno) is currently developing an LFI for the detection of the capsular polypeptide of *Bacillus anthracis*, the causative agent of anthrax. This project is in collaboration with InBios and a pre-IDE for the diagnostic has recently been submitted. The goal will be to combine these two FDA-approved tests to produce an LFI that has the ability to diagnosis both a melioidosis or anthrax infection. The AuCoin laboratory is also teaming with InBios to develop a diagnostic product for tularemia and plague. Therefore, the final product could potentially be a rapid LFI that can identify all of the Tier 1 bacterial pathogens as outlined by the Federal Experts Security Advisory Panel – Recommendations Concerning the Select Agent Program (June 13, 2011; Department of Health and Human Services).

As part of our contract with DTRA we are in the process of evaluating the portable Deki Reader (Fio Corp). This enables image analysis of the rapid tests in cassettes, determines positivity and negativity and stores clinical data in the cloud. Information and benefits on the Deki reader is provided below. Fio Corp has provided InBios two Deki Readers to evaluate the AMD LFI strips in a cassette supplied by Fio. (See attached letter of support).

Fio Technology Overview - Fionet System: The Fionet system represents a significant advance in point-of-care (POC) rapid testing and patient case management. Fio's technologies provide healthcare providers faster, more accurate, and more cost effective rapid test results. Fionet makes it possible to collect and monitor diagnostic and clinical data in real time, to support fast, targeted and economic health interventions.

The Fionet system integrates mobile diagnostic devices with cloud information services. Fionet is comprised of Deki, a companion system for health workers, airFio**,** a secure cloud data system, and Spiri, a web-based gateway to information services for health managers. Deki mobile software applications and companion devices for health workers guide clinical workflow and facilitate digital data capture at the point of care. Deki is designed for use with standard Android phones ("Deki Phones") and tablets ("Deki Tablets"), and the Deki Reader. Deki captures and transfers critical patient information to airFio, giving managers high-quality data.

airFio aggregates and stores data transmitted by Deki units (readers, tablets and phones) over wireless mobile phone or WiFi networks. Spiri controls access to airFio and provides two-way communication between Deki units and Spiri. airFio and Spiri provide real-time access to aggregated data from all deployed Deki Readers and generate automated reports for health program monitoring, evaluation and surveillance. In addition, Spiri enables managers to monitor and communicate with health workers, and to disseminate workflow protocols, custom survey forms and software updates.

The Deki Reader is a portable, rugged, rechargeable battery-powered rapid diagnostic test (RDT) reader with diagnostic software developed on Google's Android operating system. The user interface consists of a liquid crystal display (LCD) display with a touch screen, and a drawer that holds the RDT cassettes (or dipsticks) in place during image analysis. The Deki Reader is programmed to function with a predefined menu of commercially available RDTs that have been validated for use with the reader. Hence, the Deki Reader is a universal RDT reader that can accommodate the majority of RDT cassette formats.

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1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2013) The global distribution and burden of dengue. Nature 496: 504-507.
2. Limmathurotsakul D, Dance DA, Wuthiekanun V, Kaestli M, Mayo M, et al. (2013) Systematic review and consensus guidelines for environmental sampling of Burkholderia pseudomallei. PLoS Negl Trop Dis 7: e2105.
3. Limmathurotsakul D, Peacock SJ (2011) Melioidosis: a clinical overview. Br Med Bull 99: 125-139.

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7. Salam AP, Khan N, Malnick H, Kenna DT, Dance DA, et al. (2011) Melioidosis acquired by traveler to Nigeria. Emerg Infect Dis 17: 1296-1298.
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OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix: [redacted]
* First Name: [redacted]
Middle Name:
* Last Name: [redacted]
Suffix: [redacted]

2. Human Subjects

Clinical Trial? ☒ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix: [redacted]
* First Name: [redacted]
Middle Name:
* Last Name: [redacted]
Suffix:

* Phone Number: [redacted]
Fax Number: [redacted]
Email: [redacted]

* Title: [redacted]

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Street2:
* City: Seattle
County/Parish:
* State: WA: Washington
Province:
* Country: USA: UNITED STATES
* Zip / Postal Code: 98104-3829

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4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/research/registry/. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

OMB Number: 0925-0001

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)		Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	1247-Bp Phase II SA.pdf	Add Attachment	Delete Attachment	View Attachment
3. *Research Strategy	1248-Bp Phase II RS final.p	Add Attachment	Delete Attachment	View Attachment
4. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
5. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

6. Protection of Human Subjects	1249-Protection_of_ Human_Su	Add Attachment	Delete Attachment	View Attachment
7. Inclusion of Women and Minorities	1250-Inclusion_of_Women_Min	Add Attachment	Delete Attachment	View Attachment
8. Targeted/Planned Enrollment Table		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Children	1251-Inclusion_of_Children_	Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

10. Vertebrate Animals		Add Attachment	Delete Attachment	View Attachment
11. Select Agent Research	1252-Select_Agents_ Research	Add Attachment	Delete Attachment	View Attachment
12. Multiple PD/PI Leadership Plan	1253-Multiple PI Melioidosis	Add Attachment	Delete Attachment	View Attachment
13. Consortium/Contractual Arrangements	1254-UNR consortium letter.p	Add Attachment	Delete Attachment	View Attachment
14. Letters of Support	1255-Letters of support fina	Add Attachment	Delete Attachment	View Attachment
15. Resource Sharing Plan(s)	1256-Resource Sharing Plan M	Add Attachment	Delete Attachment	View Attachment

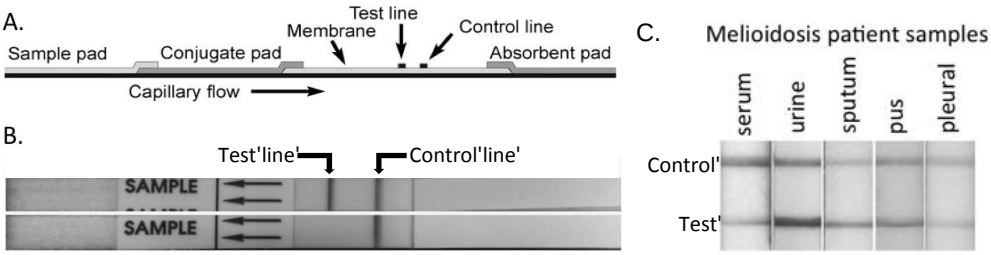
16. Appendix Add Attachments Remove Attachments View Attachments

List of Research Plan Attachments

SPECIFIC AIMS

Melioidosis is a devastating tropical disease caused by the Gram-negative bacterium *Burkholderia pseudomallei*. The clinical manifestations of melioidosis are broad and include disseminated disease with organ abscesses, severe sepsis, and mild infection of the skin and soft tissue. The highest number of reported cases occurs in endemic regions of Thailand and Australia. Of major concern is that the global distribution of melioidosis is expanding as laboratories become more familiar with identifying *B. pseudomallei*.

Isolation of *B. pseudomallei* by culturing clinical samples remains the diagnostic gold standard, unfortunately this takes 3-7 days. Culture is routinely performed on multiple sample types (blood, urine, pus, sputum etc.) and isolation of *B. pseudomallei* from any one of these samples is diagnostic for melioidosis. A major roadblock is that many diagnostic laboratories misidentify *B. pseudomallei* through lack of experience or validated diagnostic reagents. Any delay in diagnosis is critical as *B. pseudomallei* is resistant to many antibiotics. A rapid diagnosis is essential so patients can be treated with effective antibiotics.



Prototype Active Melioidosis Detect lateral flow immunoassay.

For the past two years InBios and the AuCoin laboratory have been developing the Active Melioidosis Detect™ lateral flow immunoassay (AMD LFI) under an NIAID Phase I STTR award (Figure). The AMD LFI is an immunochromatographic strip assay that detects the capsular polysaccharide (CPS) of *B. pseudomallei* within a variety of melioidosis patient samples. Phase I Specific Aims focused on producing a library of anti-CPS monoclonal antibodies (mAbs), optimizing the LFI components and sample preparation, and performing a feasibility study on archived melioidosis patient samples. The objectives of this Phase II proposal is advanced development and validation of the prototype AMD LFI. The Phase II Specific Aims are as follows:

1. **Scale up production of purified CPS and lead candidate monoclonal antibody 4C4 (Year 1)**
2. **Optimize the 4C4 AMD LFI for detection of CPS in all relevant clinical matrices**
   a. Spike all available sample matrices with CPS and determine analytical sensitivity (limit of detection) in each sample (Year 1)
   b. Fine-tune and standardize LFI prototype for compatibility with all matrices by finalizing selection of buffer, pads, membranes, blockers etc. (Year 1-2)
   c. Provide prototype tests to collaborators for further testing of all matrices and cultures using archived specimens. Develop diagnostic algorithm (Years 1 - 2)
   d. Seek pre-submission guidance from FDA for studies required for 510(k) submission (Year 2)
3. **Produce multiple lots of the 4C4 AMD LFI under document and design control and GMP for large preclinical evaluation of performance characteristics with archived patient samples**
   a. Complete in house validation following pre- submission guidance from FDA; Studies include: analytical sensitivity, reactivity, and specificity,cross reactivity, reproducibility/precision studies at 3 sites, interference and stability studies (Years 2 & 3)
   b. Validate AMD LFI in field studies at two geographical sites using archived sera and other matrices to confirm diagnostic algorithm and compare with current gold standard diagnostic (Years 2 & 3)
   c. Prevalence studies in melioidosis high and low endemic countries (Years 2 & 3)
4. **Determine the *in vitro* and *in vivo* expression and secretion profile of CPS**
   a. Determine variation of CPS expression across genetic diversity of *B. pseudomallei* (Years 1 & 2)
   b. Evaluate 4C4 AMD LFI with mouse and NHP aerosol model of melioidosis (Years 2 & 3)
   c. Quantify CPS in human sample with the qCPS ELISA (Years 2 & 3)

This is a mature project that has developed from i) identification of a melioidosis biomarker, ii) construction of an promising prototype immunoassay, iii) successful feasibility studies in endemic sites and iv) assembly of an optimized lateral flow immunoassay ready for a larger preclinical analysis.

RESEARCH STRATEGY

Significance

***B. pseudomallei* is a major cause of community-acquired sepsis in endemic areas.** The clinical manifestations of melioidosis include mild infection of the skin, disseminated disease with organ abscesses and severe sepsis [1]. The highest numbers of cases occur in endemic regions of Thailand and Australia. Rising incidence rates have been recorded in Thailand between 1997-2006 during which the average mortality rate was 42.6% [2]. In 2006, melioidosis and tuberculosis mortality rates in northeast Thailand were equivalent and second only to HIV/AIDS for infectious disease deaths [2]. In regions of northern Australia, where intensive care treatment is more readily available, the mortality rate is still alarmingly high at 20% [3,4].

The global distribution of melioidosis is expanding. Melioidosis is highly endemic to northeast Thailand and northern Australia, but more recent evidence indicates a much wider distribution that includes the India, southern China, Hong Kong, and Taiwan [6]. There are also an increasing number of reports of possible endemic disease in the Caribbean, Central and South America and East and West Africa [1,6-10]. In these regions melioidosis be overlooked because diagnostic confirmation relies on microbiological culture, which is often unavailable in resource-restricted regions. Even with such facilities, *B. pseudomallei* is often dismissed as a culture contaminant, or misidentified as a *Pseudomonas spp.* or other organisms by identification methods including API 20NE and automated bacterial identification systems. [redacted]

***B. pseudomallei* has been classified as a Tier 1 select agent.** Health and Human Services has compiled a list of roughly forty-five biological select agents and toxins (BSATs). The Federal Security Advisory Panel [11] was recently tasked with classifying the most dangerous BSATs as Tier I. Ten BSATs were classified as Tier I; *B. pseudomallei* is included on this list. Tier I classification is based on the ability to produce a mass casualty event or devastating effects to the economy, communicability, low infectious dose, and a history of or current interest in weaponization based on threat reporting [11].

Melioidosis presents with many diagnostic challenges. Isolation of *B. pseudomallei* from clinical samples remains the "gold standard" against which other melioidosis diagnostics are compared [13]. Culture is routinely performed on multiple sample types such as blood, urine, pus, sputum etc. [12,14]. A critical barrier to accurate diagnosis is the highly variable efficiency with which the bacterium is cultured from different clinical samples. Table 1 summarizes quantitative culture results from a variety of melioidosis patient samples. What is clear is blood contains the lowest levels of *B. pseudomallei* (~ 1 CFU/ml). In endemic areas blood cultures are routinely performed and nearly half are positive.

Table 1. *B. pseudomallei* culture results from 414 melioidosis patients[a]

Sample (# collected)	Median CFU/mL	% Culture positive
Blood (414)	1.1	49%
Urine (268)	1.5×10^4	21%
Sputum (120)	1.1×10^5	78%
Pus (28)	1.1×10^7	82%

[a] modified from [12]

An additional barrier is culturing of clinical samples takes 3-7 days [15]. A problem that is compounded by the fact that many diagnostic laboratories misidentify *B. pseudomallei* [16]. *B. pseudomallei* requires therapy with ceftazidime or a carbapenem drug, which are not agents of choice for empirical therapeutic regimes. Taken together, these factors point to a clear need for a simple and rapid diagnostic test for accurate identification of *B. pseudomallei* directly on clinical samples or cultures.

The current rapid technologies for diagnosing melioidosis are unacceptable. Many laboratories in endemic areas have developed "home-brew" assays in an attempt diagnose melioidosis prior to culture results becoming available. PCR has been developed but is not in routine practice because it is limited by low sensitivity, stemming from the low concentration of *B. pseudomallei* in blood and the co-purification of PCR inhibitors with target DNA [17-19]. The indirect hemagglutination assay (IHA) is a rapid and inexpensive method used to detect *B. pseudomallei* antibodies. However, a large percentage of healthy individuals in endemic areas are seropositive [20,21]. This point is underscored by the fact that nearly 70% of children in northeast Thailand are seropositive for *B. pseudomallei* antigens [22,23]. Consequently, the IHA (or any serological test for melioidosis) has limited clinical utility in the endemic setting [1,24].

Antigen detection by immunofluorescence assay (IFA) or latex agglutination is commonly used in endemic areas. IFA is used in northeast Thailand for rapid diagnosis directly from patient samples containing high levels of *B.*

pseudomallei (sputum, pus, urine and respiratory secretions) [25,26] and from blood cultures [27]. The main drawback of IFA is the requirement for a fluorescent microscope and the requisite expertise, which is not feasible in most endemic settings. In addition, although specificity of the IFA is high, the sensitivity ranges from 45-48% when used directly on clinical samples [25]. Latex agglutination is an inexpensive technique that is effective at identifying *B. pseudomallei* from cultures of patient samples grown on agar plates or within liquid broth [28-31]. The agglutination assay is able to detect *B. pseudomallei* at concentrations of 1-2 x 10^6 CFU/ml; this limits its utility to cultured patient samples or colonies isolated on solid agar [30,31].

Development of this test provides an opportunity to test for Burkholderia pseudomallei in a broad spectrum of clinical samples. This has the potential to lead to an improved diagnostic algorithm for testing for melioidosis. Availability of the test will also enable clinicians worldwide to assess the true prevalence rate of the disease. Our approach to commercializing the product is outlined in the commercialization plan. In addition in the plan we discuss the opportunity to couple the simple field test with a portable Deki reader which allows for scanning of tests, data analysis of line intensity and storage of clinical information and test data in the cloud. This would greatly improve the interpretation and record keeping at remote sites.

Innovation

A new addition to the diagnostic algorithm for melioidosis. As mentioned above, diagnosis of melioidosis is challenging. There are advantages and disadvantages to all of the rapid melioidosis assays mentioned, however the AMD LFI was designed to offer a significant improvement over the current rapid techniques. **We strongly feel that the AMD LFI can replace all unapproved rapid tests thus, simplifying the melioidosis diagnostic algorithm**. Lateral flow devices are inexpensive, rapid, sensitive, and stable at room temperature. In addition, LFIs do not require expensive equipment and they can accept multiple sample matrices, two characteristics that are essential for the diagnosis of melioidosis in resource poor settings.

InMAD is a novel methodology for the identification of microbial biomarkers. The innovation of this project began with determining which *B. pseudomallei* antigens are detectable within patient samples that contain an overwhelming amount of host proteins. In an attempt to identify circulating bacterial antigens that possess diagnostic potential, we developed a platform technology termed "In vivo Microbial Antigen Discovery" or "InMAD" [32,33]. The process involves immunizing a mouse with filtered serum collected from an animal model of melioidosis. The immunized mouse develops an antibody response to the shed/secreted *B. pseudomallei* antigens present in the filtered serum. The serum from the immunized mouse is used to probe a *B. pseudomallei* lysate by Western blot. Reactive antigens are identified as candidate diagnostic biomarkers. We identified a number of biomarkers, however the CPS was the most encouraging target [32]. Research and development performed in Phase I confirmed that the CPS was indeed shed into melioidosis patient samples, thus validating the InMAD procedure. Since then we are using the InMAD procedure to identify diagnostic biomarkers for invasive aspergillosis (IA) under the auspices of NIAID R21/R33 award: "Innovative Approaches to Target Identification and Assay Development for Fungal Diagnosis".

InBios and UNR have refined the selection of antibodies that should be used for diagnostic assays. Two practical characteristics of antibodies have been discovered during the Phase I period. First, we have determined that IgG3 antibodies may not be an ideal choice of mAb for LFI applications due to their ability to self-associate [34-36]. Our first prototype AMD LFI incorporated IgG3 mAb 3C5 as both the gold-labeled and test line mAb. The self-association properties of IgG3 mAb 3C5 made it difficult to label with gold particles for LFI conjugate production. In addition, when increased volumes of melioidosis negative patient samples were tested on the LFI self-association of the gold-labeled mAb and test line mAb occurred. This produced a small percentage of false positive results. We were able to measure this interaction via surface plasmon resonance (SPR). IgG3 mAb 3C5 was linked to a SPR sensor chip and the same mAb was allowed to flow over the chip. The data indicated that the antibodies were binding, however, other IgG subclasses did not bind to the IgG3 mAb that was linked to the chip. We also determined that IgG3 mAbs

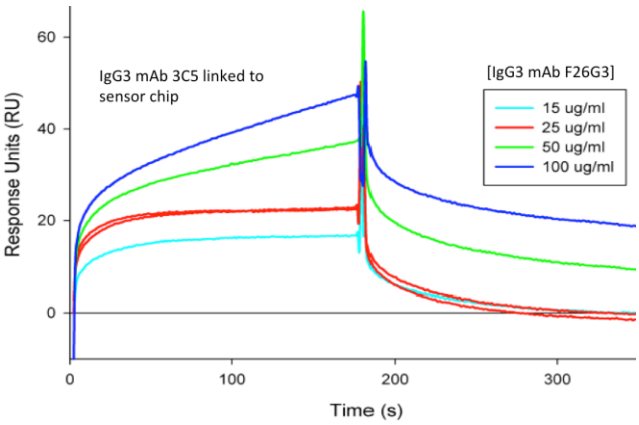


Figure 1. IgG3-IgG3 interaction. IgG3 mAb 3C5 was linked to a Biacore sensor chip. IgG3 mAb F26G3 (specific to the capsule of *B. anthracis*) was then flowed over the chip at the concentration listed. The increase in response units indicates interaction of mAbs.

that bind to different epitopes also associated. Fig. 1 illustrates that IgG3 mAb 3C5 interacts with IgG3 mAb F26G3 (specific to the capsule of *Bacillus anthracis)*.

To avoid the self-association roadblock we isolated mAb 3C5 subclass switch antibodies (IgG1, IgG2b, and IgG2a) from the IgG3 hybridoma culture. There is a low frequency of subclass switching of hybridoma cells in culture. We developed a technique to isolate subclass variants that was initially used for *B. anthracis* mAbs

[37]. As shown in Table 2, these subclass switch antibodies had considerably lower affinity than the parent IgG3 mAb and did not improve assay performance. This drastic shift in affinity occurs even though the subclass switch mAbs have identical variable regions. Our working hypothesis is that the IgG3 mAbs "self-associate" and produce higher affinity through avidity effects. This may benefit a mouse trying to survive a bacterial infection but it does not improve the performance of lateral flow immunoassays.

Approach

Research and development efforts during the Phase I period began with a prototype AMD LFI in hand. The prototype was constructed with mAb 3C5 on the test line and as the gold-labeled conjugate antibody. Our approach was to optimize the 3C5 AMD LFI in the laboratory and at endemic sites, while simultaneously trying to isolate a library of high affinity CPS mAbs. Our Phase I STTR Specific Aims and Results are listed below; we are confident all or our goals were met.

Table 2. Characteristics of CPS specific mAbs

mAb	Subclass	KD (nM)	Improvement over 3C5 IgG3*
3C5	IgG3	73	-
3C5	IgG1	1460	No
3C5	IgG2b	788	No
3C5	IgG2a	377	Same
4C4	IgG1	12	Improvement
2A5	IgG1	43	Same
1A2	IgG1	36	Same
3B4	IgG1	139	No
1D3	IgG1	Low	No
2B3	IgM	Low	No
1D4	IgM	Low	No

*As determined by ELISA and/or LFI

1. Produce and characterize a library of high-affinity CPS mAbs. mAb 3C5 performed well when incorporated into the 1st generation AMD LFI. However, we believed we could improve performance by incorporating higher affinity CPS specific mAbs into the LFI. As described above an additional reason for producing a library of CPS mAbs relates to the characteristics of mAb 3C5, which is a murine IgG3 antibody. IgG3 antibodies are difficult to label with gold and tend to self associate. We anticipated that incorporating high affinity, non-IgG3 mAbs into the LFI would simplify process development and produce a more accurate and sensitive assay.

Listed in Table 2 are the anti-CPS mAbs that were isolated during Phase I. mAb 3C5 IgG3 was our first and only CPS mAb isolated by immunization of BALB/c mice with heat killed *B. pseudomallei*. The IgG1, IgG2b, and IgG2a 3C5 mAbs were isolated from the parent 3C5 IgG3 hybridoma cell line. Interestingly, the affinities of these switch mAbs were 10-50 fold lower than the IgG3 3C5 mAb. The only subclass that was comparable to the IgG3 subclass via antigen-capture ELISA was the IgG2a.

The additional mAbs listed in Table 2 were isolated by immunization with a BSA-CPS glycoconjugate that was provided to the AuCoin laboratory by Dr. Paul Brett (University of South Alabama). As expected the glycoconjugate induced a robust IgG response in the immunized mice. We were able to isolate two IgM mAbs and five IgG1 mAbs. The IgM mAbs did not perform well, however two of the IgG1 mAbs (1A2 & 2A5) were comparable to mAb 3C5 as determined by ELISA. Additionally, one mAb (4C4) performed better via ELISA (data not shown). These mAbs were then incorporated into prototype LFIs. Each mAb was tested as the gold conjugate and test line mAb. Different combinations of mAbs were used to determine the best performing prototype. Fig. 2 is an example of one of the mAb combination experiments. InBios determined that mAb 4C4 was superior as the gold conjugate and the test line mAb. Therefore, a prototype 4C4 AMD LFI was developed and

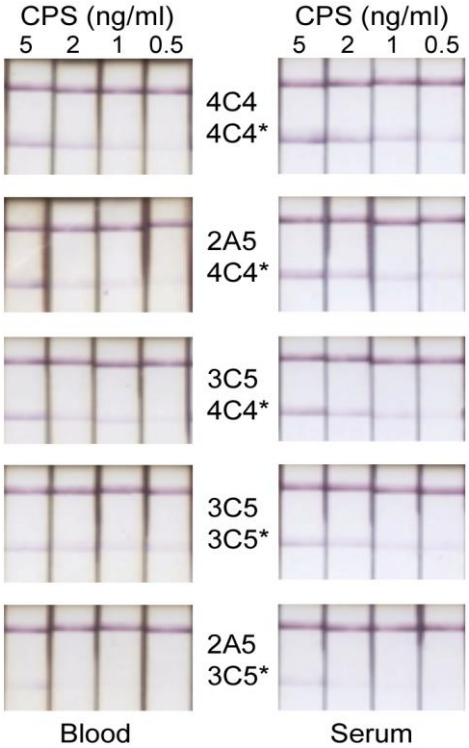


Figure 2. Optimization of mAb combinations. Example of testing different prototypes with gold-labeled mAb (indicated with *) along with a test line mAb.

tested. As shown in Fig. 3 the 4C4 AMD LFI produces a limit of detection (LOD) of 0.04 ng/ml when CPS is diluted in 150 μl of chase buffer (the chase buffer is a component of the LFI kit). This is lower than the 3C5 AMD LFI, which achieved a LOD of 0.2 ng/ml. In addition, the 4C4 AMD LFI test line appears more intense at all CPS concentrations.

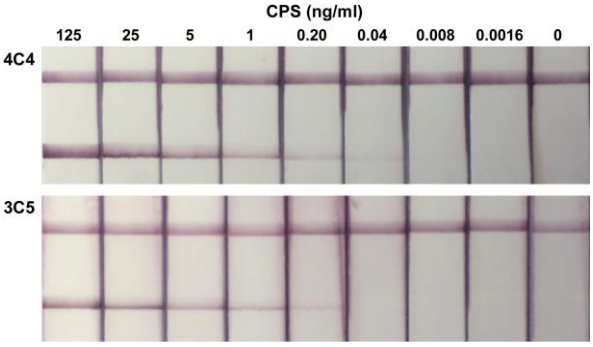


Figure 3. LOD comparison between the 3C5 and 4C4 AMD LFIs. A five-fold serial dilution of CPS was spiked into chase buffer followed by application to the sample pad.

While producing a library of CPS mAbs we concurrently tested the analytical reactivity and specificity of the 3C5 AMD LFI against a panel of *B. pseudomallei*, *B. mallei,* and near neighbor species. This provided valuable data on the extent of conservation of CPS among *Burkholderia* species. Strain panels tested included isolates selected by the Stakeholder Panel on Agent Detection Assay (SPADA) Working Group. A large panel of *B. mallei* was also tested as it has recently been shown to produce the identical manno-heptose capsule as *B. pseudomallei* [38]. We have previously shown mAb 3C5 reactivity to *B. mallei* CPS by Western blot [32].

The LFI testing was performed at the Centers for Disease Control and Prevention (CDC). The CDC requested to test the assay for possible inclusion in the Laboratory Response Network which is the network of State Health Laboratories in the U.S. Three colonies from each isolate were tested separately on the LFI. Of the *B. pseudomallei* isolates tested 76/77 (98.7%) were positive and 30/33 (90.9%) of the *B. mallei* isolates were also positive. In addition, 35/36 (97.2%) of near neighbor species were negative by LFI. Eight *B. thailandensis* isolates were tested, seven were negative. Other near neighbor species where also tested, including *B. humptydooensis sp. nov.*, *B. oklahomensis* and *Burkholderia* cepacia complex species, all were negative.

Both the *B. pseudomallei* negative and *B. thailandensis* positive LFI results can be explained through sequencing analysis. The negative isolate originated from a patient with a persistent asymptomatic *B. pseudomallei* infection in Australia. A frameshift mutation was identified within the *wcbR* gene of this isolate [39]. The *B. thailandensis* LFI positive isolate was recently shown to encode the CPS operon [40,41].

The 4C4 AMD LFI was recently tested for reactivity to *B. pseudomallei* and to other near neighbor species. A panel of 40 *B. pseudomallei* isolates was acquired from the laboratory of Dr. Paul Keim (Northern Arizona University). The 4C4 AMD LFI was positive for 39/40 of these isolates. mAb 3C5 was reactive with 38/40 of the isolates via antigen-capture ELISA. In addition, 6 Gram-negative bacteria and 9 Gram-positive bacteria were tested for reactivity against the 4C4 AMD LFI, and all were negative. In addition to the testing mentioned, the 4C4 AMD LFI will be tested at UNR against the panel of bacterial isolates identical to the panel used by the CDC. The Department of Homeland Security (DHS) will provide this panel (see letter of support).

***2.* Optimize LFI components for enhanced detection of CPS in serum and urine samples.** Phase I began with fine-tuning the 3C5 AMD LFI to achieve the lowest analytical sensitivity (limit of detection) without causing background reactions (false positive tests). When the LFI was tested with 20 different serum and urine samples a small percentage of false positive reactions were occurring. This occurred only when increased volumes of sample (50 - 100 μl) were analyzed and did not occur when 25 μl of sample was used. Ideally, we wanted the option of testing higher volumes (100 μl) on the LFI; this would be ideal for blood and urine samples that contained low levels of CPS. This is a fairly common problem and often relates to the pH of individual samples. However, we believed two possible factors could be exacerbating the false positive reactions. First, as described the gold-labeled IgG3 mAb 3C5 could be binding to IgG3 mAb 3C5 on the test line. Second, human anti-mouse antibody (HAMA) reactions could be occurring. This can occur in blood or serum samples that contain human antibodies that bind to mouse antibodies. Thus, the human antibody binds to the gold-labeled mouse mAb in addition to the mouse mAb on the test line.

We took two approaches to eliminate the false positive reactions. First, non-IgG3 CPS specific mAbs were tested (Fig. 2). In addition, commercially available HAMA blockers were added as a component of the sample pad. Initial work focused on IgG1 mAb 2A5 since mAb 4C4 had not been isolated. An LFI composed of mAb 2A5 deposited on the test line and a gold-labeled mAb 3C5 was able to accept a higher volume of sample, however the LOD was not acceptable (Fig. 2). When mAb 4C4 was incorporated into the optimized LFI as the gold-labeled and test line mAb 75 μl of sample could analyzed with virtually no false positive reactions. The sample pad was then exchanged to accept whole blood and additional sample volume. The current 4C4 AMD

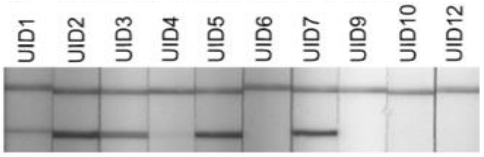
LFI is able to accept 100 μl of blood or urine. It also produces the highest signal, improved LOD and clean background when tested with multiple normal sera and whole blood samples (heparin, EDTA and citrate).

3. Evaluate optimized LFIs and quantify CPS in a variety of melioidosis patient samples. During the Phase I period we used our quantitative CPS (qCPS) ELISA [32] to analyze filtered serum and urine samples collected from patients with culture-confirmed melioidosis in Thailand (Table 3). Quantitative cultures were performed on urine samples prior to testing and are reported as colony forming units/ml (CFU/ml). Blood cultures were also tested however the CFU/ml was not determined (too low). Each serum (isolated from blood) and urine sample was filtered to remove intact bacterial cells prior to shipment. CPS was detected in 6/10 filtered urine samples at concentrations ranging from 0.78 – 448 ng/ml. The 3C5 AMD LFI was positive for all of the urine samples were positive by the qCPS ELISA (below Table 3). As expected, the concentration of CPS was higher in samples that contained a higher CFU/ml. CPS was detected in 5/10 filtered serum samples at concentrations ranging from 0.85 to 6.7 ng/ml. This is encouraging since the mean concentration of *B. pseudomallei* in patient blood is ~1 CFU/ml [12,14]. We anticipate that CPS may be shed from internal abscesses into blood; so theoretically, even if the CFU/ml is low the concentration of CPS may be within the detectable range of the LFI. Also, if the samples were not filtered we believe more would have been positive. Patient serum samples were not tested on the LFI due to insufficient volumes, but half contained concentrations of CPS that could be detected by the AMD LFI.

Table 3. Quantitation of CPS in filtered melioidosis patient samples

Urine[a]			Serum		
Sample	CFU/mL	[CPS] ng/ml	Sample	Culture result[b]	[CPS] ng/ml
UID1	2.3×10^4	2.7	MSID1	+	5.4
UID2	$>1 \times 10^5$	448	MSID2	+	0
UID3	7.5×10^4	20	MSID3	+	6.7
UID4	1.2×10^4	0.78	MSID4	+	3.3
UID5	$>1 \times 10^5$	66	MSID5	+	0
UID6	3.5×10^3	0	MSID6	+	0.85
UID7	$>1 \times 10^5$	187	MSID7	+	0
UID9	$<1 \times 10^3$	0	MSID8	+	0
UID10	1×10^3	0	MSID9	+	1.6
UID12	1×10^3	0	MSID10	+	0

[a] Serum and urine were collected from different patients
[b] Blood cultures (serum) were all positive (+)



Menzies School of Health Research (MSHR) in Darwin, Australia performed a pre-clinical evaluation of the prototype 3C5 AMD LFI over the past 18 months. In total, 81 archived samples from 38 melioidosis and two non-melioidosis patients were tested. Many of these samples were tested on the LFI multiple times in order to optimize sample preparation. This was not a trivial task. An optimized sample preparation protocol listed in Table 4. This protocol will be used to test the 4C4 AMD LFI.

In addition to sample preparation MSHR compared the performance of the 3C5 AMD LFI to both the current gold standard (culture) and real-time PCR for detection of *B. pseudomallei* specific Type III secretion system (TTS1) [42]. While all samples have been tested with LFI not all clinical specimens have been assayed with all three detection methods. Forty-eight of the samples have culture results allowing direct comparison of the sensitivity of AMD LFIs to the gold standard, with a subset of samples verified as negative or positive using the TTS1 assay.

Urine specimens (17 samples): A total of 17 melioidosis patient urine samples were evaluated. Our results show good correlation with culture results. We obtained the best correlation by first centrifuging the cellular components in the urine followed by use of the supplied lysis buffer. Initially, results indicated a decreased sensitivity of the LFIs in comparison to culture with two culture positive samples negative with the LFI tests. However, following optimization of our methods we saw an increase in LFI sensitivity and interestingly in two cases (both melioidosis patients) we saw an LFI positive while the culture results were negative.

Sputum (12 samples): Sputum performs the quite well with the LFI with strong correlation between culture results. 13 matched with culture results (11 positive, 2 negative) with one false negative. The main difficulty we encountered with sputum was the ability for the specimen to migrate along the LFI strip, necessitating variations in dilution based on sputum viscosity. The ability to diagnose melioidosis from sputum by LFI would be particularly useful in a bioterrorism setting since aerosolized *B. pseudomallei* would cause a severe pulmonary infection.

Serum (8 samples): Eight out of eight LFI tests correlated with the blood culture results (seven positive and one negative) showing promising sensitivity. Blood is a notoriously difficult clinical sample type for use in diagnosis. In this pre-clinical experimentation we saw increased sensitivity with the LFIs in comparison to the TTS1 PCR assay. This is a important result since over half of melioidosis patient blood cultures are positive.

Other Sample types: Pus, CSF and lung swabs were also tested. Four pus samples were analyzed, all showed direct correlation with culture. The one culture positive CSF sample tested failed to give a positive LFI result. The false negative result is probably indicative of the low bacterial load present in the CSF. All three lung fluid samples showed positive culture and positive LFI results.

Phase II - Specific Aim 1: Scale up production of purified CPS and lead candidate monoclonal antibody 4C4

Rationale: Our first prototype AMD LFI incorporated mAb 3C5 as the conjugate and test line mAb. During Phase I studies a significant amount of time was spent characterizing this mAb and the LFI. The mAb and the LFI performed well, however, based on our preliminary data, mAb 4C4 is now the current lead antibody. It has a higher affinity than mAb 3C5, it is a non-IgG3 antibody, and it performs better that mAb 3C5 when incorporated into the AMD LFI (Fig. 3). For these reasons we will scale up and validate large batches of mAb 4C4. In addition, we will purify CPS, an indispensible reagent for AMD LFI production.

Table 4: Optimized sample preparation protocol

Sample type	Sample preparation
Whole blood, serum, and plasma	Combine 35 μl with 3 drops of chase buffer
Urine & Culture fluid (concentrated)	Centrifuge 1-10 ml, 3000 x g, 10 min
	Resuspend cell pellet in 2 drops of lysis buffer
	Combine 20μl with 3 drops of chase buffer
Urine (straight)	Combine 50 μl with 3 drops of chase buffer
Pus	Combine 20 μl with 2 drops of lysis buffer, vortex
	Add 20 μl of lysate to sample pad followed by 3 drops of chase buffer
Sputum (viscous)	Combine 20 μl with 3 drops of lysis buffer; vortex
	Add 20 μl of lysate to sample pad followed by 3 drops of chase buffer
Sputum (non-viscous)	Combine 50 μl with 2 drops of lysis buffer; vortex
	Add 20 μl of lysate to sample pad followed by 3 drops of chase buffer
Bacterial colonies	Resuspend a single bacterial colony in 2 drops of lysis buffer
	Apply lysate to sample pad followed by 3 drops of chase buffer

Monoclonal antibody purification. We have begun scaling up production of mAb 4C4 in order to produce a large lot of 4C4 AMD LFIs for pre-clinical testing. We are currently growing mAb 4C4 hybridoma cells in CELLine bioreactors (Integra Biosciences); production from each bioreactor is 5-10 mg/week. mAb is purified via affinity chromatography over protein-A Sepharose followed by concentration. A number of quality control steps will be followed to verify batch purity and mAb performance. First, a non-denaturing Coomassie gel will be used to confirm the mAb has not fragmented during the purification process. Next, the batch is evaluated for binding to purified CPS via ELISA. The binding affinity (K_D) of each batch will also be evaluated by surface plasmon resonance (SPR). To perform the affinity analysis CPS must first be conjugated to biotin, which allows for binding to a streptavidin coated SPR sensorchip. The binding affinity (K_D) between mAb and CPS can then be calculated as a function of the kinetic constants (on- and off-rates). This analysis was performed for each of the mAbs listed in Table 2 and is commonly performed in the AuCoin laboratory. Each lot of mAb will be compared to previous lots to ensure there is no variation.

Purification of CPS. Purified CPS is an essential quality control (QC) reagent that is required to validate AMD LFI performance. The AuCoin laboratory has been optimizing the purification of CPS during the Phase I period. Purification of CPS is being performed as previously described [32,43]. The *B. pseudomallei* LPS O-antigen and CPS co-purify by the method described, therefore, an O-antigen-deficient mutant isolate of *B. pseudomallei* (DD503) is being used. The CPS is isolated from overnight cultures of DD503 by a modified hot aqueous-phenol procedure. The structure and purity of CPS will be verified by the Complex Carbohydrate Center (University of Georgia). Both mAb and CPS will be produced under GLP. A senior technician, who has completed FDA GLP training for pre-clinical testing, will oversee GLP requirements in the AuCoin laboratory.

Limitations and alternative strategies. This aim is fairly straightforward. Purification of mAb 4C4 and CPS has been initiated during the Phase I period. In addition we will produce 100 mg of each of the alternative mAbs. The alternative mAbs are 3C5, 1A2, and 2A5. These mAbs performed well via ELISA and LFI and will be available if needed. These mAbs may be incorporated into the LFI if by rare chance mAb 4C4 does not meet design specifications described in Specific Aims 2 & 3. For example, we strongly feel that the 3C5 AMD LFI would be an acceptable alternative to the 4C4 AMD LFI.

Timeline and benchmarks for success. We will produce 100 mg of purified CPS and 1 gram of mAb 4C4 by the end of Project Year 2. Success will be measured by purifying enough mAb and CPS needed to support the large preclinical analysis that will occur in Project Year 3.

Specific Aim 2: Optimize the 4C4 AMD LFI for detection of CPS in all relevant clinical matrices

Rationale. An LFI must be constructed that can accept a variety of melioidosis patient samples. LFI components and buffers have been optimized for the 3C5 AMD LFI. This Aim will determine whether these components will be compatible with the 4C4 AMD LFI. We feel if we can optimize the 4C4 AMD LFI to perform well in blood products (that contain low CFU/ml) this test should work well in other matrices that contain higher CFU/ml (Table 1).

Fine-tune and standardize LFI prototype for compatibility with all matrices by finalizing selection of buffer, pads, membranes, blockers etc. The components of the 3C5 AMD LFI were optimized and tested in endemic sites during the Phase I period. We expect that many of the same components will be used for the finalized 4C4 AMD LFI. As summarized in Table 5, these components can be modified to improve the performance of the LFI. Importantly, the 3C5 AMD LFI was originally designed with a sample pad that did not accept whole blood. The 4C4 AMD LFI contains a sample pad that accepts whole blood and a careful evaluation of the pad will be performed.

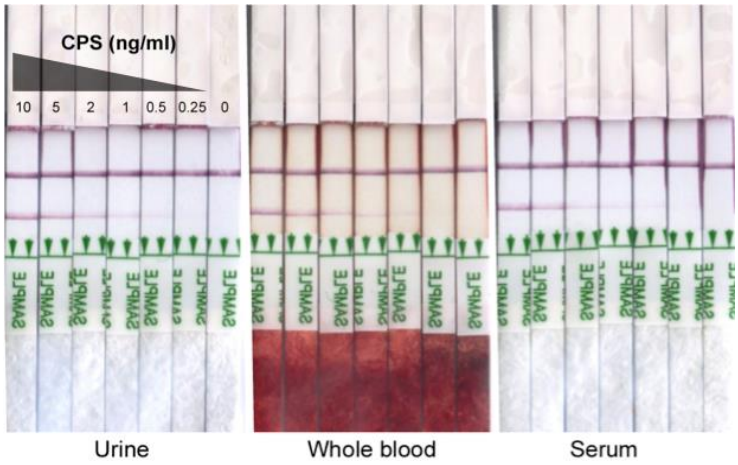


Figure 5. Optimization of the 4C4 AMD LFI in a variety of sample matrices. Purified CPS was spiked into control urine, whole blood and serum at the indicated concentrations. The same CPS concentrations were used to spike each sample type.

Spike all available sample matrices with CPS and optimize detection. 4C4 AMD LFI components and buffers will be selected based on the ability to detect the lowest concentration of CPS in all sample matrices. This will be performed by spiking blood, serum, urine, sputum, pus with a concentration range of purified CPS. This work has been initiated at InBios with the 4C4 AMD LFI prototype. Shown in Fig. 5 CPS was spiked into either 35 μl of control urine, whole blood and serum followed by addition of chase buffer. In each of these sample matrices CPS is detectable at 0.25 ng/ml.

Provide prototype tests to collaborators for further testing of all matrices and cultures using archived specimens. Our collaborators in Thailand and Australia have access to a melioidosis patient population that is not available in the US. Therefore, Drs. Currie and Limmathurotsakul will evaluate the prototype 4C4 AMD LFI on de-identified melioidosis patient samples (see support letters). As summarized in Table 4, sample preparation guidelines for the 3C5 AMD LFI have been developed by the Currie laboratory. The goal is to determine whether these protocols are valid for the 4C4 AMD LFI and make adjustments to the procedures if necessary. Batches of 50 LFIs will be shipped to each endemic location for evaluation.

The same archived samples that were used to evaluate the 3C5 AMD LFI will be used to evaluate the 4C4 AMD LFI (as volume permit). This should provide valuable performance data of the 4C4 LFI compared to the 3C5 LFI (same samples and sample preparation). If performance of the 4C4 LFI is not optimal, adjustments to sample preparation or to the components of the LFI (Table 4) can be made at this stage. Following feedback from endemic sites additional batches of 50 LFIs will be shipped to each site. We anticipate that 250 LFIs per site will be needed to validate 4C4 LFI performance and sample preparation.

Routine practice within endemic areas is to culture samples from suspected melioidosis patients in broth for seven days and plate samples from this broth on solid agar on day 2 and day 7. The agar plate is then incubated for another 24-72 hours at which time a latex agglutination test or immunofluorescence assay (IFA) can be performed from a bacterial colony. Our collaborators stationed Laos, Drs. Dance and Newton (Lao-Oxford University-Wellcome Trust-Research Unit), strongly believe the AMD LFI will be extremely effective at identifying *B. pseudomallei* at the very early stages of the culturing process. Therefore, they will evaluate the 4C4 LFI on culture broth (see letter of support). Experiments will include spiking blood culture bottles with a range of locally relevant pathogens and testing the 4C4 AMD LFI for reactivity over specific time points during the incubation period. Following validation of this method they will perform a similar study with archived samples collected from patients diagnosed with febrile illness of unknown origin. This group performed an identical study with a diagnostic test specific for *Salmonella enterica* serovar *Typhi* [45] and determined this was a rapid, inexpensive and accurate technique. Interestingly, the study also found that of 196 clinically significant Gram-negative rods that were identified following culture 34 (17.3%) were eventually identified as *B. pseudomallei*.

Table 5. Optimization of components of a lateral flow immunoassay.	
Sample pad	The sample pad is used for buffering samples for optimal reaction with gold-labeled mAbs (conjugate) and the test antibody immobilized on membranes. The LFI for detection of CPS in a drop of blood will require a sample pad with filtering properties to remove erythrocytes. The pad will also incorporate an optimized buffer that will need to neutralize a variety of sample types.
Sample volume	Optimal samples volume to be determined; - typically 10-100 μl sample volumes are used. A number of factors to be considered: • pH and buffer capacity of samples will be carefully considered (e.g. if respiratory secretions and urine samples are found to be viable matrices • Depending on the concentration of analytes, a concentration step will be devised in place
Chase buffer	The chase buffer must i) buffer all samples; especially urine, which can cause false positive reactions due to pH variations and (ii) enhance product shelf life.
Conjugate pad	The conjugate pad must stabilize the gold-labeled mAb over its entire shelf life and must consistently release the conjugate from the pad to avoid variation in assay performance.
mAb labeling	Colloidal gold is the label of choice. A number of other labels may be selected including latex particles. Labeling efficiency can vary depending on the diameter of the label and the method of labeling. Europium based detection systems will also be explored to achieve greater sensitivity, especially in situations where very low concentrations of CPS is present and may enable detection without concentration.
Membrane	A variety of nitrocellulose membranes from a number of sources will be evaluated. Membranes will be screened based on their pore size, flow rate, and consistency. Also, nitrocellulose must be converted from a hydrophobic to hydrophilic membrane through treatment with surfactant formulations.
Test line mAb	Repeating epitopes on CPS allows use of the same mAb for the conjugate and the immobilized test line mAb. Using different mAbs for the conjugate and test line that bind to different epitopes may improve sensitivity.
Absorbent pad	The only function of the absorbent pad is to serve as a sink for the liquid that is processed through the LFI. The size and composition of the absorbent pad will be adjusted following a determination of the sample size needed to achieve sensitivity targets.

Seek pre-submission guidance from FDA for studies required for 510(k) submission. A major goal of the proposal is to submit a 510(k) by the end of Project Year 3. The FDA approval process can be quite daunting. Fortunately, InBios International has a well-established history of product development with four approved medical diagnostics. InBios will take the lead establishing the initial interaction with the FDA. The goal is to understand FDA requirements, regulations, and guidance documents and define the needs for a 510(k) submission. Please see "Commercialization Plan" for more details regarding interaction with the FDA.

Limitations and alternative strategies. Ideally, the 4C4 AMD LFI performs optimally with the LFI components and the sample preparation protocols used for the 3C5 AMD LFI. However, we cannot be sure of this. To date the 4C4 AMD LFI is performing well when CPS is spiked into blood, serum and urine in the laboratory setting (Fig 5). The real test will be to evaluate how the LFI performs in the endemic setting with multiple sample matrices. During the Phase I period we were receiving real time feedback form the endemic locations on sample preparation and LFI performance. This will also occur during the Phase II period and we feel this is essential for timely optimization of the 4C4 AMD LFI.

Timeline and benchmarks for success. We will finalize the LFI components and buffers by the middle of Project Year 2. Evaluation of the 4C4 AMD LFI at endemic sites will be completed by the end of Project Year 2. Success will be measured by i) finalizing optimized LFI components, ii) developing a validated set of sample preparation guidelines and iii) producing a 4C4 AMD LFI with improved performance over the 3C5 AMD LFI. Also, from the pre-submission we will have a clear understanding of the data needed to support a 510(k) submission by the end of Project Year 3 based on archival samples or the need for future prospective studies.

Specific Aim 3: Produce multiple lots of the 4C4 AMD LFI under document and design control and GMP for large preclinical evaluation of performance characteristics with archived patient samples

Rationale: In house validation of multiple GMP lots of LFIs will be performed. Studies will include analytical reactivity/specificity, reproducibility/precision, interference and stability analysis. These studies will be followed by evaluation of GMP LFIs at endemic sites. The list of collaborators requesting the AMD LFI is expanding (see Commercialization Plan). Multiple lots of optimized 4C4 AMD LFIs need to be produced and validated prior to shipment to collaborators in endemic sites. We anticipate requiring 5000 4C4 AMD LFIs produced in multiple lots to complete the large pre-clinical analysis during Project Year 3. These potential collaborators can substantially increase the knowledge of the presence of Melioidosis worldwide.

Production of 4C4 AMD LFIs under GMP. InBios is a FDA registered and an ISO (International Organization for Standardization) certified company who has established a set of general manufacturing principles in compliance with Good Manufacturing Practice (GMP) ensuring that products are consistently produced and controlled

according to quality standards. InBios manufacturing processes are clearly defined, controlled and documented. All critical processes are validated to ensure consistency and compliance with design specifications. All operators are trained to complete and document procedures. Deviations are investigated and documented. Records of manufactured goods are retained in an accessible form, which enables the complete history of a batch to be traced. The entire InBios manufacturing staff is trained for GMP practices. The manufacturing team includes an engineer, wet chemistry application specialists and a team of assemblers.

Analytical sensitivity – Limit of Detection (LOD): LOD is the concentration of CPS that yields a positive result 50% of the time and a negative result 50% of the time. We will follow the LOD protocol that has been established by the Clinical and Laboratory Standards Institute (CLSI) entitled, *User Protocol for Evaluation for Qualitative Test Performance* [44]. LOD will be determined on GMP produced LFIs. We will run a number of replicates to accurately determine the LOD for each sample type. LOD determination for CPS spiked into blood, serum and urine will be determined at InBios and UNR. We will rely on our collaborators in Australia and Thailand to spike patient samples, including pus, sputum, and other biological samples that are not archived at InBios and UNR. Deki Readers (Fio corporation) will also be implemented at this stage (see letter of support). The Deki Reader is a CE marked medical device that can read LFI test strips. The unit digitally captures LFI test and control line data and mobile Fionet software stores test results in a secure cloud network. Deki Readers will be used at InBios, UNR, Menzies and Mahidol to facilitate interpretation of the LFI data. Please see the commercialization plan for more information regarding the Deki Reader.

Analytical reactivity and specificity testing. Preliminary testing on the 4C4 AMD LFI produced results identical to mAb 3C5. A panel of 40 *B. pseudomallei* isolates were acquired from the laboratory of Dr. Paul Keim (Northern Arizona University). The 4C4 AMD LFI was positive for 39/40 of these isolates. mAb 3C5 was reactive with 38/40 of the isolates via antigen-capture ELISA. In addition, 15 clinically relevant bacteria (6 Gram-negative and 9 Gram-positive) bacteria were not reactive with the 4C4 AMD LFI.

As described, the 3C5 AMD LFI was tested against a large panel of *B. pseudomallei*, *B. mallei*, and *Burkholderia* near neighbor species at the CDC. The same panel of isolates will be acquired from DHS (see letter of support) in order to establish the analytical reactivity and specificity for the 4C4 AMD LFI and the alternative anti-CPS mAbs. The panel consists of 77 *B. pseudomallei*, 33 *B. mallei*, and 36 near neighbor strains. Additional isolates will include a panel of common community and hospital-acquired bacteria in addition to 7 bacterial biothreats.

Our collaborators at Menzies, Mahidol and Laos will test the LFI against *B. pseudomallei* and other bacterial pathogens previously isolated from patients with severe sepsis. It is expected that there will be more than 400 strains of *B. pseudomallei* and 300 strains of other bacteria isolated yearly. Therefore, they will test a large panel of these isolates during the process of evaluation and optimization of the 4C4 AMD LFI.

Analytical reproducibility (test replicates at different agent concentrations, at different sites, etc.): Three sites will be used to test the reproducibility of each optimized LFI lot, Deki Readers will be used to quantify results. Reproducibility studies shall be performed according to the CLSI document, EP15-A2 (Clinical and Laboratory Standards Institute, 2005, User Verification of Performance for Precision and Trueness; Approved Guideline—Second Edition (EP15-A2)). A panel of samples shall be prepared in relevant analyte-negative matrices containing CPS antigen at different concentrations. The CPS concentration selected shall ensure that the analyte concentration in the specimens represents assay specification. The test panel shall include high negative, low positive and moderate positive samples. Positive and negative control in a stable buffer system shall be provided along with the reproducibility panel package. Before LFIs will be used in pre-clinical evaluations concordant results will need to be obtained at each site.
Data analysis. The Reproducibility (Precision) study uses a QC panel that is tested at various sites by multiple users. The precision is analyzed by following NCCLS guideline EP5-T2, “Evaluation of Precision Performance of Clinical Chemistry Devices”. This will establish within-run, day-to-day, site-to-site and total standard deviations and coefficients of variation.

Ruggedness: Ruggedness testing shall examine the behavior that results from small changes in environmental and/or operating conditions, such as temperature of samples or of reagents, accuracy of obtaining sample quantity, time to test once collected, sample throughput rate and time to result, concentration of reagents, age of reagents, mixing time (if required), temperature of analysis, flow rates, variations in measuring apparatus. We shall have a detailed protocol describing how different parameters shall be studied.
Data analysis. Ruggedness testing will evaluate a QC panel and directly compare samples/tests that have undergone the ruggedness protocols and compare these to a control group. Paired student’s t-tests will be used to evaluate for statistical significance with the control group.

Stability and shelf life: The typical shelf life for rapid tests prepared with the proposed LFI format is 24 months at room temperature. However, that shall depend on active components used in the assay. Therefore, the final assay design shall be evaluated using three different lots of test strips, according to the same SOP. Reagent shelf life is routinely measured by a combination of real-time and accelerated stability testing.
Data analysis. The stability study shall evaluate a QC panel and directly compare tests that have undergone both accelerated and real-time aging to a control group and to expected values. Results shall be analyzed with a paired student's t-test to evaluate for significance with the control group.

Validate AMD LFI in field studies at two geographical sites using archived sera and other matrices to confirm diagnostic algorithm and compare with appropriate gold standard for melioidosis confirmation

Clinical sensitivity and specificity. By the end of Project Year 2 an optimized 4C4 AMD LFI and validated sample preparation protocols will be available. A large lot of LFIs will be produced and distributed to endemic sites. However, a large number of archived samples will be available given the melioidosis rates that occur in Thailand and Australia every year. Our collaborators estimate that a minimum of 300 culture-confirmed melioidosis cases will occur in these regions per year. An average of three patient sample types are collected from each patient. Therefore, the total number of samples to be evaluated will be 900.

Collaborators in Thailand (Mahidol) and Australia (MSHR) will compare all LFI results with culture results. In addition, LFI results will be compared with common diagnostic procedures that are used in each endemic region. The LFI will be used to test de-identified samples from patients with culture confirmed melioidosis and also patients presenting with sepsis. At Mahidol alone there are at least 1000 suspected cases of melioidosis per year of which 100 are confirmed by culture. Over the last 2.5 years MSHR has confirmed 254 melioidosis cases by culture. Therefore, a variety of sample types will be available from melioidosis confirmed and non-melioidosis patients presenting with sepsis to optimize the LFI.

In addition to culture, the LFI will be compared to two rapid diagnostics used for melioidosis. MSHR will test the TTS1 real-time PCR assay [46]. Mahidol will compare LFI results with an immunofluorescence assay (IFA) that is routinely performed directly on clinical samples from suspected melioidosis patients. Taken together the LFI will be methodically compared to the current gold standard and two commonly used rapid techniques in endemic regions.

Further studies will test ≥100 human whole blood samples (capillary, venous blood and plasma) obtained through commercial suppliers. InBios and the AuCoin laboratory maintain a large bank of such samples. These samples include more than 100 random blood donors, as well as samples from patients with unrelated Third World diseases and various febrile diseases such as tuberculosis, *T. cruzi,* leishmania, West Nile virus, Dengue virus, and Japanese encephalitis virus infections. Preliminary studies indicate that the 4C4 AMD LFI does not produce false positive reactions when tested against a panel twenty sera and urine samples. However, in case some samples do react in the AMD, further studies will be undertaken to characterize the response and optimize the assay to prevent it. All results shall be expressed in a specificity table summarizing analysis of normal samples and cross-reactivity; appropriate statistical methods will be used for data analysis.

The % sensitivity and % specificity will be calculated based on the number of AMD-positive or negative samples that correlated with culture positive or negative data. Samples that are AMD-positive and culture-negative may occur in patients who are diagnosed with "clinical melioidosis", have internal abscesses on the liver and spleen, and/or respond to antibiotics effective against *B. pseudomallei*. The diagnosis of "clinical melioidosis" is described in detail in a published article by our collaborator Dr. Limmathurotsakul [47].

With collaborators from other regions where melioidosis may be prevalent it is anticipated that prevalence rates may be assessed and compared to known rates in Thailand and Northern Australia enabling a prediction of expected cases in these regions.

Table 6. PRODUCT SPECIFICATION

• ***Product Identification:***	Active Melioidosis *Detect*™, device not yet classified by FDA
• ***Device:***	Active Melioidosis *Detect*™ (AMD; Lateral flow immunoassay (LFI)
• ***Intended Use:***	In vitro diagnosis of active melioidosis for visual and rapid qualitative detection of the *Burkholderia pseudomallei* capsular polysaccharide (CPS) antigen
• ***Analyte:***	Whole blood (venous and/or capillary blood), blood, urine, pus, and respiratory secretions
• ***Antibodies to be used:***	Proprietary antibodies to capsular polysaccharide antigen CPS *Burkholderia pseudomallei*
• ***Detection agent:***	Selected antibody(s) to CPS conjugated with gold
• ***Buffer system:***	Chase buffer that should not require cold storage
• ***Instrument size/weight:***	No additional instrumentation required.

Performance input:

• ***Sensitivity***	Pus > 98% Sputum > 98% Urine > 95% Blood > 90%
• ***Specificity***:	≥95%
• ***Analytical sensitivity:***	Limit of detection ≤ 1 ng/ml
• ***Comparative Methods***	PCR, Immunofluorescence, Culture
• ***Sample preparation time:***	None (depending on the matrix to be used)
• ***Reading time (time to result):***	5 to 20 minutes ***(TBD)***
• ***Sample throughput rate:***	Unlimited. Many samples can be run in parallel on separate strips.
• ***General assay characteristics:***	Clear test and control lines

Shelf life: 24 months at room temperature (≤30°C)

Matrix comparison: Matrix comparison for serum and plasma, whole blood (venous and/or capillary blood), urine, pus, and respiratory secretions matrices will be compared. The purpose of this study is to determine which of the matrix or matrices will be most suitable for the current LFI format. For this purpose, we will spike purified CPS and/or CPS containing clinical samples into the above matrices at concentrations close to LOD of the AMD system and test. The matrix study will be performed in-house or by our collaborators before clinical studies can begin. The results will be reported in the FDA application.

Limitations and alternative strategies. The GMP produced LFIs need to perform consistently as outlined in above. If major problems arise (high false positive rates, poor sensitivity, etc.) then InBios will make the proper modifications to the LFI and produce additional GMP lots for testing. We do not anticipate changes will need to be made to sample preparation guidelines, however, this can also occur during this larger LFI analysis.

Timeline and benchmarks for success. Analytical reactivity and specificity testing is currently being performed and will be a major focus throughout the grant period. Large lots of 4C4 AMD LFIs will be ready for testing at the start of Project Year 3. The large preclinical analysis will occur throughout Project Year 3. Product specifications and performance targets are listed in Table 6. There is currently no approved rapid diagnostic for melioidosis. However, there are a number of non-validated rapid diagnostics that have been evaluated (latex agglutination, immunofluorescence, PCR, etc.). The 4C4 AMD LFI will need to be superior to any current rapid diagnostic for melioidosis while performing as well as culture.

Specific Aim 4: Determine the *in vitro* and *in vivo* expression and secretion profile of CPS

Rationale. Development of a CPS detection immunoassay for the diagnosis of melioidosis will require a detailed understanding of the expression and secretion profile of CPS. To this end, the qCPS ELISA will be used to establish the concentration of CPS within (i) in vitro grown cultures, (ii) a variety of de-identified melioidosis patient samples, and (iii) within samples from animals challenged via the aerosol route with *B. pseudomallei*. The advantage of the animal studies is that the exact time of infection is known and the concentration of CPS within a variety of samples can be determined at defined time points post-exposure.

Determine variation of CPS expression across the genetic diversity of B. pseudomallei. *Burkholderia* encode very complex and variable genomes that have developed from its ability to acquire sequences from other bacteria [1]. However, we have shown that expression of CPS is highly conserved among *B. pseudomallei* isolates. We will determine if there is variation in the expression and secretion profile of CPS over a diverse panel of isolates that includes representatives from each the major clades of *B. pseudomallei*. The isolates will be selected from the panel of *B. pseudomallei* supplied by DHS. Isolates will be grown in culture broth and a time-course of samples will be collected. Quantitative cultures will be performed on each sample and the production of CPS/bacterial cell and CPS/ml of supernatant fluid will be determined with the qCPS ELISA. Purified CPS will be used to create a standard curve in order to determine the concentration of CPS within the samples.

Evaluate 4C4 AMD LFI with mouse and NHP aerosol model of melioidosis. The AuCoin laboratory has established a collaboration with Dr. Simon Funnell (see support letter) who is the Scientific Leader/Program Coordinator of Microbiology Services for Public Health England (PHE, Porton Down, United Kingdom). PHE has secured funding from NIAID for two projects in which BALB/c mice and rhesus macaques will be used to study the effects of aerosol challenge with *B. pseudomallei*. The PHE team is interested in determining if *B. pseudomallei* CPS is detectable in samples taken during the BALB/c and macaque studies of infection. A pilot study was recently performed with the 3C5 AMD LFI. Initially, LFI performance was confirmed with macaque urine (naïve and spiked with CPS), followed by testing urine collected from a macaque on day 4 post *B. pseudomallei* infection (Fig. 5). The macaque sample was positive by LFI indicating accumulation of CPS and/or

B. pseudomallei in urine. Based on the pilot study PHE has requested the use of the 4C4 AMD LFI and the qCPS ELISA.

Figure 5. Detection of CPS in macaque urine. Urine sample was collected at day 4 post *B. pseudomallei* infection. Sample was diluted 1:1 in chase buffer and tested on the 3C5 AMD LFI.

The BALB/c study involves challenge with the K92643 strain of *B. pseudomallei*. The rhesus macaques will be challenged with a different laboratory strain of *B. pseudomallei* (NCTC 13392). For each of these studies, urine, blood and oral swabs (macaques) will be collected prior to and following infection at defined time points. Although we would expect CPS concentrations to be correlated to CFU/ml of *B. pseudomallei* in the animal samples, it may be possible to detect CPS in the absence of bacteremia. The AuCoin laboratory will provide the reagents to run the qCPS ELISA to enable PHE to determine the concentration of CPS in the collected samples. In addition, the 4C4 AMD LFI will also be tested on as many samples as possible (as volumes permit).

Quantify CPS in human samples with the qCPS ELISA. Filtered urine and serum samples from Thailand were recently shipped to the AuCoin laboratory and analyzed with the qCPS ELISA (Table 3). The data was quite encouraging with six out of ten urine samples containing readily detectable levels of CPS. Serum as expected, contained less CPS however; in five out of 10 samples CPS was detectable. The AuCoin laboratory will continue to perform a qCPS ELISA on samples tested by LFI in endemic areas. Small aliquots of de-identified patient samples will be shipped to the AuCoin laboratory and the qCPS ELISA will be performed in a BSL-3 laboratory. The samples will not be filtered, unlike the samples used to generate the data in Table 3. Culture (CFU/ml) will have been previously performed; this data will be compared with the concentration of CPS in each sample. We anticipate detection of higher concentrations of CPS in patient samples when whole bacterial cells are not removed by filtration. Also, since we have shown that CPS is a shed/secreted antigen, there is a good chance that although a blood culture is negative it still may contain CPS that is shed from a distant site, e.g. an abscesses, which is a hallmark characteristic of melioidosis. Therefore, blood and urine samples that are culture negative from melioidosis positive patients will be evaluated for the presence of CPS.

Limitations and alternative strategies. The qCPS ELISA will be performed on as many de-identified patient samples as possible. It may be the case that not every patient sample tested by LFI will be tested with the qCPS ELISA due to volume limitations. However, we are confident that enough samples will be tested by ELISA to understand the accumulation of CPS in a variety of patient samples. Another potential problem is that the concentration of CPS in blood samples may be too low to routinely detect with the prototype qCPS ELISA. This potential problem may be addressed with use of mAb 4C4 instead of mAb 3C5 as part of the qCPS ELISA. Additionally, our laboratory has recently estimated the concentration of antigens in patient samples by SPR. This will involve linking mAb 4C4 to a SPR sensor chip followed by flowing patient samples over the chip. The advantages of this method are that low volumes (~10 μl) of samples are analyzed and detection limits are far below those seen with ELISA. Another potential problem is whether the qCPS ELISA will perform consistently performed in Funnell laboratory in the UK. We will also ship the appropriate control CPS samples to ensure that results are consistent prior to testing the animal samples. We have previously shipped the qCPS ELISA to the laboratory of Dr. Gregory Bancroft (London School of Hygiene and Tropical Medicine, UK) where the assay was used to successfully identify CPS in tissue from mice infected with *B. pseudomallei*.

Timeline and benchmarks for success. We will perform the in vitro CPS expression and secretion studies and the animal model studies during Project Year 1 & 2. Quantification of CPS in patient samples will occur during the Project Years 1-3. Success will be measured by having a clear understanding of the expression and secretion profile of *B. pseudomallei* CPS across its genetic diversity and during infection.

Protection of Human Subjects

a. Human Subjects Involvement and Characteristics: The diagnostic assay developed in the course of this project will be applicable to both genders, all age groups, and all ethnicities and races. The studies described in this proposal will utilize only archived and commercial human samples without personal identifiers from patients with symptoms of particular disease. Subjects will not be selected based on age, gender, or ethnicity. The targeted racial and ethnic mix composition will be reflective of demographic regions of sample origin.

*Fetuses, neonates, pregnant women, prisoners or institutionalized individuals will not be used at any time in this project***.**
The proposed research is in compliance with DHHS and NIH regulations on protection of human subjects (HHS regulations at 45 CFR 46.102(f), and NIH regulations on inclusion of women, minorities, and children. This research project will use small quantities (less than 1 ml) of human plasma/serum, urine or respiratory secretions obtained through collaborations and via commercial sources. These will be archived samples with no personal identifiers collected as part of routine clinical procedures and not specifically for these studies. Since these are archival samples we are requesting Exemption 4. Testing of the LFI will be done with a variety of human samples, however i) no specimens will be collected specifically for this study and ii) there are no subject identifiers.

b. Source of Materials:

University of Nevada. Serum and urine samples from controls are maintained within our laboratory at UNR. The consenting process included informing subjects that the samples will be used for a wide variety of diagnostic studies. The samples have been de-identified. Specifically, i) studies will be limited to existing archived samples, and ii) the samples will be de-identified in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects. These samples will be used as negative controls for the antigen-capture ELISA for detection of CPS.

InBios. InBios maintains a bank of specimens from normal subjects and patients with a variety of infectious diseases. Consent for use of these samples for a variety of diagnostic studies has been obtained. These samples contain no subject identifiers. These samples will be used as negative controls and will also be spiked with purified CPS to determine the LOD of the LFI. No melioidosis samples will be tested at the InBios facility.

Menzies School of Health Research. All samples are coded and tested delinked to patient name. Written informed consent is obtained for sample collection from the patients and the collection has been approved by the Human Research Ethics Committee (IRB) of the NT Dept of Health and the Menzies School of Health Research (HREC 04/09). The samples are not obtained for a specific study and are used for a variety of diagnostic studies. The samples will be used to test optimized LFIs and determine proper sample preparation.

Mahidol University. The melioidosis laboratory at Sappasithiprasong Hospital received a number of specimens from patients with suspected melioidosis for rapid diagnostic tests, including immunofluorescent assays, and confirmatory tests, such as latex agglutination tests. Therefore, they are not collected for a specific study. All samples used in this proposal will be previously coded and tested delinked to patient names and identifiers.

c. Potential Risks: No potential risks to patients are expected as we will use only archived and commercial human samples. Normal universal precautions for handling samples will be adhered to.

d. Potential benefits of the proposed research to the subjects and others and importance of the knowledge to be gained:

The archived and commercial samples obtained for this project will be used to assist the design of a novel diagnostic directed to expedite the early diagnosis of Melioidosis. These samples will be used to evaluate performance of assay(s), which will help to select successful assay prototypes for further studies directed to assembly of the full diagnostic panel. The development of proposed diagnostic assay(s) can assist in developing an improved diagnostic algorithm for active melioidosis.

InBios is already approved as a site of medical research using human subjects by the NIH and has both IRB and FWA registration (FWA00001139).

INCLUSION OF WOMEN and MINORITIES

INCLUSION of WOMEN

1. A description of the subject selection criteria and rationale for selection in terms of the scientific objectives and proposed study design. In this study we will use archived samples only. Melioidosis affects both men and women; therefore, the study samples will not be gender or age specific. Samples included in this study will be from male and female individuals having symptoms of melioidosis, as well as subjects with other febrile illnesses, and normal individuals.

2. A compelling rationale for proposed exclusion of any sex/gender or racial/ethnic group. There will be no selection/exclusion based upon racial/ethnic group.
The proposed dates of enrollment (beginning and end). Archived samples will be used shortly after the grant is funded and continue throughout the grant period.

3. A description of proposed outreach programs for recruiting women and minorities in clinical research as subjects. An outreach program specifically for recruiting women and minorities will not be done as this study will not conduct recruitment. Samples will be archived and include women and minorities. Most probably the positive samples will represent racial/sexual mix representative of the total population in the specified endemic areas.

INCLUSION OF MINORITIES

1. A description of the subject selection criteria and rationale for selection in terms of the scientific objectives and proposed study design. Melioidosis is not a disease that affects a particular racial or ethnic background. Therefore, subjects used in this study will include individuals from all racial groups. Clinics providing samples typically serve an ethnically diverse population and will encompass samples from individuals with African-American, Asian, Hispanic and whites that present at that particular geographic area.

2. A compelling rationale for proposed exclusion of any racial/ethnic group. There will be no selection/exclusion based on racial/ethnic group.

3. Proposed dates of enrollment (beginning and end). The acquisition of archived samples will begin shortly after the grant is approved for funding and will continue throughout the grant period.

4. A description of outreach programs for recruiting minorities in clinical research as subjects. An outreach program specifically for recruiting minorities will not be done as this study will not conduct recruitment.

INCLUSION OF CHILDREN

If children will be included, please describe your plans for including children. We will use archived samples only. Archived samples will include children. Special recruitment of children, however, will not take place.

If children will be excluded from the Research, please provide an acceptable justification for the exclusion. Children are not excluded from this study.

Select Agents Research

InBios International

No work with Select Agents will be done at InBios.

University of Nevada, Reno

Select agent to be used. The select agent to be used is *Burkholderia pseudomallei and Burkholderia mallei*. The Department of Homeland Security (DHS) will ship large *B. pseudomallei* and *B. mallei* strain panel to the BSL3 facility at UNR. Also, if needed the Currie laboratory will supply additional *B. pseudomallei* strains in order to test the analytical reactivity and specificity of the CPS specific mAbs.

Provide the registration status of all entities where Select Agent will be used. Select Agent Registration at the University of Nevada, Reno is done under the auspices of the Department of Environmental Health and Safety at the University of Nevada, Reno (UNR). The Select Agent Registration Number for the UNR campus is C20110303-1196, effective until March 3, 2014.

Provide a description of all facilities where the Select Agent will be used. Work for the proposed study will be done in a newly constructed and CDC certified BSL-3 laboratory located within the Center for Molecular Medicine (CMM), a new $78 M biomedical research facility on the medical school campus. The BSL-3 laboratory is located adjacent to the AuCoin laboratory in the CMM. The BSL-3 laboratory has been designed and constructed from its inception for use of Select Agents. The BSL-3 was designed through consultation with Ted Traum of World BioHazTec. Mr. Traum is a world authority on design and commissioning of BSL-3 laboratories and is one of the co-authors of the NIH document for *Biosafety Level 3 Laboratory Certification Requirement*s. Under Mr. Traum's supervision, a team from World BioHazTec has conducted a certification of the BSL-3 laboratory and confirmed it meets NIH and BMBL facility performance requirements.

Procedures that will be used to monitor possession, use and transfer of Select Agent. Records of all *B. pseudomallei* and all Select Agent possession will be maintained in the form of an inventory log. A Select Agent suite (314G, see attached BSL-3 map) has been designated within the BSL-3 and contains a locked Select Agent freezer. Bacterial strains will be stored upon arrival in the Select Agent freezer within suite 314G. Access to suite 314G and the freezer within is allowed only to select agent approved laboratory employees. Information to be noted on the Select Agent freezer inventory log includes: user name, agent, date and time removed from freezer, date and time returned to freezer, purpose of use, and date and method of disposal. The inventory list is kept locked in the BSL-3 laboratory, accessible only to approved microbiologists and administrative personnel. A monthly inventory is made of all Select Agents in storage to determine if documentation of amounts in storage are correct. All materials to be discarded will be autoclaved at the end of every work session and the sterilization verified. Other reusable equipment will be decontaminated at the end of every work session. Destruction/disposal of the Select Agents is documented on a log sheet.

Plans for appropriate biosafety, biocontainment, and security of the Select Agent.

Biosafety and biocontainment considerations. All work with viable *B. pseudomallei* will take place in the CMM BSL-3. Any open cultures will handled within a biosafety cabinet within the BSL-3 suite 314G. Biosafety Class II cabinets are used, with HEPA filtration of single pass hood air to the outside, in addition negative pressure ventilation of the BSL-3 area is functional. Every reasonable precaution is taken to ensure that these organisms are handled safely in an environment designed to minimize the risk to laboratory personnel. All personnel working with open cultures of *B. pseudomallei* will be required to where appropriate PPE including a powered air-purifying respirator (PAPR).

Security measures. Eight video surveillance cameras are mounted and in use to monitor the BSL-3 area.

These are monitored at a central campus location, and can be accessed for investigation if tampering with, or removal of agents is suspected or detected. There is no outside facing windows and the BSL3 is located on the third floor of the CMM. Card-key access along with a pass-code is needed to enter the BSL-3, all entries and exits are logged in real-time. There are four suites located within the BSL-3, suite 314G has been designated a Select Agent suite. All of the Select Agents are stored in a -80°C freezer within suite 314G. Card key access to suite 314G is given to Select Agent approved employees only (as defined in the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 [42 CFR]). The select agent freezer within 314G is secured with a combination lock, thus limiting access to only those persons previously designated as approved persons. No visitors are permitted into the laboratory unless accompanied by a Select Agent approved laboratory employee. All routine maintenance and janitorial services are carried out during customary working hours of the laboratory. No casual traffic is allowed in the BSL-3 laboratory. By laboratory policy, only approved employees are allowed entrance into that area; no employee may use his/her card-key to give access to an unapproved employee, family member, or any other non-approved persons. “Piggy-backing,” the practice of two or more employees (even if approved for access to the BSL3 area) entering upon use of a single card-key, is not permitted. Each person must individually use his or her card to enter the area. Video surveillance of the area is in place to detect any violations of these policies.

Menzies School of Health Research - Royal Darwin Hospital (Menzies)

The select agent to be used is *Burkholderia pseudomallei*. Menzies *Burkholderia* repository has over 3000 isolates for use in various projects.

Registration at the Menzies School of Health Research, Darwin, Australia is through the Biosafety Committee and Laboratory Safety Committee (AQIS registration/Australian Standard AS2243, Safety in Laboratories). BSL3 laboratory registration is by The Office of the Gene Technology Regulator (OGTR) a part of the Australian Commonwealth Department of Health and Ageing. Menzies has select agent approval in the United States of America (USA) from the National Institute of Allergy and Infectious Diseases/NIH to work in collaboration with the select agent.

Menzies School of Health Research is an independent research organization located on the grounds of the Royal Darwin Hospital. Menzies operates three BSL3/PC3 suites located within a BSL2/PC2 laboratory. Select agent work is subject to approval from the Biosafety Committee and Laboratory Safety Committee (AQIS registration/Australian Standard AS2243, Safety in Laboratories) Menzies conforms with IATA Dangerous Goods Regulations and they are subject to rulings of the Office of the Gene Technology Regulator.

B. pseudomallei and all Select Agent possession will be monitored in the form of inventory log sheet documentation. The Select Agent will only be worked on within the BSL3/PC3 suite rooms. Bacterial strains will be stored in the locked Select Agent freezer. Access to the BSL3 laboratory and freezer is allowed only to select agent approved laboratory employees. Information to be recorded on the Select Agent freezer inventory log includes: user name, agent, date and time removed from freezer, date and time returned to freezer, purpose of use, and date and method of disposal. The inventory list is kept locked in the BSL-3 laboratory, accessible only to approved microbiologists. A quarterly inventory is made of all Select Agents in storage to determine if documentation of amounts in storage are correct. All materials to be discarded will be autoclaved at the end of every work session and the sterilization verified. Other reusable equipment will be decontaminated at the end of every work session. Destruction/disposal of the Select Agents is documented on a log sheet.

Work with viable *B. pseudomallei* will take place in the BSL-3/PC3 suites. Any open cultures will be handled within a biosafety cabinet within the BSL3/PC3 suites. Biosafety Class II cabinets are used, with HEPA filtration of single pass hood air to the outside; in addition the BSL3 is under negative pressure. Every reasonable precaution is taken to ensure that these organisms are handled safely in an environment designed to minimize the risk to laboratory personnel. All personnel working with open cultures of *B.*

pseudomallei will be required to where appropriate PPE including a powered air-purifying respirator (PAPR).

Mahidol-Oxford Tropical Medicine Research Unit, Mahidol University, Bangkok Thailand

Dr. Limmathurotsakul and collogues have full access to a new 5,400 sq. ft. microbiology laboratory which includes a 1216 sq. ft. BSL3 laboratory. The laboratory is located on the second floor of the building. The BSL3 laboratory can accommodate up to eight people at the same time, and is equipped with eight -80°C freezers, one -20°C freezer and six Class II biosafety cabinets. Two biosafety cabinets are dedicated to *B. pseudomallei* work. One -80 freezer is dedicated to select agents including *B. pseudomallei*. This freezer is locked and access is given to authorized personnel only. Freezerworks, the sample tracking system complying with international conference on harmonization-good clinical practice (ICH-GCP), is used for storage and tracking all specimens. We will perform all bacterial culture within the BSL3 laboratory, under an established standard of procedures, and with the approvals from the Ministry of Public Health, Thailand. Mahidol-Oxford Tropical Medicine Research Unit is also enrolled in a Select Agent Program developed by CDC/NIH.

Sappasithiprasong Hospital, Ubon Ratchathani, northeast of Thailand

Dr. Limmathurotsakul and collogues have an office within Sappasithiprasong Hospital, Ubon Ratchathani. The hospital laboratory is very familiar with *B. pseudomallei* as it is one of the most common bacteria isolated from clinical specimens, and safety measures of this laboratory are at the highest standard required by the Ministry of Public Health, Thailand. *B. pseudomallei,* which is isolated from clinical specimens from patients, will be transferred to the BSL3 laboratory of Mahidol-Oxford Tropical Medicine Research Unit under appropriate containment using a high level biosafety standards. Freezerworks, the sample tracking system, has been set up at Sappasithiprasong Hospital and will be used to track the records of specimen transportation.

Multiple PD/PI Leadership Plan:

The rationale for choosing the multiple PD/PI approach relates to the unique abilities of the Co-PIs. is well-established investigator who has extensive experience immunoassay development. is the UNR Hybridoma Laboratory Director who has produced many mAbs specific to protein and polysaccharide antigens. This collaboration works well since InBios does not have a hybridoma laboratory and UNR lacks the skills needed to bring an immunoassay to market. Both investigators will contribute to all aspects of the project; however, each investigator has a distinct role in making the final decisions on scientific direction. The organizational structure of the leadership team is as follows:

is the Contact PD/PI for the project and is responsible for communication with NIH, assembling application materials, and coordinating progress reports. He will direct the studies of LFI optimization and production and evaluation studies for FDA clearance.

is a PD/PI for the project and will oversee production of the 4C4 mAb (or others as needed). He will also direct the evaluation of and Quality control of monoclonal antibodies by Biacore and studies involving quantification of CPS in patient or NHP samples by antigen-capture immunoassay. He will also be responsible for scale up and purification of CPS. In addition, he will guide experiments performed in the BSL3 laboratory relating to testing prototype LFI against a large panel of *B. pseudomallei* and *B. mallei* isolates. Finally, Dr. AuCoin will assist Dr. in assembling manuscripts and progress reports and in data analysis. Both PIs will interact with Fio Corp with regards evaluating LFIs with the Deki reader.

To enhance communication among the team the InBios and the AuCoin laboratory will arrange a conference call every two weeks to discuss progress and future directions. Also, monthly conference calls or other forms of communication (Skype) with collaborators will take place to discuss LFI evaluation. Also, will travel to InBios twice per year for one-day meetings to review data and plan future experiments. The meetings will also allow time to resolve any conflicts that have arisen between researchers. Conflicts that are particularly difficult to resolve will be decided by consensus of the PD/PI's and collaborators.

The PD/PI's will both have access to project funds through separate accounts. A subaward will be issued from InBios to the AuCoin laboratory. will make the budget decisions for InBios while will make the budget decisions for UNR.

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[redacted]

University of Nevada, Reno

December 4, 2013

[redacted]
InBios International
[redacted]

Re: STTR Certification
Project Title: **Antigen Dectection Assay for the Diagnosis of Melioidosis**
UNR PI(s): Dr. David AuCoin
UNR SP No.: 1400380

Dear Ms. Fisher,

The University of Nevada, Reno (UNR) will be pleased to participate in the proposed STTR project entitled "Antigen Dectection Assay for the Diagnosis of Melioidosis" that InBios International (Company), a small business concern, and University of Nevada, Reno (UNR), a research institution, have jointly prepared.

The small business concern and the research institution certify jointly that: (1) the proposed STTR project will be conducted jointly by the small business concern and the research institution in which not less than 40 percent of the work will be performed by the small business concern and not less than 30 percent of the work will be performed by the research institution ("cooperative research and development"); (2) the proposed STTR project is a cooperative research or research and development effort to be conducted jointly by the small business concern and the research institution in which not less than 40 percent of the work will be performed by the small business concern and not less than 30 percent of the work will be performed by the research institution ("performance of research and analytical work"); and (3) regardless of the proportion of the proposed project to be performed by each party, the small business concern will be the primary party that will exercise management direction and control of the performance of the project

Furthermore, UNR hereby provides its standard intellectual property agreement terms in the event your company receives an award under the Prime Sponsor's SBIR/STTR program:

Patents and Inventions. The Company shall own all right, title and interest in all inventions and improvements conceived or reduced to practice by the Company in the performance of the research which is the subject of this proposal. The University shall own all right, title and interest in all inventions and improvements conceived or reduced to practice by University or University personnel in the performance of the Research (hereinafter collectively "Invention") and may, at its election, file all patent applications relating thereto. In consideration of Company's support of University in performance of the Research, University grants to Company an option for an exclusive license. The rights to said option shall expire three months after University has provided written notice to Company of any such invention, improvement, application or patent ("Option Period"). Upon execution of the option in writing, the parties will meet within thirty (30) days to begin negotiating the terms of the license. The parties agree to negotiate in good faith and the terms of the license will be reasonable in relation to licenses in the field and industry. In the event a license is not executed within three (3) months from the exercise of the option,

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license will be reasonable in relation to licenses in the field and industry. In the event a license is not executed within three (3) months from the exercise of the option, or the option is not exercised within the Option Period, the University shall be free to license the Invention to others in the University's sole discretion. All Inventions that are jointly conceived or reduced to practice by both parties to this agreement shall be owned jointly by the parties. Exclusive rights to jointly owned Inventions shall be negotiated in good faith. Inventorship shall be determined under U.S. patent law.

In the event this project is funded, please send the award documents to this office, issued to our legal name:

Board of Regents, Nevada System of Higher Education, on behalf of the University of Nevada, Reno
Sponsored Projects/Mailstop 325
University of Nevada, Reno
Reno, NV 89557-0240

Attn:
If by email:

For questions of a technical or program nature, please contact the project director. For contractual or business questions, please contact this office and refer to the above SP number.

We look forward to a successful collaboration with your company on this project.

Sincerely,

Manager, Pre-Award, Sponsored Projects

[redacted]

menzies
school of health research

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discovery for a healthy tomorrow

November 4, 2013

[redacted]

Dear David,

I am drafting this letter in full support for your STTR Phase II proposal "Antigen Detection Assay for the Diagnosis of Melioidosis". A rapid, inexpensive, point-of-care immunoassay for the detection of *B. pseudomallei* antigen is greatly needed in endemic areas in order to properly treat melioidosis.

Your collaboration with InBios International has produced a prototype AMD LFI for the detection of CPS. My research group has been testing small batches of the prototype LFIs on de-identified patients samples. We are GREATLY encouraged by the results to date. As you know we have done an extensive analysis on patient sample preparation. We feel we have developed a validated set of protocols for the 3C5 AMD LFI. We would like to now test the sample preparation protocols on the 4C4 AMD LFI.

The LFI is adaptable to test many patient samples (blood, urine, pus, respiratory secretions) directly and also has the ability to test cultured patient samples. This is essential for the diagnosis of melioidosis. There is no doubt this simple test should be included in the melioidosis diagnostic algorithm. Therefore, I look forward evaluating optimized LFIs and to continue our established collaboration.

Good luck with the proposal,

[redacted]

[redacted]

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Mahidol Oxford

WELLCOME TRUST - MAHIDOL UNIVERSITY-OXFORD
TROPICAL MEDICINE RESEARCH PROGRAMME

[redacted]. November 4, 2013

Dear David,

This letter is to confirm my role in the development of the Active Melioidosis Detect lateral flow immunoassay (LFI) for the rapid diagnosis of melioidosis. We fully support the submission of the STTR Phase II grant "Antigen Detection Assay for the Diagnosis of Melioidosis" that will be submitted to NIAID. The Phase II you are drafting along with InBios will hopefully lead to continued support for this encouraging project. As you know we have tested a small number of prototype LFIs for the detection of *B. pseudomallei* CPS. We are excited with the results we have seen so far and look forward to testing optimized LFIs in the future.

As we discussed we are preparing for a large pre-clinical analysis of melioidosis patient samples and we would like to request as many as 2000 4C4 AMD LFIs.

My laboratory will continue to test de-identified samples and optimize sample preparation. We test a number of samples in order to diagnose melioidosis (blood, urine, pus, and respiratory secretions). Most often we need to rely on the "gold standard" of culturing for diagnosis, unfortunately this can take 3-7 days and the sensitivity of the culture is only about 60%. Therefore, we anticipate that the melioidosis LFI will fill a much-needed gap.

In addition, I a personally excited about the use of the lateral flow immunoassay to explore the prevalence of melioidosis across the globe. I have no doubt this assay will be useful all over Southeast Asia, India, and Africa.

Kind Regards,

[redacted]

wellcome trust
Laos Oxford
LOMWRU

Lao-Oxford University-Mahosot Hospital-Wellcome Trust-Research Unit,
Microbiology, Mahosot Hospital,Vientiane, Lao PDR. Tel/Fax:[redacted]

12 November 2013

To whom it may concern

We would like to express our support for the Phase II study being planned by Dr David AuCoin and InBios to develop and evaluate their lateral flow tests for the detection of *Burkholderia pseudomallei*. We are a research group based in Microbiology Laboratory of Mahosot Hospital in the Lao People's Democratic Republic, where melioidosis is emerging as an important public health issue. In the past 10 years we have seen more than 670 patients with culture-positive melioidosis in our hospital in Vientiane, but it is likely that this is just the tip of the iceberg as there are very few other laboratories across the country that are capable of making the diagnosis. This is important, as melioidosis does not respond to the antibiotics used routinely for the empirical treatment of sepsis and pneumonia in Laos and so, by analogy with northeast Thailand, many hundreds of patients are likely to be dying without a diagnosis or appropriate specific treatment. New diagnostic tools are urgently needed.

One of the research interests of our group is the evaluation of new diagnostic approaches appropriate to a developing country such as Laos, where there are currently few clinical microbiology laboratories. We are especially interested in simple techniques that do not need highly trained and skilled technical staff, such as lateral flow tests. We have had success using commercially available rapid diagnostic tests for typhoid on blood culture fluids (Castonguay-Vanier *et al*. Evaluation of a simple blood culture amplification and antigen detection method for diagnosis of *Salmonella enterica* serovar Typhi bacteremia. J Clin Microbiol 2013; 51: 142-8) and are keen to evaluate similar approaches for other bacteria of public health importance in this region, such as *B. pseudomallei*. We have agreed to collaborate with Dr AuCoin and his colleagues in an evaluation of the use of their lateral flow test in a real-life diagnostic setting. Initially we plan to validate of the method with simulated blood culture bottles and a range of locally relevant pathogens, as we did for the S. Typhi RDTs. We would then follow this up with a prospective study on real samples during the rainy season, when *B. pseudomallei* is one of the commonest clinically significant isolates we see in blood cultures. Since urine is a readily accessible and non-invasive sample, and urinary antigen detection has revolutionized the diagnosis of some infectious diseases such as legionellosis, we

[redacted]

would also like to investigate the use of the *B. pseudomallei* lateral flow test on urine samples from patients with febrile illness in our hospital.

Yours sincerely

[redacted]

[redacted]

[redacted]

Public Health England

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[redacted]

27 November 2013

Dear David

Re: Letter of Intent

I am writing to confirm our continued enthusiastic support for your Phase II STTR proposal entitled "Antigen Detection Assay for the Diagnosis of Melioidosis". Based on your data and our preliminary findings it appears that the *Burkholderia pseudomallei* capsular polysaccharide (CPS) maybe a valuable antigen that can be targeted for a rapid diagnosis of acute melioidosis.

As you know, we have secured funding for aerosol challenge studies in which primates and Balb/c mice will be infected via the pulmonary route with *Burkholderia pseudomallei*. These studies will provide a unique opportunity to collect and store diagnostic samples pre-infection and at defined time-points following infection. As part of our study we would like to determine if there are significant amounts of *B. pseudomallei* antigens shed into samples from infected animals. Therefore, we would like to perform the following experiments at our research facility:

- Continue to evaluate your prototype CPS LFI with animal blood, urine, and possibly respiratory secretions
- Use or to adapt if necessary, your quantitative ELISA to determine the concentration of CPS within animal samples
- Determine if CPS can be detected in the absence of viable *Burkholderia pseudomallei* in animal samples

This collaboration is very likely to enhance our current study and your proposed study. We look forward to participating in the continued success of your research. Good luck with the proposal.

Yours sincerely

Simon [redacted]

Simon Funnell
Scientific Leader/
Programme Co-ordinator
[redacted]

Victoria [redacted]

Victoria Graham
Senior Immunologist
Project Team leader
[redacted]

Miles [redacted]

Miles Carroll
Deputy Director
Head of MS Research
[redacted]

[redacted]

DEPARTMENT OF MICROBIOLOGY
THE KASTURBA MEDICAL COLLEGE
MANIPAL – 576 104, KARNATAKA, INDIA

प्रज्ञानं ब्रह्म
Manipal
INSPIRED BY LIFE

[redacted]

Ref. No. : ..... Date : .....
November 29, 2013

[redacted]

Dear David,

This letter of support is to confirm our collaboration to explore the presence of melioidosis in India through the use of your Active Melioidosis Detect™ lateral flow immunoassay (AMD LFI). My colleagues strongly agree that melioidosis is under-diagnosed in India, therefore I am also in full support of your Phase II STTR application entitled "Antigen Detection Assay for the Diagnosis of Melioidosis".

Ideally, with your assistance we will adopt the same initiative used for Africa - Detection of Melioidosis in Africa Working Group (DMAWG). The disease was long considered under-recognized in India but has now gained the status of emerging infectious disease. Increasing isolation of *Burkholderia pseudomallei*, the causative agent of melioidosis, has been encountered in recent years multiple geographic locations within India. What is desperately needed is a rapid and accurate diagnostic test to identify *B. pseudomallei* directly from patient samples and from cultures of patient samples.

My collaborators and I look forward to establishing a more accurate picture of the true incidence of melioidosis in India. We feel the currently recognized cases are merely the "tip of the iceberg".

Good luck with your Phase II application,

[redacted]

[redacted]

World Health Organization

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Tel. direct: [redacted]
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E-mail :

In reply please refer to: EZD/EB

Your reference:

[redacted]

25 November 2013

Dear David,

This letter of support is to confirm the World Health Organization's interest in the Active Melioidosis Detect™ Lateral Flow Immunoassay (AMD LFI) and your Phase II STTR application entitled, "Antigen Detection Assay for the Diagnosis of Melioidosis".

I would personally like to thank you for your contribution to the Detection of Melioidosis in Africa Working Group (DMAWG). The goal of the DMAWG is to determine if *Burkholderia pseudomallei*, the causative agent of melioidosis, is an underreported cause of infection in Africa. Within the framework of an exploratory multicenter study, hospital/microbiological facilities in Africa will be offered by DMAWG for the use of the prototype AMD LFI developed by your laboratory at the University of Nevada and InBios International to rapidly identify *B. pseudomallei* isolates.

As you know, melioidosis is highly endemic to northeast Thailand and northern Australia, but more recent evidence indicates a much wider distribution that includes the Indian subcontinent and southern China, Hong Kong, and Taiwan. There are also an increasing number of reports of possibly endemic disease in the Caribbean, Central and South America and East and West Africa. The extent of melioidosis in Africa remains uncertain.

I look forward to collaborating with your team.

Yours sincerely, [redacted]

[redacted]

Fio Corporation

November 12, 2013

Dear Ray and David:

Thank you for considering Fio Corporation and the possible use of our Deki Reader and Fionet system in your STTR Phase II submission entitled "Antigen Detection Assay for Melioidosis". The Deki reader is a CE marked medical device that has successfully been used in evaluating a malaria test in Tanzania (http://www.malariajournal.com/content/12/1/141) as well as Dengue tests. The instrument can read both cassettes but also test strips such as you are proposing using our reusable cartridge. Fio provides a Mobile Deki reader with digital data capture, mobile software applications and access to a secure cloud data system. This enables the collection and storage of information obtained from rapid field tests by small community health workers in resource poor areas. This will facilitate interpretation of rapid tests and improve the quality of diagnosis.

We wish you success in the review process of your grant and we look forward to the possibility of evaluating your test strips with the Deki reader and software with the view to their potential field use. Please keep us informed as to the status of the application

Regards

Fio Corporation
111 Queen St. E., Suite 500
Toronto, Ontario M5C 1S2 Canada

[redacted]

November 25, 2013

[redacted]

Dear David,

I wanted to extend my support and interest in your Phase II STTR application, "Antigen Detection Assay for the Diagnosis of Melioidosis". As you know I have been tasked with leading the 24 Month Diagnostics Challenge for the U.S. Defense Threat Reduction Agency (DTRA). The diagnostic assay development includes pathogen targets for antigen detection of dengue, malaria, melioidosis and plague. We are extremely interested in the progress you have made through your Phase I STTR to develop the Active Melioidosis Detect lateral flow immunoassay with InBios International because there are limited reagents for the detection of *Burkholderia pseudomallei*.

I am also following the progress of your contract to supply DTRA with a melioidosis/dengue multiplex lateral flow immunoassay. We provided funds for your group through the Naval Research Laboratory to optimize the melioidosis assay to work in combination with a dengue test developed by InBios International. The test needs to simultaneously detect both pathogens from within blood only. This is quite different from your singleplex melioidosis assay that can be used with multiple patient samples.

The contract was based on a purchase price of roughly $12 per melioidosis/dengue test. We have requested 1000 combined tests for delivery in November and 5-10,000 tests for delivery starting January of 2014. I understand that these tests were purchased as "Research Use Only" and will be evaluated by one of our Navy laboratories.

If your assay performs well there is the possibility for Next Generation Diagnostics System Increment 2, a program of record for the Medical Countermeasure Systems Joint Program Executive Office. If this technology was selected, the Department of Defense would be a potential acquisition partner in future years.

We look forward to working with your team in the future,

[redacted]

[redacted]

U.S. Department of Homeland Security
Washington, DC 20528

December 4, 2013

[redacted]

Dear David,

We at the Department of Homeland Security (DHS) wanted to express our full support for your Phase II STTR application, "Antigen Detection Assay for the Diagnosis of Melioidosis". As you know DHS is interested in the Active Melioidosis Detect lateral flow immunoassay. The project aligns with the mission of the Department of Homeland Security Science and Technology Directorate to deter, detect and mitigate a biological attack on the nation.

DHS can supply a panel of *Burkholderia pseudomallei* and near-neighbor strains. This panel is identical to the one used to evaluate the Active Melioidosis Detect LFI that incorporated mAb 3C5 at the Centers for Disease Control and Prevention. These bacterial isolates will be essential to develop an inclusivity and exclusivity panel for the new anti-capsular mAbs you have isolated during the STTR Phase I. Also, the panel will be useful in your DHS project to produce antibodies to *Burkholderia mallei* specific diagnostic targets.

DHS has experience in navigating diagnostics products through the FDA approval process. We are currently taking the lead on an FDA approval process for a lateral flow immunoassay for the detection of *Bacillus anthracis* in blood samples. This is a project that InBios International and your laboratory in collaboration with Dr. Thomas Kozel are currently involved in. Therefore, when the time is appropriate we would like to offer assistance in the FDA approval process for your Melioidosis assay.

Good luck with your proposal.

Sincerely,

[redacted]

[redacted]

DSO NATIONAL LABORATORIES
DMERI@DSO
27 Medical Drive
[redacted]

November 27, 2013

[redacted]

Dear David,

My research team from Singapore heard your presentation at the World Melioidosis Congress in Bangkok just recently and we are very interested in your Active Melioidosis Detect lateral flow immunoassay (AMD LFI). We also wish you good luck for your Phase II STTR application entitled, "Antigen Detection Assay for the Diagnosis of Melioidosis".

I would like to personally thank you for agreeing to send a batch of your AMD LFI to assist in our soil-sampling project described below:

Our project is an environmental sampling study focused on isolating *B. pseudomallei* from soil. The project covers 10 locations in Singapore, including military training grounds, nature reserves and parks. At each location, we take 100 samples as per protocol recommended by the *Detection of Environmental Burkholderia pseudomallei Working Party (DEBWorP)*. Currently we have completed 4 locations (400 samples). Water samples are collected at various sites, largely correlating to floodwaters. We have yet to commence on our air sampling yet. This project is will continue into 2016.

Kind Regards,

[redacted]

Resource Sharing Plan

Research results will be presented at national and international meetings and published in a timely fashion. MAbs produced as a result of this grant will be made available to academic investigators or licensed to commercial sources should a product arise. LFIs will be provided to academic investigators initially as Research Use Only or For Export Only.

We do not anticipate the development of any model organisms and are not generating genome-wide association studies.