**CRB Project No. 5300-141_**

# CITRUS RESEARCH BOARD

## PROJECT PLAN - RESEARCH GRANT PROPOSAL FOR FY2010-2011

**Fiscal Year:** _2010___ **Anticipated Duration of Project:** _3 years_____

**This Project is:** _____**New or** ___X___ **Ongoing (Year __2__ of _3__)**

**Project Leader:** _R. Bruce Cary, Ph.D._____
Name
**Location:** Mesa Tech International, Inc., 2778 Agua Fria St., Santa Fe, NM 87507___
Address *(University department, if applicable)*
**Mailing Address** (if different): _____

**Phone:** __505-424-1114__**FAX:**_505-424-4441____ **E-Mail:**_rbcary@mesabiotech.com_____

**Cooperating Personnel:** Norman W. Schaad, USDA/ARS, Fort Detrick, MD 21702

**Project Title: An Integrated Low Cost Nucleic Acid Analysis Platform for the Rapid Detection of Plant Pathogens**

**Keywords:** Molecular diagnostics, lateral flow, microarray, nucleic acids, sample preparation, pathogen detection

**Abstract** *(limit 200 words):(clearly and succinctly state what your project is about why you are doing it and expected out come and how the industry will use these outcomes)*

To address the need for field deployable nucleic acid detection technologies that also offer the multiplex and information capacity required to interrogate multiple genetic markers, we developed a novel microarray technology that enables rapid hybridization-based nucleic acid detection using an easily visualized colorimetric signal [1,2]. One primary hurdle to the realization of truly facile nucleic acid tests suitable for field use has proven to be the processing of complex biological samples into amplification ready DNA or RNA [5]. In prior work, we developed an inexpensive yet efficient method of removing amplification inhibitory compounds from crude plant tissue extracts [6, 7]. Thus our prior work has overcome two significant hurdles to the transition of nucleic acid testing to the field: rapid sequence-specific colorimetric detection and instrumentation free sample preparation. The realization of a highly simplified system for nucleic acid testing suitable for field use will require the integration of sample preparation, amplification and detection into a low cost device that eliminates the current requirements for user manipulations. To address these requirements we propose the development of disposable hardware systems for isothermal amplification support and the integration of sample preparation, amplification and detection sub-systems into an easily used low cost device.

**Problem and its Significance*:** *(include literature review)*

*Literature Review*

The wide spectrum of sample contaminants encountered in the collection and analysis of crude biological samples for nucleic acid testing presents a significant challenge to the development of general and robust processing protocols [5]. Although reliable nucleic acid isolation methods applicable to such samples have been reported for both DNA and RNA [8-10], such methods are labor intensive, dependent upon laboratory instrumentation and require hours to complete rendering them of little utility for rapid field assays. In recognition of these shortcomings, microfluidic devices have been proposed or demonstrated that enable the isolation of nucleic acids from crude samples [11-14]. Unfortunately, many microfluidic methods remain reliant upon instrumentation, user expertise and relatively costly disposable components subject to fouling. Moreover, highly miniaturized

*Use as much space as necessary; attach additional pages as needed; budget info and signatures will appear on last page.

systems lack the capacity to process adequate sample volumes required to assure detection of scare or trace biological constituents critical to the early detection of viral or bacterial infection. Thus, a pressing need exists for methods capable of rendering crude samples, potentially dilute with respect to the agents of interest, suitable of nucleic acid amplification and detection. Down-stream enzymatic manipulations, such as polymerase chain reaction (PCR) and nucleic acid sequence-based amplification (NASBA), can be adversely impacted by the presence of matrix constituents inhibitory to enzymatic activity, rendering reliable sample preparation indispensable [5, 15]. Small molecules and complex polysaccharides are commonly encountered in plant samples while additional inhibitory compounds such as humic acids often accompany environmentally collected samples containing soil, plant material or decaying mater [5, 16, 17]. Additionally, the trace nature of many analytes in complex samples together with the abundance of closely related but non-probative constituents contributes significantly to analytical challenges [18-22].

Lateral flow immuno-chromatography is well established and has been used for the detection of proteins and small molecules for many years [23, 24]. Indeed, immuno-capture during lateral flow is the basis for rapid hand-held immuno-assays that have found widespread use in the point-of-care (*e.g.* group A Streptococcal antigen) and in the home (*e.g.* pregnancy tests). While these assays make use of immuno-capture during lateral flow as a detection end-point, we have shown that the same principles can be employed as a means of attaining rapid and efficient immuno-capture as a first step in a sample preparation strategy designed to enable the recovery of scarce targets (cells, viruses, spores) from complex plant tissue samples. Once captured in the stationary phase, these targets can then be subjected to further processing for nucleic acid isolation and amplification. Thin-layer chromatography systems have been employed for several decades as solid supports for rapid hand-held immuno-assays [23]. The simplicity of thin-layer chromatography renders it an attractive approach for field deployable and/or rapid screening assays. However despite their speed and simplicity, the discrimination of closely related microbial species can be difficult or impossible with immunological assays alone (see [15] and references therein). Reliance upon direct detection via antibody sandwich assays also limits the sensitivity of traditional hand-held assays [15]. Although these factors constrain the applications where simple lateral flow immuno-assays are most ideally deployed, it should nonetheless be recognized that the thin-layer membrane substrates employed in these systems provide the capacity for *bona fide* preparative chromatographic separations. In fact, the lateral flow immuno-assay is essentially a highly efficient immuno-chromatographic separation that we have shown can be exploited not only for direct immunologically mediated detection but also as a means of attaining rapid immuno-capture for concentration of target organisms prior to washing, lysis and nucleic acid amplification. Rapid immuno-capture methods provide a powerful means of concentrating and immobilizing microbial flora from complex mixed environmental sample matrices. By concentrating biological constituents of interest from a mixed sample into a defined spatial zone of a solid substrate, subsequent manipulations to render the sample amenable to nucleic acid amplification are greatly facilitated. More recently, we have extended thin-layer chromatographic methods to enable the direct isolation of nucleic acids from complex samples to eliminate the need for immunological reagents. These methods have been shown to be capable of superior removal of amplification inhibitory sample matrix derived contaminants compared to widely used laboratory methods such as Qiagen spin columns (see for example Figure 2 below).

Retaining assay sensitivity while circumventing requirements for thermocyclers and fluorescence detection hardware also presents a significant challenge to the field deployment of molecular diagnostics. The use of simple colorimetric detection schemes, which circumvent the requirements for complex instrumentation, require an upstream amplification strategy to attain suitable sensitivity. Recent advances in isothermal DNA amplification technologies promise to eliminate the need for thermocycling to achieve exponential amplification of nucleic acids (for review see [25]). Indeed, such methods as nucleic acid sequence based amplification (NASBA) [26-28], strand displacement amplification (SDA) [29-31], helicase displacement amplification (HDA) [32, 33], loop-mediated amplification (LAMP) [34], recombinase polymerase amplification (RPA) [35] and EXPAR [36] provide alternative methods for DNA amplification with no thermocycling requirements.

Several chromatographic lateral flow assays have been described for the detection of amplified nucleic acid sequences. Early work made use of cumbersome enzymatic detection strategies that relied on time consuming

manipulations of dipsticks following introduction of the PCR amplified sample [37, 38]. More recently, lateral flow-based detection of PCR products has been reported using standard immunological methods for lateral flow detection of antigen-labeled amplicons [39]. Other lateral flow assays for DNA detection have been described that make use of PCR amplification and colorimetric detection using nanogold conjugates and biotin-streptavidin capture, an approach that provides rapid single-plex detection of amplification products yet remains linked to the hardware requirements of PCR [40]. The appeal of lateral flow detection in the context of a PCR-based assay is limited by the fact that real-time PCR detection would offer similar hardware complexity and less sample handling than an assay requiring post-thermocycling introduction of PCR reactions on to a lateral flow detector. This scheme requires each PCR reaction to be interrogated with a separate dipstick thus increasing sample handling and decreasing throughput. In addition to the hardware requirements of PCR, these devices have employed schemes poorly suited to multiplexed detection, a fact that further limits their utility to single-plex PCR assays.

Strategies to eliminate PCR amplification prior to lateral flow nucleic acid detection have sought to either detect unamplified nucleic acid targets or to employ isothermal amplification techniques. Enabled by the use of up-converting phosphor reporters, unamplified *Streptococcus pneumoniae* DNA sequence has been detected using a lateral flow assay format [41]. Up-converting phosphor technology, while sensitive, remains dependent upon hardware required to detect phosphor emission [42]. Isothermal nucleic acid amplification coupled with lateral flow detection has been reported for assays making use of cycling probe technology (CPT, [43]), recombinase polymerase amplification (RPA, [35]) and nucleic acid sequence-based amplification (NASBA, [26-28])[1-4, 44-47]. While the work by Fong *et al.* and Piepenburg *et al.*, made use of a lateral flow immuno-assay for DNA detection, the NASBA amplified products generated in the work from Cary's and from Baeumner's groups were detected using a lateral flow system that facilitates rapid sequence-specific hybridization capable of supporting multiplexed detection [1, 48]. Although Baeumner's work employed liposome encapsulated dye and a sandwich hybridization assay similar that reported by [49], refinements by Cary's group have led to the development of stabilized detection particles and supporting methods that enable high information density microarrays, or lateral flow microarrays (LFMs) as we refer to them here, to be fabricated and stored at ambient temperature for up to one year (unpub. obs.).

LFM technology makes use of DNA microarray-like patterning of a small lateral flow chromatography strip allowing multiple nucleic acid sequences to be detected in a single assay. The reduced surface area of the device confers several advantages over traditional lateral flow device form factors. Sample volumes are reduced to 10 µL resulting in reduced reagent consumption as well as reduced sample transport times. Moreover, hybridization times exhibited by the LFM are significantly reduced compared to standard glass substrate microarrays, which typically are allowed to hybridize with sample for several hours, as well as more complex microarray implementations that make use of microfluidic systems to facilitate more rapid hybridization [1, 4, 50]. The convective fluid movement through the lateral flow substrate as well as the open-ended pores of the membrane substrates employed result in superior chromatography performance compared to bead-based column chromatography [51-54]. These factors result in hybridization-based detection of < 250 amol of analyte in 2 minutes [1]. The LFM platform has been shown to detect RNA from as few as 2-3 bacterial cells when present in a complex nucleic acid background consisting of 2 million-fold excess of non-probative nucleic acid [1]. The reported LFM approach made use of standard laboratory methods for RNA isolation and an isothermal RNA amplification scheme known as nucleic acid sequence based amplification (NASBA) [26-28].

Integration of LFM-based detection with lateral flow sample processing and isothermal amplification offers several potential advantages for processing and screening of plant tissue samples. Lateral flow sample processing provides the capacity to concentrate target analytes from a wide range of dilute sample volumes. Once immobilized at the device's affinity capture zone, amplification and detection of assay targets are greatly facilitated. The use of passive capillary wicking to drive sample and buffer transport through the system eliminates the need for complex and costly moving parts and electrical requirements. Coupled with passive buffer exchange systems consisting of specific geometric configurations of bibulous substrates, low cost but highly effective sample processing, amplification and detection systems could be integrated to realize a simple field deployable nucleic acid testing device.

**CRB Project Plan – Research Grant Proposal for FY 2010-2011**

**Project Title: An Integrated Low Cost Nucleic Acid Analysis Platform for the Rapid Detection of Plant Pathogens**

---

*Background*

In prior work supported by the CRB (Contract #5300-134, "Novel Immunocapture Technology for Field Deployable Nucleic Acid-Based Detection of Plant Pathogens"), we developed a completely passive immunocapture method for enriching pathogenic agents from crude complex plant samples such as macerated plant leaf tissue. The method provides a readily field deployable approach to sample processing to enable subsequent nucleic acid based assays for highly sensitive and sequence-specific detection of pathogens. The benefits of the newly developed technology over existing sample processing methods are several fold: 1) the use of a proprietary passive buffer exchange system enables multiple sample processing buffers to be employed without increasing the complexity of assay execution from the perspective of the end user, 2) by employing low cost absorbent materials easily fabricated using standard and widely available manufacturing systems, devices employing the approach will be readily fabricated for very low cost, 3) the passive nature of the technology eliminates requirements for external instrumentation such as pumps, controllers or centrifuges. Although initial embodiments of the technology focused on the use of immunological ligands for pathogen enrichment, we recognized that such ligands are not available or are not of suitable affinity for all pathogens of interest. For example, the fastidious bacteria associated with citrus greening (HLB) presents specific challenges that may render an immunocapture-based sample preparation strategy disadvantageous.

To overcome reliance upon immunological reagents, we have also devised and demonstrated nucleic acid affinity capture methods that make use of the same simple, low cost and easily used passive buffer exchange system. Use of this approach for direct isolation of nucleic acids from plant tissue provides an effective and field deployable alternative to widely used methods, such as Qiagen spin columns, that offers similar performance without laboratory instrumentation requirements. Mesa Tech's sample processing methods are based on thin-layer chromatography substrates and offer chromatographic separation of pathogen targets and their nucleic acids from sample matrix contaminants confounding to critical downstream assay manipulations such as nucleic acid amplification. Moreover, the use of chromatographic approaches based on capillary wicking through absorbent materials provides a sample preparation method amenable to integration with rapid and sensitive colorimetric detection schemes such as those employed in our lateral flow microarray (LFM) platform. The LFM is now a robust and relatively mature sequence-specific nucleic acid detection method well suited for field deployable assays. Prior studies have demonstrated the detection of as few as 250 amol of analyte by sequence-specific hybridization in a microarray format in less than 2 minutes. This level of sensitivity, representing the detection of $1.5x10^8$ molecules, is an important milestone for the development of exquisitely sensitive assays incorporating exponential nucleic acid amplification methods. Methods for the amplification of specific nucleic acid sequence targets from complex mixtures of nucleic acid have been shown to provide amplification on the order of $10^9$ fold. Therefore, the presence of a single target in a sample is, theoretically, sufficient to result in the production of $10^9$ amplicon molecules during amplification, well above the $1.5x10^8$ molecules required for detection by the LFM. Although the most widely employed exponential amplification approaches are currently dependent upon relatively costly instrumentation, *i.e.* the thermocyclers used for polymerase chain reaction (PCR), more recently devised amplification schemes, known generically as isothermal amplification reactions, offer similar levels of amplification without the need for complex thermal manipulations. Taken together, rapid instrumentation free methods for sample processing, amplification and detection could provide the basis for a highly sensitive and specific diagnostic suitable for use under field conditions. Indeed, we have shown that as few as 2 bacterial cells can be detected in a complex nucleic acid mixture using a combination of isothermal nucleic acid sequence based amplification (NASBA) and LFM detection. Moreover, the low cost and simplicity of the approaches we have developed will assure accessibility of the technology to a broad base of end users.

Despite the simplicity and excellent performance characteristics of LFM detection, lateral flow sample processing and isothermal amplification methods; these technologies currently exist as independent steps of an analytical procedure. Although our prior work has resulted in adaptations of each requisite process that are amenable to field deployment, the manipulations required, though simple, are poorly suited for execution under field conditions. A truly useful field assay for nucleic acids will require a level of subsystem integration not currently

---

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

available. However, the methods we have developed were devised with the longer-term vision of integration into a low cost disposable device suitable for use under field conditions with little or no end user training. By formulating thin-layer chromatography-based methods for each stage of assay performance we have greatly facilitated the ultimate integration of existing component technologies into an easily used sample-to-answer diagnostic tool.

**Objectives*: (***succinctly state each objective and milestone, ie the time expected to successfully complete an achieve each objective)*

The fundamental goal of this project is to develop and test an integrated device incorporating the requisite steps for nucleic acid-based detection of citrus pathogenic agents. Specifically, we will:

**1. Develop and test isothermal amplification support systems suitable for integration with existing thin layer sample processing and LFM detection technologies in a low cost disposable device.** Hardware support for isothermal amplification will be developed and tested. Efforts will focus on the development of extremely low cost battery operated systems that can be incorporated into a disposable device.

**2. Devise and optimize an integration approach to couple sample preparation, isothermal amplification and detection in a thin layer chromatographic system.** Methods for nucleic acid sample preparation, isothermal amplification and amplicon detection have been devised that are based upon or compatible with incorporation into thin-layer chromatographic systems. The passive capillary flow characteristics of these thin-layer chromatography systems have been exploited in the development of a novel passive, yet efficient, buffer exchange/fluid flow control system that is completely independent of moving parts, valves, or electrical requirements. The availability of this technology will significantly simplify the integration of all requisite steps of a nucleic acid test into a low cost disposable device similar in simplicity and ease of use to a hand-held immuno-assay such as a home pregnancy test. Prototype development will prioritize methods that remain compatible with low cost manufacturing and widely available lateral flow device assembly techniques.

**3. Conduct initial field-testing of the first generation integrated device prototype.** An iterative approach will be employed for prototype development, testing and refinement. Initial prototypes will be laboratory tested and optimized. The resulting prototypes will be field-tested and findings from field studies used to refine initial prototype designs.

**Project's Benefit to the Industry*: (***How will the industry utilize your research results or product)*

The generic nature of the technology developed will simplify the addition or alteration of the pathogens targeted by the resulting assays. In this way, the platform technology can be rapidly adapted to the detection of important disease agents as threats emerge. Assays for *Xylella fastidiosa* (Citrus Variegated Chlorosis (CVC) strain) and *Xanthomonas axonopodis* pv. *citri* (Citrus Canker) have been developed and tested in our laboratories (patent applied for) and are now being transferred to the USDA for external testing in collaboration with Laurene Levy (USDA). Additionally, we have identified signature sequences and sequence specific reagents for Citrus Tristeza Virus (CTV) and are currently collaborating with Norm Schaad (USDA) to develop and test *Candidatus* Liberibacter assays for citrus greening (HLB) detection. LFM assays for HLB will be developed making use of existing rRNA markers as well as novel markers predicted to emerge from ongoing sequencing efforts. Additionally, it should be noted that the multiplex capacity of the LFM platform will ultimately enable the inclusion of multiple confirmatory nucleic acid signatures allowing the use of not only pathogen derived sequences but also host biomarkers induced by infection. Taken together, the simplicity, flexibility and information capacity of the LFM combine to offer a unique testing system for use under field conditions, thus addressing the current needs of the citrus growing community to monitor and respond to infectious diseases before they gain a foot hold in California orchards.

**Research Collaboration*** (*be specific*):

We will collaborate with Norm Schaad's group (USDA/ARS, Foreign Disease-Weed Science Research Unit) to obtain materials and target sequences for the development and testing of HLB assays (*Candidatus* Liberibacter). Additionally, we will collaborate with both Schaad's group and Laurene Levy's (USDA) groups for

external evaluation of our assay prototypes. Levy's group will evaluate *Xylella fastidiosa* (Citrus Variegated Chlorosis (CVC) strain) and *Xanthomonas axonopodis* pv. *citri* (Citrus Canker) detection assays while Schaad's group will evaluate *Candidatus* Liberibacter (HLB) assays.

**Plans and Procedures*** *(use this section to describe your experimental design site location(s)and elaborate on objectives and milestones)*

**1. Develop and test isothermal amplification support systems suitable for integration with existing thin layer sample processing and LFM detection technologies.**

Existing LFM assay methods make use of an isothermal amplification technology known as nucleic acid sequence based amplification (NASBA). This method is robust and, owing to its isothermal nature, well suited for a field diagnostic tool. Additionally, it is an RNA amplification scheme capable of direct amplification of viral RNA or mRNA targets without an intermediate reverse transcription step. Perhaps most significant for applications employing sequence specific hybridization-based detection, such as the methods employed for LFM detection, the products of the NASBA reaction are single-stranded and thus suitable to direct hybridization based detection without and intervening denaturation of amplicon. Existing NASBA methods require the use of an external incubation in a temperature control apparatus capable of maintaining an optimal amplification temperature of 41 ˚C. Development of an integrated device for sample preparation, amplification and detection will need to support the maintenance of NASBA reaction temperatures for the duration of the amplification step. To address this need we will develop and test low cost, disposable heating systems. Surface mount resistors and inexpensive integrated circuit digital controls can be used as inexpensive heating systems costing less than $1.00 in limited quantities and less than $0.50 in larger quantities required for manufacturing.

To test the suitability of low cost heating solutions for the support of NASBA reaction conditions we will test battery powered resistor-based heating devices integrated into disposable plastic housings. Toward this end we have developed breadboard devices that will be tested for NASBA amplification support (Figure 1). A single 9V battery will power these simple heating systems. It should also be noted that heating from such low power, low cost heaters could also be exploited for maintaining controlled temperatures during hybridization-based detection on the LFM component of an integrated diagnostic device. This approach will assure the ability to obtain reliable sequence specific amplification under diverse ambient temperatures during field use of the device.

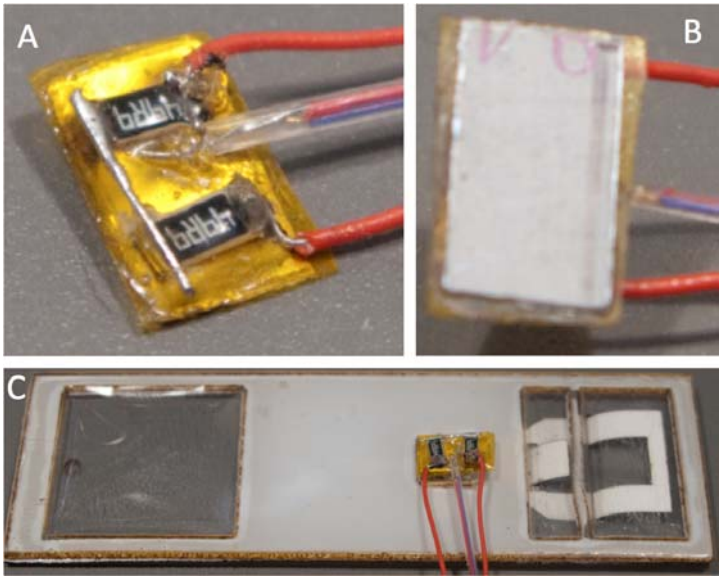


***Figure 1.*** *(A) Surface mount resistors embedded in an epoxy-based potting agent on a sheet aluminum substrate provides a small heat source capable of maintaining optimal NASBA reaction temperatures. (B) The reverse side of the heater shown in part A. The aluminum sheet mediates even heat distribution and conduction to the amplification chamber. (C) Resistive heating element mounted under the amplification chamber of a breadboard device. The amplification chamber is formed by the lamination of polycarbonate sheet housing material to a thin plastic layer.*

**2. Devise and optimize an integration approach to couple sample preparation and isothermal amplification in a thin layer chromatographic system.**

Lateral flow nucleic acid isolation methods together with LFM hybridization-based detection developed previously (patents applied for) will form the basis for an integrated disposable diagnostic platform. These approaches make use of a passive buffer exchange method that will be exploited not only for buffer exchange during nucleic acid isolation but also for introduction of amplification reagents (enzymes, reaction components and primer oligonucleotides) and detection reagents (detection oligonucleotides and dyed microspheres). This approach will eliminate the need for user intervention during assay performance while retaining other attributes required for a field deployable system, namely

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

independence from instrumentation and external power source requirements. Additionally, the proposed approach will make use of materials easily manufactured and assembled at low cost allowing the production of inexpensive test devices.

To help assure the success of integration efforts we are pursuing two basic strategies for coupling lateral flow sample preparation with subsequent isothermal amplification and lateral flow-based colorimetric detection. These methods differ primarily in their reliance on either simple user interventions or alternative, more elaborate, implementations of the passive buffer exchange strategies we have successfully employed for sample preparation wash buffer exchange. Laboratory and field-testing studies will be used to evaluate the impact of user interventions and passive fluid control schemes on both assay robustness and ease of use. The generic nature of the methods assures that they will be suitable for use with any isothermal amplification reaction suitable for generating LFM detectable amplicon. This independence from specific amplification reaction chemistries will allow their development in parallel with less mature amplification technologies.

One integration strategy to be pursued will be based on the nucleic acid transfer method employed in existing sample preparation breadboard prototypes. Specifically, we will design and test device prototypes employing a simple punch through system for physically transferring nucleic acid affinity matrix material directly into amplification reaction mix containing primers and enzymes for isothermal amplification. This method, although it requires a single user intervention following the completion of final sample preparation wash buffer exhaustion, is low risk and robust with respect to the exact timing of user intervention. Owing to the stability of nucleic acids on the affinity matrix, sample punch through to the amplification reaction chamber can be executed at any time following the completion of final wash buffer transport. Figure 2, illustrates a sample preparation card before and after liberation of nucleic acid by elution in a crude implementation of the punch through strategy. The device in Figure 2 makes use of a tuberculin syringe to introduce elution buffer to the punch through harvested nucleic acid affinity matrix in an under lying chamber (formed by an awaiting well of a 96-well titer plate in this breadboard level example). This same strategy is readily adapted from elution of nucleic acids as shown to the direct introduction of amplification reagents in lieu of elution buffer. By devising a simple devoted system of buffer chambers for sample input, sample preparation wash buffers and amplification reagents a low cost and easily designed and fabricated device can be developed. A prototype employing this strategy will be developed using a custom fabricated punch and amplification reagent reservoir to more elegantly incorporate the punch into an integrated device (Figure 3).

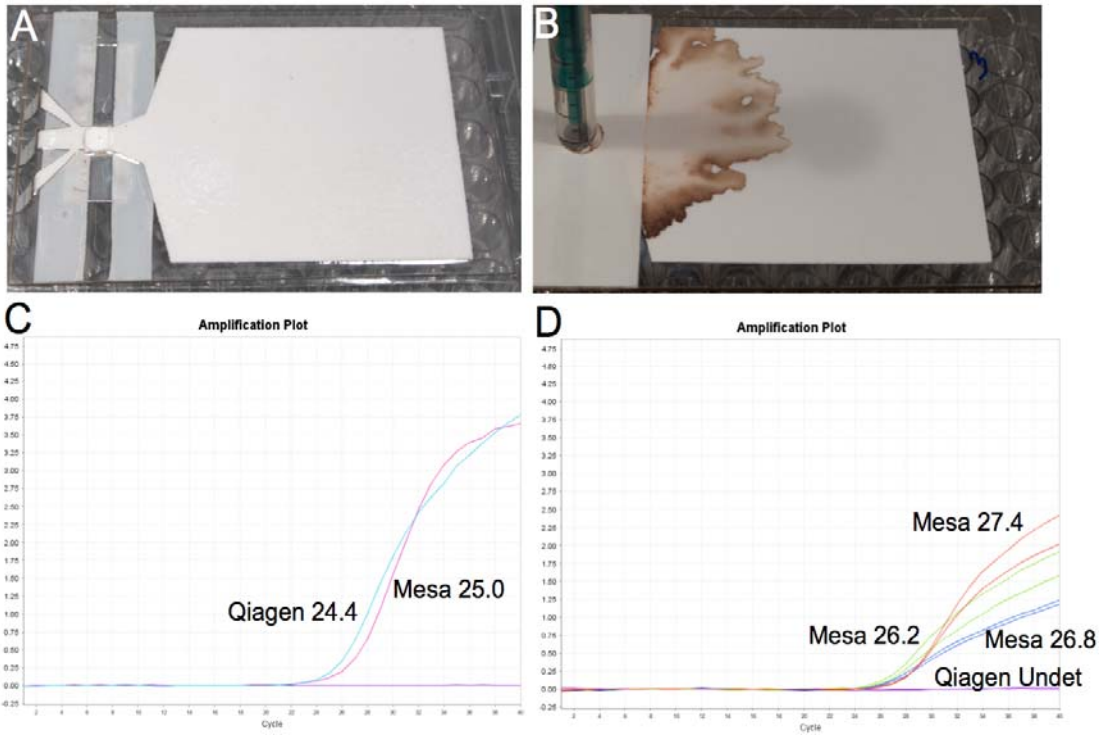


***Figure 2.*** *(A) Laminated lateral flow sample preparation cards were fabricated on polycarbonate sheet supports to which were affixed a passive buffer exchange structure cut from backed nitrocellulose (Millipore, HiFlow HF-90) in lateral flow contact with a nucleic acid affinity matrix (GF/B glass fiber filter, Whatman) in turn*

*placed in contact with an absorbent pad comprised of chromatography paper (3MM, Whatman). A 96 well titer plate was used as a buffer reservoir. Wash buffers were added, prior to sample addition, to corresponding plate wells. Sample in lysis buffer (100 µL 4M guanidinium thiocyanate, 25 mM sodium citrate pH 7.5) was introduced directly to the affinity matrix. (B) Following completion of capillary flow of sample and wash buffers (Wash 1: 250 µL 2M guanidinium thiocyanate, 25 mM tris pH 7.5; Wash 2: 500 µL 80% ethanol) the glass fiber filter material was collected from the device by punching the filter material through a hole in the polycarbonate support and into an underlying plate well using a tuberculin syringe containing 100 µL of elution buffer (H2O). (C) PhiX174 dsDNA was diluted to 5025 copies/µL into pristine lysis buffer and subjected to lateral flow through the sample preparation device shown in part (A). 5 µL of the 100 µL elutate was assayed for PhiX174 by real-time PCR generating a Ct value of 25.0. Identical sample and wash protocol was employed using a Qiagen QiaAMP spin column following the centrifugation procedure recommended by the manufacturer. Real-time PCR of the Qiagen elutate exhibited a Ct value of 24.4. (D) Lateral flow sample preparation devices or Qiagen QiaAMP spin columns were challenged with PhiX174 spiked DCTLE generated in lysis buffer (~33 mg tobacco/mL) and elutates assayed for PhiX174 by real-time PCR. PhiX174 was undetectable in QiaAMP elutates using the same wash protocols employed for lateral flow. Lateral flow device elutates were positive for PhiX174 with Ct values between 26.2 and 27.4 using three different wash buffer conditions.*

Nucleic acids liberated into amplification reaction mix will be incubated at constant temperature using an integrated disposable heater comprised of surface mount resistors and a low cost digital controller (see above). Juxtaposition of the heating element with a thin plastic film laminated to the underside of the amplification chamber forms a low cost and easily manufactured design that enables efficient heat transfer to the amplification reaction vessel. Alternatives, should more complex hardware support systems be found necessary, could make use of a reusable docking station incorporating a more costly heating system. However, given the simple heating requirements of isothermal amplification methods under consideration, we do not anticipate the need for a reusable component.

Following isothermal amplification incubation, amplification reaction mix will be introduced to lateral flow detector substrates to liberate dried detection particles for detection by LFM hybridization. To eliminate significant manipulations of amplified material and to maintain a closed system that eliminates the potential for cross contamination of other assays with amplicon, introduction of the amplification reactions to the detector strip will be accomplished by simply inverting the device to allow reaction mix to flow onto the LFM sample input zone. This approach is compatible with a completely enclosed system to reduce the risk of cross contamination. Alternative approaches will also be explored, including the use of microcontroller triggered fluid movement following the completion of amplification reactions.

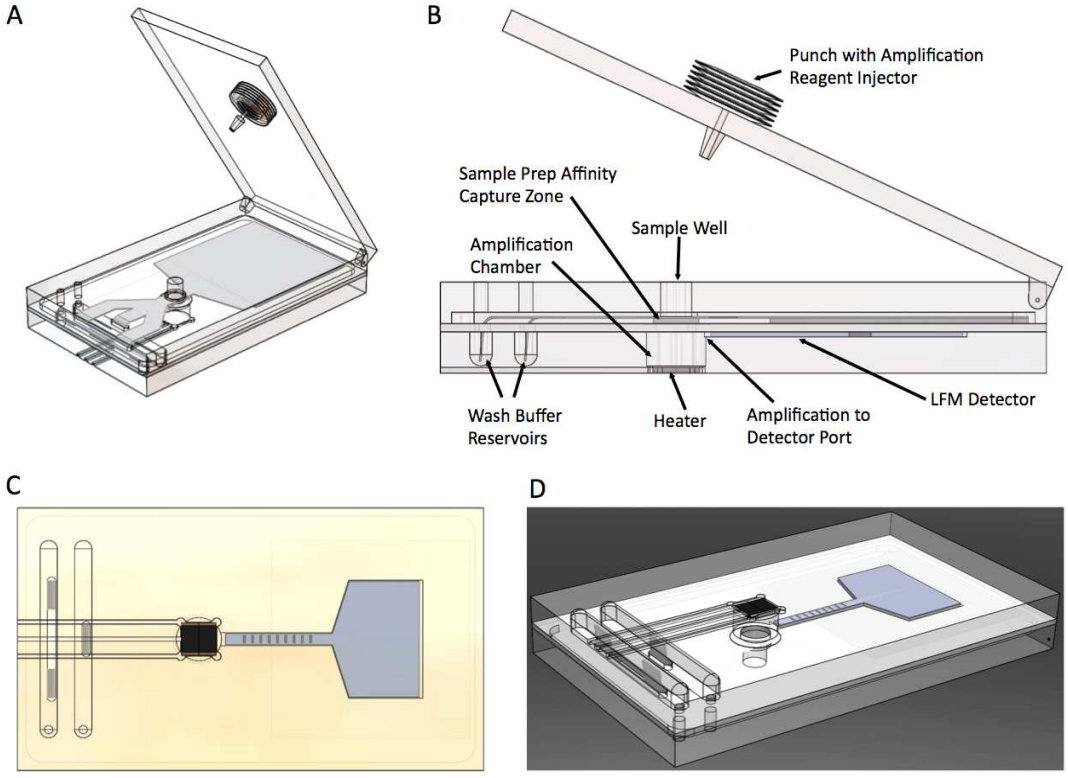


***Figure 3.*** *(A) Perspective view of a proposed device design employing a punch based method for transferring isolated nucleic acids to a heated reaction chamber. (B) A side view of the device with key components labeled. (C) Bottom view of the device depicted in parts A&B. (D) In inverted/detection position the device allows amplification reaction mix to flow into a small input port in communication with an LFM detector.*

The above described system makes use of extremely simple and low cost device designs while assuring a low risk path to the integration of all requisite assay manipulations by employing methods already shown to offer effective solutions to key integration challenges. However, the approach makes use of two user interventions: one following the completion of sample nucleic acid isolation and a second following the completion of isothermal amplification. Given the stability of nucleic acids on the affinity matrix and in the amplification reactions, the method should prove relatively insensitive to the exact timing of user interventions. Coupled with simple indicators to alert the user to the earliest time point at which the next intervention can be executed, this approach should be capable of providing a robust and easily used system.

Ideally, an integrated sample-to-answer nucleic acid testing device would eliminate the need for any user intervention following sample introduction and allow assay results to be collected at an appropriate time following assay initiation. This goal will require a more elaborate fluid control system to facilitate the transport of amplification reagents to isolated nucleic acids and to enable the detection of amplicon. Systems designed to achieve this objective will rely on geometric structures cut or ablated into absorbent substrates shown to enable the sequential introduction of different buffer solutions to specific zones of solid phase immobilized nucleic acid and to liberate, in turn, the required reagents in a completely user independent fashion. This approach will exploit refinements to our existing passive buffer exchange thin-layer chromatography methods currently employed for sample preparation (Figure 2). By using this passive approach low fabrication costs are maintained by obviating the need for valves or other moving parts while providing a means of timing the exposure of isolated nucleic acids to amplification and detection reagents (Figure 4). Though more complicated in design, such systems should support more facile assay execution from the perspective of the end user. Significantly, the fabrication of more elaborate fluid flow control geometries in absorbent substrates does not increase manufacturing costs due to our prior development of rapid high resolution laser cutting and laser ablation methods for constructing complex two dimensional structures in materials appropriate for these applications. Nonetheless, this approach represents a higher degree of risk given the need to develop and test novel fluidic designs.

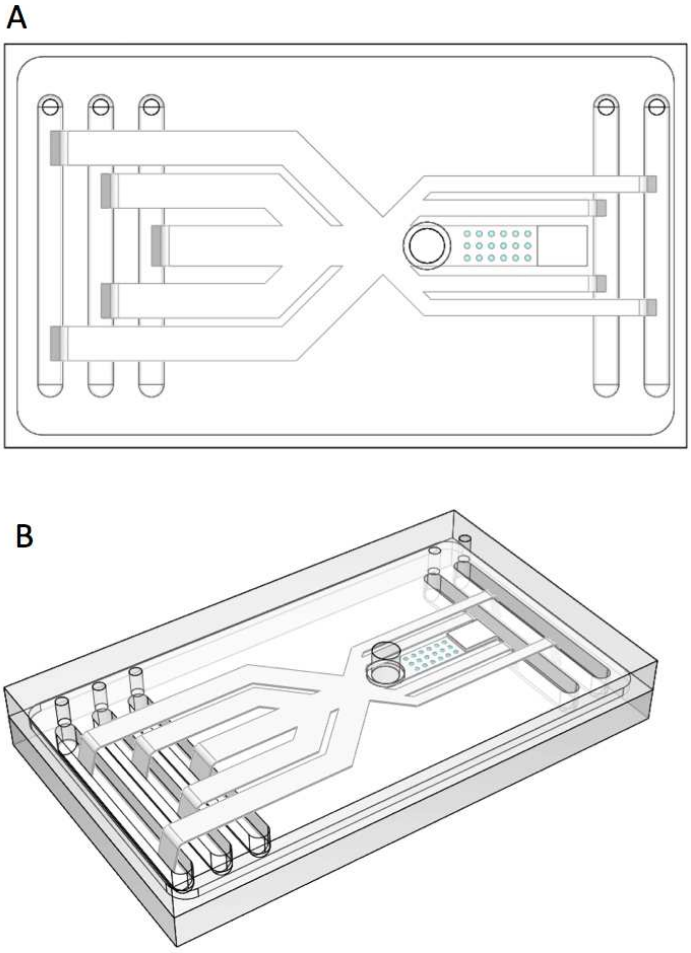


***Figure 4.*** *(A) Top view of an integrated device design employing passive buffer exchange structures to accomplish sample preparation, amplification and detection. Sample preparation wash buffers are introduced in the left most wells while elution and amplification buffers are introduced to wells on the right of the device. A centrally located well accepts crude sample. LFM detection occurs as amplicon is generated during lateral flow and passes through the capture features. (B) A perspective view of the integrated device. A heating system (not visible) maintains constant temperature for both amplification and hybridization-based detection.*

Pursuing the development of two integration approaches, each representing a balance of compromises in assay execution simplicity or device design complexity, reduces the risks associated with the development of a useful diagnostic tool for deployment under field conditions. Additionally, this approach helps assure that a first generation system, based on low risk methods, will be available early in the design cycle to enable the evaluation of assay characteristics such as specificity and sensitivity under field conditions.

**3. Conduct initial laboratory and field-testing of the first generation integrated device prototype.**

Initial laboratory testing and early protocol optimization efforts will make use of studies conducted in

Mesa Tech's molecular biology laboratories. These internal studies will assure baseline performance evaluations and preliminary optimization. External laboratory studies will be conducted in collaboration with Norm Schaad (USDA) and Laurene Levy (USDA). These efforts will offer an independent evaluation of protocol and device implementations. Test systems found to offer reproducible performance in internal and external characterization studies will be used in initial field tests.

Device designs found, under laboratory conditions, to offer specificity and sensitivity characteristics meeting a minimum predetermined level will be evaluated under field conditions. Initial performance parameters required will be specificity of >90% with sensitivity of better than 10,000 copies/sample. These initial requirements represent significant improvements of over existing field deployable methods (e.g. hand-held immuno-assays) yet present a relaxed starting point upon which further device and protocol refinements will be based. Ultimately, the nucleic acid based nature of the tests will allow significant improvements in performance predicted to enable the reliable detection of as few as 100 copies/sample with specificity comparable to laboratory-based real-time PCR assays (>95%).

Data from field studies conducted by Mesa personnel will be used to determine those physical assay attributes requiring modification to enable more facile use under field conditions. Additionally, assay performance data will be used, in comparative studies with laboratory-based data, to determine the impact of use under diverse field conditions. Refined protocols and device designs employing refinements guided by initial field studies will under go a second round of field testing prior to transition to a limited number of growers for evaluation in the hands of the intended end users. This iterative testing and optimization approach to device development will allow us to respond to end user input and develop a robust, easily used test system.

**Other Funding Sources for this Project** *(current, pending, potential; can this project be used as matching funds for other funding sources)*

**Funded:** LATERAL FLOW MICROARRAY PLATFORM FOR THE DETECTION OF REGULATORY PLANT PATHOGENS INCLUDING SELECT AGENTS, National Plant Germplasm and Biotechnology Laboratory, APHIS PPQ CPHST BARC-East, Bldg-580, Beltsville, MD 20705. Total funding: $50,000. Cary, PI.

**Technology Transfer*** *(include any potential intellectual property issues; steps necessary for grower utilization extension/communication component):*

To facilitate the most cost effective and rapid development of a nucleic acid testing product the project leader, R. Bruce Cary along with fellow former Los Alamos National Laboratory scientists, Hong Cai and John Elling, have formed a private corporation, Mesa Tech International, Inc. Mesa Tech has been established to commercialize lateral flow molecular diagnostic technologies under an exclusive licensing option agreement with Los Alamos National Laboratory. This "spin-off" strategy will facilitate product development and testing in a scientific environment that offers greater flexibility and more cost effective research than available within the National Laboratory complex.

**Budget Justification:**

***Personnel*** (total costs: direct + indirect (F&A=55%) + fringe (F&B=33%))

*Project Leader*: 0.2 FTE to support project scientific leadership/direction and oversight. R. Bruce Cary, Ph.D. will devise integration strategies and direct sample preparation and detection systems development. Hong Cai, Ph.D. will develop rapid isothermal amplification reaction schemes and lead their development and optimization. $38,248.00/yr

*Biochemist*: 0.5 FTE to support a biochemist devoted to amplification enzyme purification, characterization and stabilization methods. $36,562.00/yr

*Engineers*: 1.0 FTE to support 40% of Pat Turner, senior engineer and 40% of Marc DeJohn mechanical engineer. The engineering team will develop and fabricate prototypes for evaluation and assist in devising and realizing subsystem integration concepts. $64,000.00/yr

**Total requested personnel support: $138,810.00/yr for three years (total cost)**

*Travel*: $5000 to support travel to CRB reviews/presentations and field testing site visits.

*Materials and supplies*: $10,000.00/yr to support reagents for assay and prototype development & testing

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

**CRB Project Plan – Research Grant Proposal for FY2010-2011**
**Project Title:** _____

_____

**TOTAL REQUESTED FUNDING: $153,810.00/year for three years.**

_____

## Project Budget

**Department Account Number:** *(if applicable)*_____

| | Year: **2010-2011** | Year: **2011-2012** | Year: **2012-2013** |
|---|---|---|---|
| **Salaries and Benefits:** | | | |
| **Postdocs/Research Assistants** | $__$93,002 ___ | ___$93,002 ___ | _____ |
| **SRA's** | _____ | _____ | _____ |
| **Lab/Field Assistance** | _____ | _____ | _____ |
| **Benefits** | __$45,808 ___ | _$45,808 ___ | _____ |
| **Supplies and Expenses:** | __$10,000 ___ | ___$10,000 ___ | _____ |
| **Equipment:** | _____ | _____ | _____ |
| **Operating Expenses and Travel:** | ___$5,000 ___ | __$5,000 ___ | _____ |
| **Lindcove Recharges:** | _____ | _____ | _____ |
| **Lindcove Packline:** | _____ | _____ | _____ |
| **Other:** _____ | _____ | _____ | _____ |
| _____ | _____ | _____ | _____ |
| **ANNUAL TOTAL:** | _$153,810 ___ | __$153,810 __ | _____ |

_____

**Specifics regarding contract** (i.e., "split" funding to more than one PI):

_____

## Signatures

**Project Leader:** _____ **Date:**_____

_____ **Date:**_____

_____ **Date:**_____

**Dept. Chair:** _____ **Date:**_____

_____

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

**Literature Cited**

1. Carter, D.J. and R.B. Cary, *Lateral flow microarrays: a novel platform for rapid nucleic acid detection based on miniaturized lateral flow chromatography.* Nucleic Acids Res, 2007. **35**(10): p. e74.
2. Cary, R.B., *MINIATURIZED LATERAL FLOW DEVICE FOR RAPID AND SENSITIVE DETECTION OF PROTEINS OR NUCLEIC ACIDS*, Application Number 11/894,910, U.S.P.T. Office, Editor. 2007.
3. Cheek, B.J., et al., *Chemiluminescence detection for hybridization assays on the flow-thru chip, a three-dimensional microchannel biochip.* Anal Chem, 2001. **73**(24): p. 5777-83.
4. Peytavi, R., et al., *Microfluidic device for rapid (<15 min) automated microarray hybridization.* Clin Chem, 2005. **51**(10): p. 1836-44.
5. Wilson, I.G., *Inhibition and facilitation of nucleic acid amplification.* Appl Environ Microbiol, 1997. **63**(10): p. 3741-51.
6. Cary, R.B., HIGHLY SIMPLIFIED LATERAL FLOW-BASED NUCLEIC ACID SAMPLE PREPARATION AND PASSIVE FLUID FLOW CONTROL, International Application Number: PCT/US2009/002809, W.I.P. Organization, Editor. 2009.
7. Cary, R.B., *HIGHLY SIMPLIFIED LATERAL FLOW-BASED NUCLEIC ACID SAMPLE PREPARATION AND PASSIVE FLUID FLOW CONTROL* U.S.P.T. Office, Editor. 2009.
8. Kuske, C.R., et al., *Small-Scale DNA Sample Preparation Method for Field PCR Detection of Microbial Cells and Spores in Soil.* Appl Environ Microbiol, 1998. **64**(7): p. 2463-72.
9. Schneegurt, M.A., S.Y. Dore, and C.F. Kulpa, Jr., *Direct extraction of DNA from soils for studies in microbial ecology.* Curr Issues Mol Biol, 2003. **5**(1): p. 1-8.
10. Saleh-Lakha, S., et al., *Microbial gene expression in soil: methods, applications and challenges.* J Microbiol Methods, 2005. **63**(1): p. 1-19.
11. Chen, X., et al., *Continuous flow microfluidic device for cell separation, cell lysis and DNA purification.* Anal Chim Acta, 2007. **584**(2): p. 237-43.
12. Easley, C.J., et al., *A fully integrated microfluidic genetic analysis system with sample-in-answer-out capability.* Proc Natl Acad Sci U S A, 2006. **103**(51): p. 19272-7.
13. Marcus, J.S., W.F. Anderson, and S.R. Quake, *Microfluidic single-cell mRNA isolation and analysis.* Anal Chem, 2006. **78**(9): p. 3084-9.
14. Witek, M.A., et al., *Purification and preconcentration of genomic DNA from whole cell lysates using photoactivated polycarbonate (PPC) microfluidic chips.* Nucleic Acids Res, 2006. **34**(10): p. e74.
15. Lim, D.V., et al., *Current and developing technologies for monitoring agents of bioterrorism and biowarfare.* Clin Microbiol Rev, 2005. **18**(4): p. 583-607.
16. Akane, A., et al., *Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification.* J Forensic Sci, 1994. **39**(2): p. 362-72.
17. Jackson, C.R., et al., *A Simple, Efficient Method for the Separation of Humic Substances and DNA from Environmental Samples.* Appl Environ Microbiol, 1997. **63**(12): p. 4993-4995.
18. Wickenheiser, R.A., *Trace DNA: a review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact.* J Forensic Sci, 2002. **47**(3): p. 442-50.
19. Elliott, K., et al., *Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides.* Forensic Sci Int, 2003. **137**(1): p. 28-36.
20. Gill, P., *Application of low copy number DNA profiling.* Croat Med J, 2001. **42**(3): p. 229-32.
21. Gill, P., et al., *An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA.* Forensic Sci Int, 2000. **112**(1): p. 17-40.
22. Jobling, M.A. and P. Gill, *Encoded evidence: DNA in forensic analysis.* Nat Rev Genet, 2004. **5**(10): p. 739-51.
23. Pristoupil, T.I., *Microchromatography and microelectrophoresis on nitrocellulose membranes.* Chromatogr Rev, 1970. **12**(2): p. 109-25.

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

24. Kohn, J., *An immunochromatographic technique.* Immunology, 1968. **15**(6): p. 863-5.
25. Schweitzer, B. and S. Kingsmore, *Combining nucleic acid amplification and detection.* Curr Opin Biotechnol, 2001. **12**(1): p. 21-7.
26. Compton, J., *Nucleic acid sequence-based amplification.* Nature, 1991. **350**(6313): p. 91-2.
27. Kievits, T., et al., *NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection.* J Virol Methods, 1991. **35**(3): p. 273-86.
28. Malek, L., R. Sooknanan, and J. Compton, *Nucleic acid sequence-based amplification (NASBA).* Methods Mol Biol, 1994. **28**: p. 253-60.
29. Walker, G.T., et al., *Strand displacement amplification--an isothermal, in vitro DNA amplification technique.* Nucleic Acids Res, 1992. **20**(7): p. 1691-6.
30. Nadeau, J.G., et al., *Real-time, sequence-specific detection of nucleic acids during strand displacement amplification.* Anal Biochem, 1999. **276**(2): p. 177-87.
31. Hellyer, T.J. and J.G. Nadeau, *Strand displacement amplification: a versatile tool for molecular diagnostics.* Expert Rev Mol Diagn, 2004. **4**(2): p. 251-61.
32. Goldmeyer, J., H. Kong, and W. Tang, *Development of a novel one-tube isothermal reverse transcription thermophilic helicase-dependent amplification platform for rapid RNA detection.* J Mol Diagn, 2007. **9**(5): p. 639-44.
33. Vincent, M., Y. Xu, and H. Kong, *Helicase-dependent isothermal DNA amplification.* EMBO Rep, 2004. **5**(8): p. 795-800.
34. Notomi, T., et al., *Loop-mediated isothermal amplification of DNA.* Nucleic Acids Res, 2000. **28**(12): p. E63.
35. Piepenburg, O., et al., *DNA detection using recombination proteins.* PLoS Biol, 2006. **4**(7): p. e204.
36. Van Ness, J., L.K. Van Ness, and D.J. Galas, *Isothermal reactions for the amplification of oligonucleotides.* Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4504-9.
37. Reinhartz, A., et al., *A novel rapid hybridization technique: paper chromatography hybridization assay (PACHA).* Gene, 1993. **136**(1-2): p. 221-6.
38. Groody, E.P., *Detection of foodborne pathogens using DNA probes and a dipstick format.* Mol Biotechnol, 1996. **6**(3): p. 323-7.
39. Kozwich, D., et al., *Development of a novel, rapid integrated Cryptosporidium parvum detection assay.* Appl Environ Microbiol, 2000. **66**(7): p. 2711-7.
40. Glynou, K., et al., *Oligonucleotide-functionalized gold nanoparticles as probes in a dry-reagent strip biosensor for DNA analysis by hybridization.* Anal Chem, 2003. **75**(16): p. 4155-60.
41. Zuiderwijk, M., et al., *An amplification-free hybridization-based DNA assay to detect Streptococcus pneumoniae utilizing the up-converting phosphor technology.* Clin Biochem, 2003. **36**(5): p. 401-3.
42. Zijlmans, H.J., et al., *Detection of cell and tissue surface antigens using up-converting phosphors: a new reporter technology.* Anal Biochem, 1999. **267**(1): p. 30-6.
43. Duck, P., et al., *Probe amplifier system based on chimeric cycling oligonucleotides.* Biotechniques, 1990. **9**(2): p. 142-8.
44. Fong, W.K., et al., *Rapid solid-phase immunoassay for detection of methicillin-resistant Staphylococcus aureus using cycling probe technology.* J Clin Microbiol, 2000. **38**(7): p. 2525-9.
45. Baeumner, A.J., et al., *Biosensor for dengue virus detection: sensitive, rapid, and serotype specific.* Anal Chem, 2002. **74**(6): p. 1442-8.
46. Baeumner, A.J., J. Pretz, and S. Fang, *A universal nucleic acid sequence biosensor with nanomolar detection limits.* Anal Chem, 2004. **76**(4): p. 888-94.
47. Hartley, H.A. and A.J. Baeumner, *Biosensor for the specific detection of a single viable B. anthracis spore.* Anal Bioanal Chem, 2003. **376**(3): p. 319-27.
48. Zaytseva, N.V., et al., *Multi-analyte single-membrane biosensor for the serotype-specific detection of Dengue virus.* Anal Bioanal Chem, 2004. **380**: p. 46-53.
49. Rule, G.S., R.A. Montagna, and R.A. Durst, *Rapid method for visual identification of specific DNA sequences based on DNA-tagged liposomes.* Clin Chem, 1996. **42**(8 Pt 1): p. 1206-9.

50. Wei, C.W., et al., *Using a microfluidic device for 1 microl DNA microarray hybridization in 500 s.* Nucleic Acids Res, 2005. **33**(8): p. e78.
51. Lonnberg, M. and J. Carlsson, *Chromatographic performance of a thin microporous bed of nitrocellulose.* J Chromatogr B Biomed Sci Appl, 2001. **763**(1-2): p. 107-20.
52. Tennikov, T.B. and F. Svec, *High-performance membrane chromatography: highly efficient separation method for proteins in ion-exchange, hydrophobic interaction and reversed-phase modes.* J. Chromatography, 1993. **646**: p. 279-288.
53. Tennikova, T.B. and R. Freitag, *An introduction to monolithic disks as stationary phases for high performance biochromatography.* J. High Resol. Chromatogr., 2000. **23**: p. 27-38.
54. Thommes, J. and M. Kula, *Membrane Chromatography-- An Integrative Concept in the Downstream Processing of Proteins.* Biotechnol. Prog., 1995. **11**: p. 357-367.

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

**United States Department of Agriculture**
Research, Education, and Economics
Agricultural Research Service

R. Bruce Cary
Mesa Tech International, Inc.
2778 Agua Fria St. BLDG C, SUITE A
Santa Fe, NM 87507

Dear Bruce:
We are pleased to collaborate with you in developing field deployable nucleic acid assays for HLB. My laboratory has developed important methods to address the threat from *Candidatus* Liberibacter including techniques for culture, nucleic acid isolation and real-time PCR detection. My group will make available our target sequences for PCR assays for you to adapt to the integrated lateral flow microarray-based platform you are proposing. In addition, we will supply adequate amounts of relevant DNAs for amplification and detection testing in your laboratory. Also, using your lateral flow devices, we will screen for specificity and sensitivity using DNA and cells of *Can.* Liberibacter and other bacteria extracted from diseased plants in our containment facility and in the field. These unique reagents will provide an important resource for developing useful field assays for the rapid diagnosis of HLB.

Norman W. Schaad
Research Plant Pathologist
USDA/ARS, Foreign Disease-Weed Science Research Unit

North Atlantic Area . Foreign Disease-Weed Science Research Unit
1301 ditto Avenue, Fort Detrick, MD 21702-5023, USA
Voice: 301-619-2847. Fax: 301-619-2880