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Rapid, Closed-tube Multiplexed Detection of Viral and Bacterial Pathogens by Isothermal Amplification with Streamlined Sample Prep

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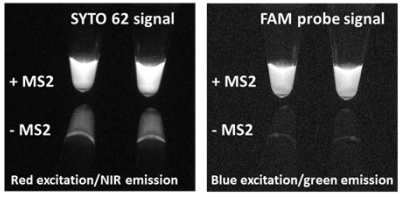
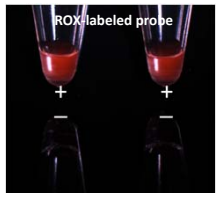
Background: Nucleic acid amplification tests (NAATs) like real-time PCR offer outstanding sensitivity and sensitivity, but generally require a well-equipped laboratory and trained staff to carry out sample prep and complex protocols. Commercial instruments for all-in-one sample-to-answer analysis are 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



West Nile Virus (WNV) RT-LAMP



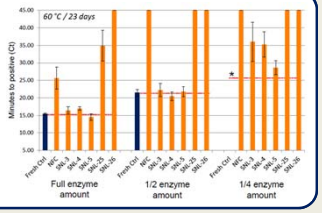
Intercalating dye: Requires heating for best discrimination; negatives are still somewhat bright

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

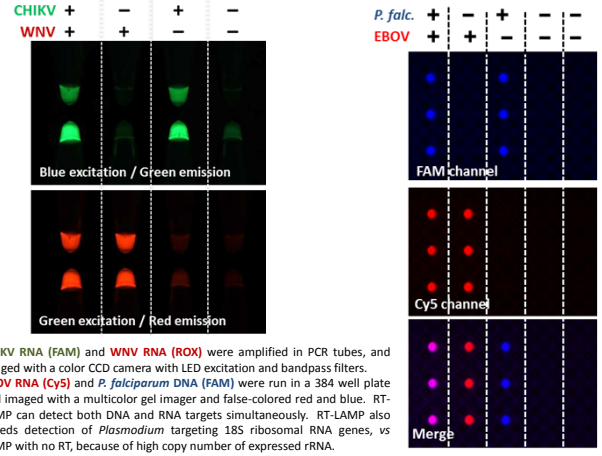
Bright signals: ROX fluorescence visualized with a green LED flashlight and red plastic film "filter"

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

Stabilized reagents: LAMP assay reagents were air-dried using stabilizing formulations developed in partnership with Biomatrica. In accelerated aging tests, reagents were stable when stored for >3 weeks at 60 ℃, even with reduced amounts of enzymes. This is expected to reduce dependence on cold chain for forward deployed assays.

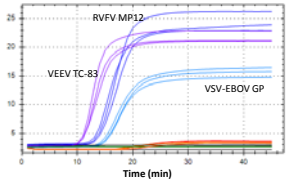


End-point multiplexing: We have demonstrated closed tube multiplexing for mosquito-borne viruses (West Nile virus, WNV and chikungunya virus, CHIKV). Our detection chemistry was easily adapted to LAMP primer sets reported in literature. We have also demonstrated closed tube multiplexing for Ebola virus (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 extraction: We and others have observed that a rigorous DNA/RNA extraction is not absolutely required for LAMP/RT-LAMP amplification. We have demonstrated amplification of intact Gram-negative bacteria, several families of enveloped RNA viruses, and non-enveloped RNA virus (MS2 phage).



Real-time monitoring of RT-LAMP amplification with several intact enveloped RNA viruses (SYTO 9 monitoring; real-time PCR machine in BSL-2 containment)

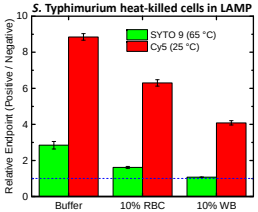
	WNV		SLEV		WEEV	
PFU/rxn	Extracted	Intact	Extracted	Intact	Extracted	Intact
10,000		+			+	+
1,000	+	+	+	–*	+	+
100	+	+	+	+	+	+
10	+	+	+	–	+	+
1	+	+	+	+	+	+
0.1	+	+	+	+	+	+
0.01	+	–	–	–	–	+
None	–	–	–	–	–	–

Sensitivity test of RT-LAMP for intact viruses *vs* equivalent amount of extracted viral RNA (closed-tube endpoint monitoring in BSL-3 containment is made feasible with our novel detection technique).

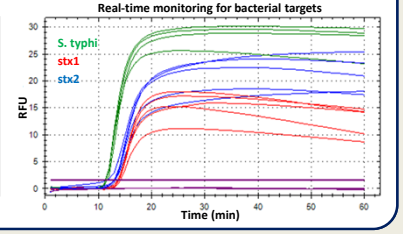
Direct LAMP for bacteria in clinical sample matrices: By monitoring LAMP with real-time-compatible dyes (*e.g.* SYTO 9, SYTO 82), we have observed that the speed of amplification by LAMP is generally not impacted by up to 10% of whole blood, serum, saliva, urine, or stool (50 mg/mL suspension, boiled) added 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 detection technique provides a stronger discrimination between positive and negative reactions (below).

LAMP target	Target Bacteria	LOD in Stool	LOD in buffer
stx1	*E. coli* O157:H7	100 cells	10 cells
stx2	*E. coli* O129:H19	100 cells	10 cells
cj0414	*C. jejuni*	100 cells	10 cells
inv	*S.* Typhimurium	100 cells	10 cells
hly	*L. monocytogene*	1,000 cells	100 cells

S. Typhimurium heat-killed cells in LAMP

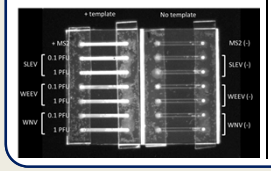


Real-time monitoring for bacterial targets

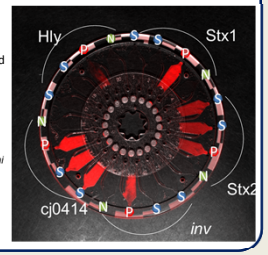


LAMP in a microfluidic sample analysis platform: The combination of sensitive, specific detection, extraction-free sample prep, tolerance to crude samples, and low operating cost makes LAMP ideal for implementation in low-resource settings. Our novel single-step closed-tube detection technique extends 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



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