Rapid, closed-tube multiplexed detection of viral and bacterial pathogens by isothermal amplification with streamlined sample prep

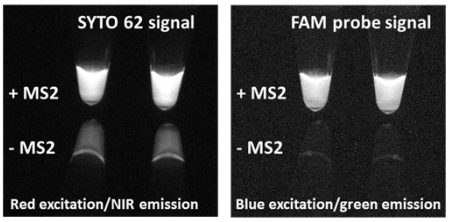
SAND2015-1184C

Robert J. Meagher[1]*, Chung-Yan Koh[1], Yooli K. Light[1], Cameron Ball[1], Sarah Wheeler[2], William Reisen[2], Lark Coffey[2]; [1] Sandia National Laboratories, Livermore, CA, USA
[2] UC Davis Center for Vectorborne Diseases, Davis, CA, USA

Background: Nucleic acid amplification tests (NAATs) like real-time PCR offer outstanding sensitivity and sensitivity, but generally require a well-equipped laboratory and trained staff to carry out sample prep and complex protocols. Commercial instruments for all-in-one sample-to-answer analysis are expensive both for equipment and consumables. Isothermal NAATs such as Loop-mediated isothermal amplification (LAMP) eliminate the need for a thermal cycler, and may be preferable to PCR for point-of-care use. However, detection techniques for LAMP are usually non-specific, or require some hands-on manipulation after the amplification step, or both. Options for multiplexing more than one target per reaction are also limited with LAMP. We demonstrate here *single step, closed tube, target specific* detection of multiple pathogens, with easily visualized duplex detection employing a novel quenched probe chemistry.

Easily visualized endpoint: The SYTO intercalating dyes (STYO 9, SYTO 82, SYTO 62) are compatible with closed-tube detection in LAMP, but are non-specific, and offer ~3-fold difference in brightness between positive and negative reactions. Our novel technique allows >10X difference in brightness and can be visualized with an LED flashlight and colored plastic filter.

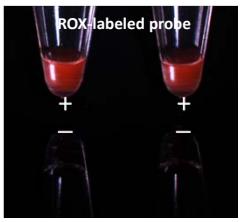
MS2 phage RT-LAMP: Positive amplification control assay



Intercalating dye: Requires heating for best discrimination; negatives are still somewhat bright

New technique: Detection at ambient temperature with obvious difference between positive and negative results

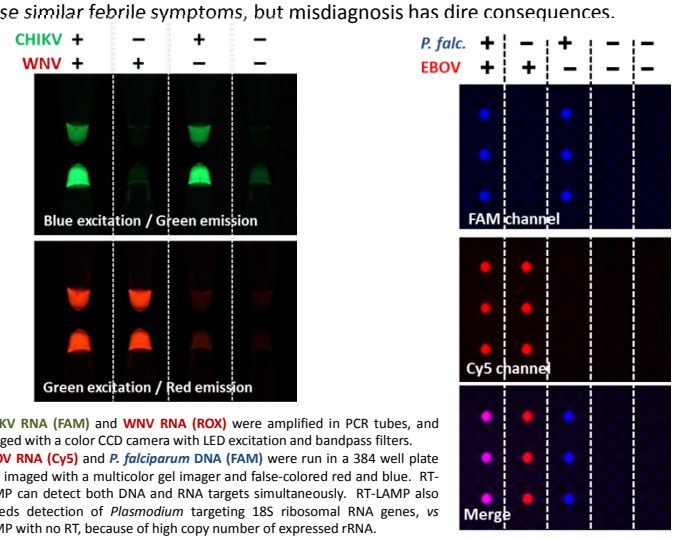
West Nile Virus (WNV) RT-LAMP



Bright signals: ROX fluorescence visualized with a green LED flashlight and red plastic film "filter"

Bright, obvious endpoint with simple equipment and no manipulations after reaction setup enables **distributed bioassays** for **biosurveillance** or **point-of-care**.

End-point multiplexing: We have demonstrated closed tube multiplexing for mosquito-borne viruses (West Nile virus, WNV and chikungunya virus, CHIKV). Our detection chemistry was easily adapted to LAMP primer sets reported in literature. We have also demonstrated closed tube multiplexing for Ebola virus (EBOV) and *Plasmodium falciparum*. For EBOV, the assay was tested with RNA from the 1976 Mayinga strain, but primers were designed for homology to isolates sequenced in 2014 from the outbreak in West Africa. Malaria and Ebola cause similar febrile symptoms, but misdiagnosis has dire consequences.



CHIKV RNA (FAM) and WNV RNA (ROX) were amplified in PCR tubes, and imaged with a color CCD camera with LED excitation and bandpass filters. EBOV RNA (Cy5) and *P. falciparum* DNA (FAM) were run in a 384 well plate and imaged with a multicolor gel imager and false-colored red and blue. RT-LAMP can detect both DNA and RNA targets simultaneously. RT-LAMP also speeds detection of *Plasmodium* targeting 18S ribosomal RNA genes, *vs* LAMP with no RT, because of high copy number of expressed rRNA.

Direct LAMP and RT-LAMP without DNA/RNA extraction: We and others have noticed that LAMP and RT-LAMP don't necessarily require extraction to amplify. In the case of RNA viruses, our novel endpoint detection technique allows closed-tube monitoring of amplification from intact, infectious RNA viruses in a BSL-3 laboratory setting where a real-time PCR machine isn't available. The following results were obtained for Western Equine Encephalitis virus (WEEV) and St. Louis Encephalitis virus (SLEV), grown in Vero cell culture. Supernatants were quantitated by plaque assay, and then added to RT-LAMP reactions, either intact or with column-based RNA extraction.

	WEEV		SLEV	
PFU/rxn	Extracted RNA	Intact virus	Extracted RNA	Intact virus
100000	pos	neg*	NT	NT
10000	pos	pos	NT	NT
1000	pos	pos	pos	neg*
100	pos	pos	pos	pos
10	pos	pos	pos/pos	neg**
1	pos/pos	pos	pos/pos	pos/pos
0.1	pos/pos	pos	neg/pos	pos/pos
0.01	neg/neg	pos/pos	neg/neg	neg/neg
0.001	neg/neg	pos/neg	neg	neg/neg
0.0001	neg	neg/neg	neg	neg
0.00001	neg	neg/neg	neg	neg
water	neg	neg	neg	neg

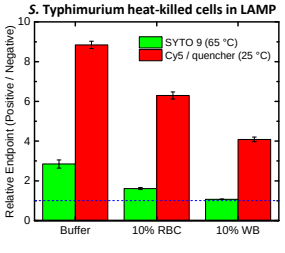
For both viruses, the 63 °C incubation of RT-LAMP is sufficient to release RNA for incubation. WEEV (an alphavirus) is more resistant to thermal inactivation than SLEV (a flavivirus). This experiment with live viruses was logistically impossible with a real-time PCR machine, and would have required extensive safety testing prior to employing a conventional "open tube" endpoint detection technique.

Direct LAMP for bacteria in clinical sample matrices: By monitoring LAMP with real-time-compatible dyes (*e.g.* SYTO 9, SYTO 82), we have observed that the speed of amplification by LAMP is generally not impacted by up to 10% of whole blood, serum, stool, saliva, or urine added directly to the reaction mixture. Blood does, however, reduce the overall signal strength and the relative difference between positive and negative reactions when using SYTO dyes for monitoring. Our novel fluorophore/quencher detection technique provides a stronger discrimination between positive and negative reactions (lower left).

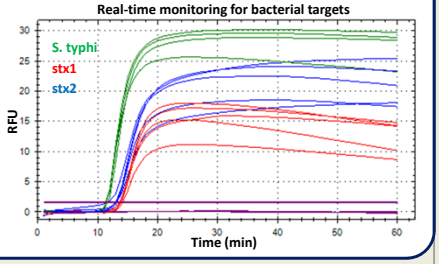
Target bacteria	Detection limit
Campylobacter *jejuni*	10 cells
Escherichia *coli* H157:O7	10 cells
Listeria *monocytogenes*	100 cells
Salmonella *typhimurium*	10 cells

Matrices	Minimum dilution needed for LAMP
Whole Blood	No dilution
Saliva	No dilution
Urine	No dilution
Stool	1:50

***S.* Typhimurium heat-killed cells in LAMP**

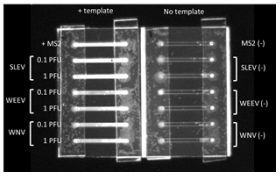


Real-time monitoring for bacterial targets

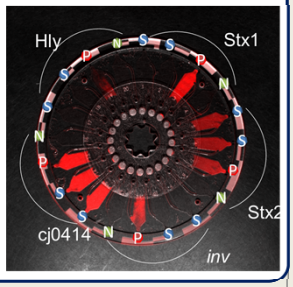


LAMP in a microfluidic sample analysis platform: The combination of sensitive, specific detection, extraction-free sample prep, tolerance to crude samples, and low operating cost makes LAMP ideal for implementation in low-resource settings. Our novel single-step closed-tube detection technique extends this capability by providing easy discrimination between positive and negative endpoints and multiplexing capability.

RT-LAMP for arbovirus RNA targets in polycarbonate microchannels (platform for vectorborne disease surveillance)



LAMP for enteric pathogenic bacteria spiked in human stool, using SpinDx centrifugal microfluidic platform



E.Coli O121:H19 + *C.jejuni*
All enteric bacteria targets
water

Acknowledgment: Funded by Sandia Laboratory Directed Research and Development (LDRD); Defense Threat Reduction Agency (DTRA); NIH-NIAID. Sandia National Laboratories is a multi-program laboratory managed and operated by Sandia Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, for the U.S. Department of Energy's National Nuclear Security Administration under Contract DE-AC04-94AL85000