Viral RNA Testing and Automation on the Bead-Based CBNE Detection Microsystem (U)

Mark S. Derzon¹, Paul Rossito², Jim Cullor², Jaime McClain¹, Cara Farrell³, Kamyar Rahimian¹, Chris Bourdon¹, Paul C. Galambos¹

¹ Sandia National Laboratories
² UC-Davis School of Veterinary Medicine
³ Summer Intern, Sandia National Laboratories

Prepared by
Sandia National Laboratories
Albuquerque, New Mexico 87185 and Livermore, California 94550

Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy’s National Nuclear Security Administration under Contract DE-AC04-94AL85000.

Approved for public release; further dissemination unlimited.
Issued by Sandia National Laboratories, operated for the United States Department of Energy by Sandia Corporation.

NOTICE: This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government, nor any agency thereof, nor any of their employees, nor any of their contractors, subcontractors, or their employees, make any warranty, express or implied, or assume any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represent that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government, any agency thereof, or any of their contractors or subcontractors. The views and opinions expressed herein do not necessarily state or reflect those of the United States Government, any agency thereof, or any of their contractors.

Printed in the United States of America. This report has been reproduced directly from the best available copy.

Available to DOE and DOE contractors from
U.S. Department of Energy
Office of Scientific and Technical Information
P.O. Box 62
Oak Ridge, TN 37831

Telephone: (865) 576-8401
Facsimile: (865) 576-5728
E-Mail: reports@adonis.osti.gov
Online ordering: http://www.osti.gov/bridge

Available to the public from
U.S. Department of Commerce
National Technical Information Service
5285 Port Royal Rd.
Springfield, VA 22161

Telephone: (800) 553-6847
Facsimile: (703) 605-6900
E-Mail: orders@ntis.fedworld.gov
Online order: http://www.ntis.gov/help/ordermethods.asp?loc=7-4-0#online
Viral RNA Testing and Automation on the Bead-Based CBNE Detection Microsystem (U)

Mark S. Derzon¹, Paul Rossito², Jim Cullor², Jamie McClain¹, Cara Farrell³, Kamyar Rahimian¹, Chris Bourdon¹, Paul C. Galambos¹

¹MEMs Technologies Department
Sandia National Laboratories
P.O. Box 5800
Albuquerque, New Mexico 87185-MS1080
²University of California at Davis, School of Veterinary Medicine

Abstract

We developed prototype chemistry for nucleic acid hybridization on our bead-based diagnostics platform and we established an automatable bead handling protocol capable of 50 part-per-billion (ppb) sensitivity. We are working towards a platform capable of parallel, rapid (10 minute), raw sample testing for orthogonal (in this case nucleic acid and immunoassays) identification of biological (and other) threats in a single sensor microsystem. In this LDRD we developed the nucleic acid chemistry required for nucleic acid hybridization. Our goal is to place a non-cell associated RNA virus (Bovine Viral Diarrhea, BVD) on the beads for raw sample testing. This key pre-requisite to showing orthogonality (nucleic acid measurements can be performed in parallel with immunoassay measurements). Orthogonal detection dramatically reduces false positives. We chose BVD because our collaborators (UC-Davis) can supply samples from persistently infected animals; and because proof-of-concept field testing can be performed with modification of the current technology platform at the UC Davis research station. Since BVD is a cattle-prone disease this research dovetails with earlier immunoassay work on Botulinum toxin stimulant testing in raw milk samples. Demonstration of BVD RNA detection expands the repertoire of biological macromolecules that can be adapted to our bead-based detection. The resources of this late start LDRD were adequate to partially demonstrate the conjugation of the beads to the nucleic acids. It was never expected to be adequate for a full live virus test but to motivate that additional investment. In addition, we were able to reduce the LOD (Limit of Detection) for the botulinum toxin stimulant to 50 ppb from the earlier LOD of 1 ppm. A low LOD combined with orthogonal detection provides both low false negatives and low false positives. The logical follow-on steps to this LDRD research are to perform live virus identification as well as concurrent nucleic acid and immunoassay detection.
ACKNOWLEDGMENTS

Sandia is a multi-program laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy’s National Nuclear Security Administration under contract DE-AC04-94AL85000. We thank the LDRD office and the Nano to Microsystems investment area at Sandia for funding this research. We also thank A. Kommandoor and K. Ortiz for their assistance and guidance.
CONTENTS

1. Introduction.................................................................................................................... 7
2. BVD Assay ..................................................................................................................... 9
3. Increased Technology Platform Sensitivity (lowering LOD)........................................ 11
4. Conclusions and Next Steps......................................................................................... 13
5. References..................................................................................................................... 14
FIGURES
Figure 1. Two-Stage BVD Hybridization Assay. Top (a) shows whole illustration of the two-stage hybridization assay that we are developing. This first phase of the development allows us to perform two critical components for nucleic acid development. The first is the establishment of the assay using primers on grown virus (performed at UCD DVM in Tulare, CA) (c – lower right) and the second was the conjugation of primer onto a magnetic bead similar to those used on the basic bead platform (b – lower left).

Figure 2. Demonstration of Biotinated Oligo binding to streptavidin coated magnetic beads (sub-assay of Figure 1(b)).

Figure 3. Sensitivity of detection technology platform demonstrated to 100 ppb.
1. INTRODUCTION

In the current threat climate pertaining to chemical, biological or nuclear weapons of mass destruction (CBN WMD) there is a need for a rugged, unobtrusive, mass deployable detector/sensor that can rapidly identify a wide variety of potential CBN agents (target molecules – toxins, proteins, cells, viruses or actinides) at low concentrations in raw samples. Such a sensor could, for instance, be used by soldiers on a battlefield to determine if they are under chemical or biological attack, or as part of an automated early warning alarm in a major city mass transit system, or as a tool for inspectors searching for evidence.

Due to the rapid propagation of disease organisms within the human body and their high lethality, early detection and classification is essential. Much of this is articulated in the February 2001 report on “Biological Detection System Technologies – Technology and Industrial Base Study,” prepared by TRW ¹ and “Sensor Systems for Biological Agent Attacks: Protecting Buildings and Military Bases”² These are detailed studies focusing on early Bio-warfare agent (BW) detection and identification technologies for the US military.

A portable, modular diagnostic system for CBN detection must rapidly and accurately identify the onset of an attack by sensing one or more of the following diagnostic markers associated with the agents:

- Agent itself/toxin, toxic industrial chemical, Antigen, Antibody, Cytokines/Chemokines,
- Radionuclide (i.e. Pu, U, etc.)
- Deoxyribonucleic Acid (DNA) or Ribonucleic Acid (RNA)

Diagnostic speed and accuracy are foremost requirements in battlefield settings subject to these WMD warfare attacks. Assay accuracy is described in terms of sensitivity and specificity to a specific diagnostic signature. Since false negatives and false positives are likely, particularly during presymptomatic stages of infection or exposure, and would directly affect the readiness and morale of a unit, it is imperative that a portable diagnostic system contain primary and secondary assays for agents of interest. It is envisioned that these assays detect and identify different features of a given biomarker, e.g., nucleic acid and peptide coat, to ensure orthogonality of the two assays.

The “Bead-based Multiplexed, Orthogonal BW/ID (BioWarfare/Infectious Disease) Detection Microsystem” was an earlier LDRD to perform proof-of-principle experiments for the concept of a raw sample, rapid assay detection scheme. This LDRD was successful: we have a crude prototype³, a detailed model⁴, and excellent results with a Botulinum Toxin surrogate (ovalbumin) in raw milk. We have extended that earlier work to include detection of actinides and lanthanides with collaborators from Argonne National Laboratory.⁵
The purpose of this LDRD was to expand the target sensitivity of the bead-based assay and technology to nucleic acids, thereby allowing orthogonal detection. The target prototype nucleic acid we have chosen is BVD (Bovine Viral Diarrhea). BVD is an RNA virus in the pestivirus (family, Arenavirus), that gives rise to a number of viral hemorrhagic fevers. Hemorrhagic fevers are the most prevalent diseases on the Category A list of the National Institutes of Health and the Center for Disease Control. RNA provides a more rigorous proof of the technology platform than deoxyribonucleic acid (DNA) due to the chemical instability of RNA.

In this LDRD we plan to test the viability of performing rapid RNA analysis on raw samples in the field using BVD as the target nucleic acid. This work begins the effort to develop nucleic acid hybridization assays on our magnetic beads. We continued our collaboration with the UC-Davis veterinary college in Tulare, CA with the Director Jim Cullor and Paul Rossitto, DVM (Department of Veterinary Medicine). Our collaborators can supply samples from persistently BVD infected animals and the proof-of-principle (POP) testing can be done in the field at Tulare.

Upon completion of the bead-based BVD assay we believe that we will have added nucleic acid processing to our technology platform. This means a distinct step towards completion of scientific proof-of-principle for a platform capable of accepting many raw samples for rapid, concurrent immunoassay and nucleic acid assay detection (orthogonal detection – low false positive). The implication is that we will have proven the basic science for a detection platform capable of meeting and exceeding the full suite of Joint Biological Agent Identification and Diagnostic System (JBAIDS)⁶ long-term mission needs.

The second focus of this LDRD was to reduce the LOD (limit of detection) achievable with our assay technology – specifically the two-wavelength optical detection system and mixing and bead/sample handling techniques used by our system platform – using the prototype substitute botulinum toxin chemistry (Ova assay, Ovalbumin/antiOvalbumin immunoassay). Our summer student, Cara Farrell, was able to reduce the LOD from 1 ppm (part-per-million) to from 50-100 ppb (parts-per-billion). This reduction in LOD involved additional fluid handling automation – automated mixing. The goal is to achieve 1 ppb LOD with a high level of system automation for assay repeatability. By lowering the LOD the likelihood of false negative errors is reduced, and by incorporating an orthogonal assay (nucleic acid assay) the likelihood of false positive errors is reduced. In this LDRD we demonstrated both a partial ability to perform nucleic acid assays (BVD), and a high level of sensitivity, low LOD (Ova assay) with our bead-based technology platform.
2. BVD ASSAY

Cost and biohazard issues set the stage for the scope of this work. For the budget available it was not feasible to either set up a local laboratory for processing live BVD virus nor to ship our system to Tulare CA to do performance testing. What was proposed was to simulate the assay in a two step process. The process is shown graphically in Fig. 1. In Fig.1(a) (top) the complete assay is shown. In Fig 1(b) (lower left) and 1(c) (lower right) the complete assay is split into two sub-assays. Sub-assay 1(b) was to be performed at Sandia National Laboratories and sub-assay 1(c) at UC Davis.

Figure 1. Two-Stage BVD Hybridization Assay. Top (a) shows whole illustration of the a two-stage hybridization assay that we are developing. This first phase of the development allows us to perform two critical components for nucleic acid development. The first is the establishment of the assay using primers on grown virus (performed at UCD DVM in Tulare, CA) (c – lower right) and the second was the conjugation of primer onto a magnetic bead similar to those used on the basic bead platform (b – lower left).

The assay reagents were purchased from Operon Biotechnologies Inc and consist of the Oligo (oligo-nucleid) (BioTEG)TAGCCATGCTTAGTAGGAC(AmC7-Q+Alexa660) (complimentary oligo to BVD) attached to biotin at one end and a dye marker Alexa-fluor600 at the other end. The biotin end of the molecule binds to our internally labeled streptavidin coated magnetic beads. This oligo, with dye attached, was simply used to validate the fact that indeed the oligo was attached to the bead. From the success of this attachment we conclude that success is highly probable for the attachment of an identical oligo, capable of binding the BVD RNA, without the dye molecule attached. The biotinated oligo specific to BVD did not arrive at Sandia National Laboratories in time
for us to perform the assay. However we were able to use a substitute biotinated Olig 
\[(5'\text{biotin})5'\text{-AATACCTTTCTTGGGGTAATACTCATCGCAATATCCTTAAGAGGGCGTTCAG C-3'}(\text{Atto-655 NHS ester dye})\] to demonstrate oligo attachment to our beads. See Figure 2.

![Bead prep 2 (fluorescently labeled oligo)](image)

**Figure 2. Demonstration of Biotinated Oligo binding to streptavidin coated magnetic beads (sub-assay of Figure 1(b)).**

Significantly higher signal was obtained with the atto655-oligo combination over both beads alone and atto655 (no oligo) indicating that the oligo did bind to the beads. The fact that there was significant signal with the beads plus atto655 case indicates that non-specific binding is an issue. Instead: This non specific binding is probably due to unbound atto655 dye not being separated for oligo bound atto-655 dye using a precise size exclusion column, such as utilized in HPLC, prior to mixing with the beads.

We did not accomplish the full two-part assay shown in Figure 1a. The part of the assay shown in Figure 1c was not accomplished because the reagents did not arrive at UC Davis in time for the live BVD virus part of the assay to be completed. The part of the assay shown in Fig. 1b was accomplished, but not with the exact oligo, rather with an substitute oligo (data shown in Fig. 2). Based on previous experience we estimate that approximately 1 month of work would be required to finish part b of the assay (bead functionalization), with a similar amount of work required to finish part c of the assay.
(virus labeling). At that point we would be ready to ship our detection system to Tulare CA and conduct the full Figure 1 assay (a two week test).

3. INCREASED TECHNOLOGY PLATFORM SENSITIVITY (LOWERING LOD)

Several steps were taken to reduce the LOD from ppm towards ppb during this LDRD. The ova-assay was used as the well understood workhorse chemistry on which to try various different technology changes. The signal/noise ratio (S/N) for the ova-assay was utilized as the figure of merit to determine the effectiveness of the different technology changes investigated. In our previous experience with the ova-assay, we achieved S/N of approximately 2:1 at 1 ppm. This was the minimum S/N that allowed identification without too high a false positive or too high a false negative. In the research summarized here we consistently achieved 2:1 S/N with a concentration of 50-100 ppb. Therefore 50-100 ppb is our new lower LOD (see Fig. 3).

The detection technology system that we utilized consists of two subsystems: 1) an optical detector, and 2) a fluid handling protocol. The optical detector consists of two excitation sources, a microscope, a sensitive CCD array, internal software, and two sets of filters that allow us to conduct two-wavelength detection of first; bead presence at a pixel and second; captured fluorescent tag at the same pixel location. This concurrent two-wavelength signal allows a higher degree of certainty that a true positive identification has occurred, indicating that the sandwich assay reaction on which detection is based has occurred. The variations in the optical part of the detection system that were investigated included; signal integration time, optical interrogation illumination intensity, and software background subtraction. The software background subtraction allows us to determine a positive signal identification for a S/N ~ 2. Improvements to the background subtraction software should allow us to extend our positive signal identification S/N to < 2, and allow us to extend our LOD < 50 ppb. However, we have not done enough repeat data points at this condition to rigorously make this claim.

The fluid handling technology was based on pipetting samples and reagents (beads and dye labeled antibodies required for sandwich assay – see Fig. 1) together into small test tube volumes (~100’s µl typical), and then mixing the sample with the reagents using a commercial mixing technology or by pipette mixing, followed by pelleting the beads, washing away the unwanted unbound sample constituents, and pipetting a drop of bead sample onto a microscope slide for optical interrogation. The key parameters that were investigated as part of this research were mixing process (two commercial mixers and hand hand pipette mixing or finger-flick mixing), mixing time (2 to 30 minutes), pellet clean-up techniques (pipette tip diameter, centrifuge rpm, magnetic hold-down), buffer concentration, and number of beads. The best combination of parameters that resulted in the 100 ppb or better (50 ppb) consisted of:

1) Rotating mixer (model m90615, Labquake™ Rotisserie) at 8 rpm rotation rate.
2) 10 minute mixing
3) Small pipette tip cleanup up with centrifuge set at 2000 rpm for 2 minutes for pelleting and magnetic assist in pellet immobilization
4) 1X buffer concentration (lower ion concentration)
5) $10^6$ beads (or greater).

To reduce the LOD even further (our goal is to achieve 1 ppb) we propose to investigate:

1) increasing the number of beads further
2) < 1X buffer concentration (lower ionic strength at pH 7).
3) Bead diameter.
4) Optimizing mixing time/method.
5) Improved pipetting/pelleting.
6) Full implementation of software improvements for background correction.

All of these factors can be automated and integrated into a single micro-to-meso scale system that should result in a consistently lower LOD (remove pipette operator variations). Currently we have consistently achieved 100 ppb, in some cases achieved 50 ppb and can potentially achieve 1 to 10 ppb with the implementation of these technology improvements.

![Figure 3. Sensitivity of detection technology platform demonstrated to 100 ppb.](image)
4. CONCLUSIONS AND NEXT STEPS

In conclusion, during this LDRD progress was made towards reaching our goal of developing the ability to conduct fully orthogonal (nucleic and immunoassay), low LOD (limit of detection) assays with our suite of technologies. This ability will allow us to rapidly process samples, and identify multiple threat agents with a high level of sensitivity (low false negatives) and specificity (low false positives). Specifically, in this research we demonstrated a new low LOD of 50 to 100 ppb with our optical and fluid handling technology; and we partially demonstrated a bead-based nucleic (oligo) assay using BVD (bovine viral diarrhea) as a target oligo threat of interest by attaching an oligo to a bead.

The remaining steps that need to be taken to reach orthogonal high sensitivity detection with our technology platform include:

1) Attach specific anti-BVD oligo to bead.
2) Attach anti-BVD to dye marker.
3) Perform simultaneous sandwich assay using BVD and items (1) and (2) of this list.
4) Reduce LOD from 50 to 100 ppb to 1 to 10 ppb using the techniques outlined in section 3.
5) Perform items (3) and (4) of this list simultaneously in the same sample.
6) Automate the detection process and technologies.
7) Explore possible collaborations that allow application of this detection technology in the dairy industry.
8) Explore other national security application for this technology platform in the CBN area.

Completion of these steps would result the application of this research to important national security concerns, thereby fulfilling the mission of the LDRD process and Sandia National Laboratories.
5. REFERENCES


3. Not published. We have performed double blind studies with *Botulinum* toxin surrogates in conjunction with Jim Cullor (Associate Dean, Director – Vet. Med. Teaching and Research Center (VMTRC) branch of UC-California at Davis in Tulare, CA).


5. Ibid.

6. The most articulate, possible and concise definition of requirements and capabilities’ the authors have seen are those articulated by the Department of Defense for JBAIDS. DHS, EPA, USDA, etc, all have requirements but for engineering and conceiving this project we use the JBAIDS mission need. If these are met the technology will then be truly multiple use. (last accessed 8/1/07, http://www.jpeocbd.osd.mil/page_manager.asp?pg=2&sub=54).
**Distribution (all electronic)**

<table>
<thead>
<tr>
<th>1</th>
<th>MS1080</th>
<th>Mark Derzon</th>
<th>17492</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS1080</td>
<td>Paul Galambos</td>
<td>17492</td>
</tr>
<tr>
<td>1</td>
<td>MS1080</td>
<td>Jaime McClain</td>
<td>17492</td>
</tr>
<tr>
<td>1</td>
<td>MS1080</td>
<td>Cara Farrell</td>
<td>17492</td>
</tr>
<tr>
<td>1</td>
<td>MS1080</td>
<td>Chris Bourdon</td>
<td>0346</td>
</tr>
<tr>
<td>1</td>
<td>MS0892</td>
<td>Kamyar Rahimian</td>
<td>1821</td>
</tr>
<tr>
<td>1</td>
<td>MS1080</td>
<td>Keith Ortiz</td>
<td>17492</td>
</tr>
<tr>
<td>1</td>
<td>MS1374</td>
<td>Kevin Seager</td>
<td>6723</td>
</tr>
<tr>
<td>1</td>
<td>Jim Culllor, UCD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Paul Rossito, UCD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>MS0899</td>
<td>Technical Library</td>
<td>9536</td>
</tr>
<tr>
<td>1</td>
<td>MS0123</td>
<td>D. Chavez, LDRD Office</td>
<td>1011</td>
</tr>
</tbody>
</table>