Highly Multiplexed, Two-Stage Isothermal Assay (RAMP) for Molecular Detection at the Point of Care

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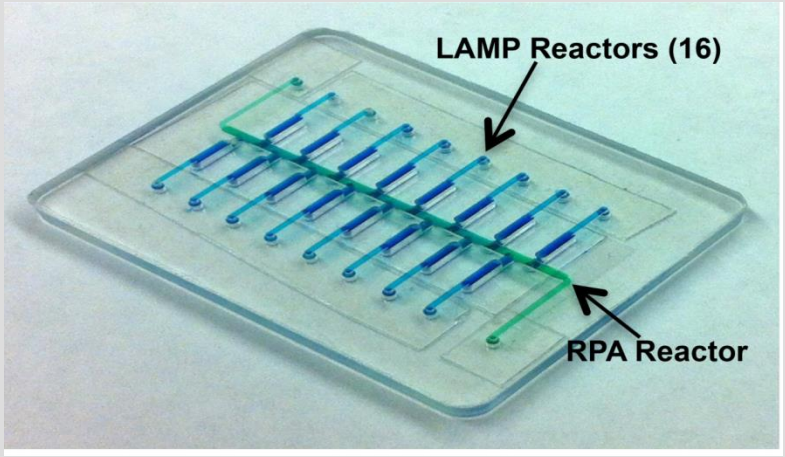

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Participating Lab Members

- Prof. Changchun Liu
- Prof. Michael Mauk
- Dr. Jinzhao Song
- Ms. Jing Peng

Collaborators

- Prof. Robert Greenberg (Vet School)
- Prof. Shelley C. Rankin
- Prof. James B. Lok

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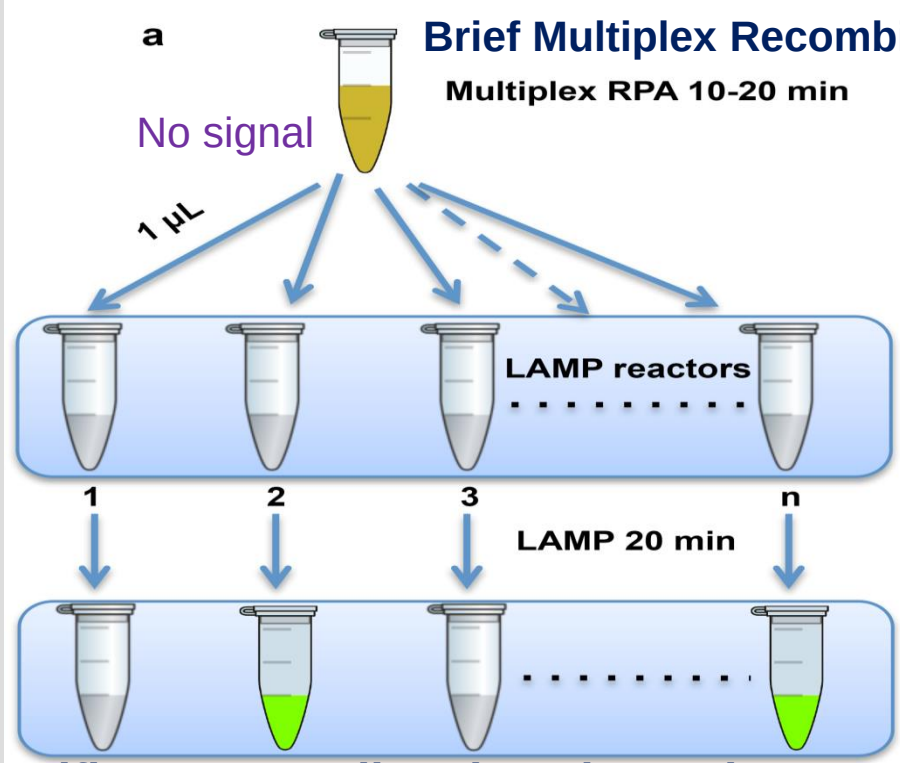
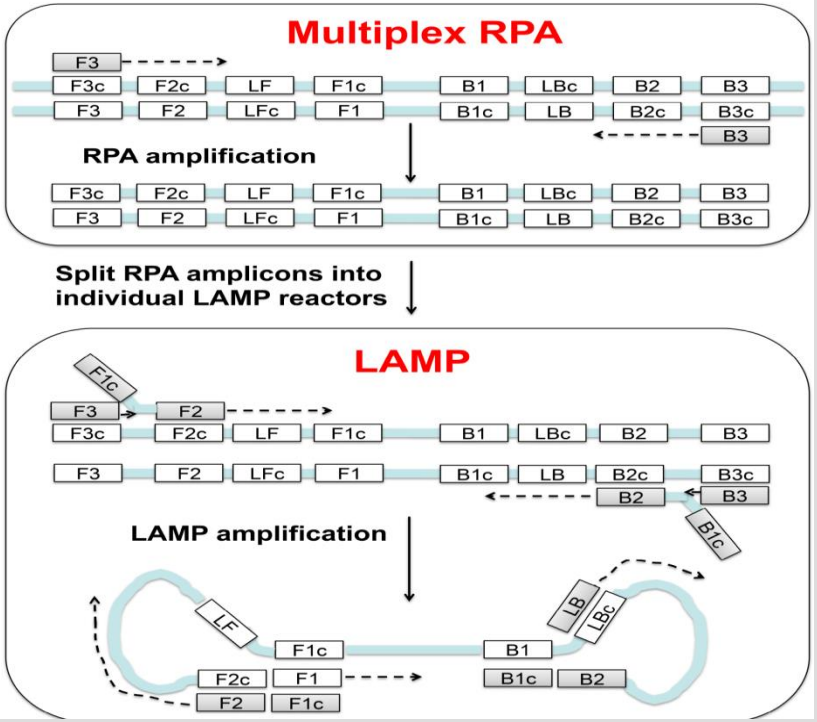
Motivation

- The wide array of pathogens responsible for infectious diseases makes it difficult to identify causative agents with single-plex tests. Different infections can cause similar overt symptoms, but require specific disease management strategies
- Pathogens are often co-endemic, making it imperative to distinguish single infections from co-infections, which can alter host responses, disease prognosis, transmission dynamics, and treatment strategies and outcomes.
- Syndromic panel tests with simultaneous detection of multiple biomarkers for infectious diseases can assist health care providers to determine effective treatments and the potential need for additional ancillary diagnostic testing.
- Although multiplex PCR detects multiple targets concurrently, it is restricted to centralized laboratories, which delays test results, depriving healthcare providers of critical, real-time information.

Method: Two-Stage Isothermal Amplification



Brief Multiplex Recombinase Polymerase Amplification (RPA) ~37°C

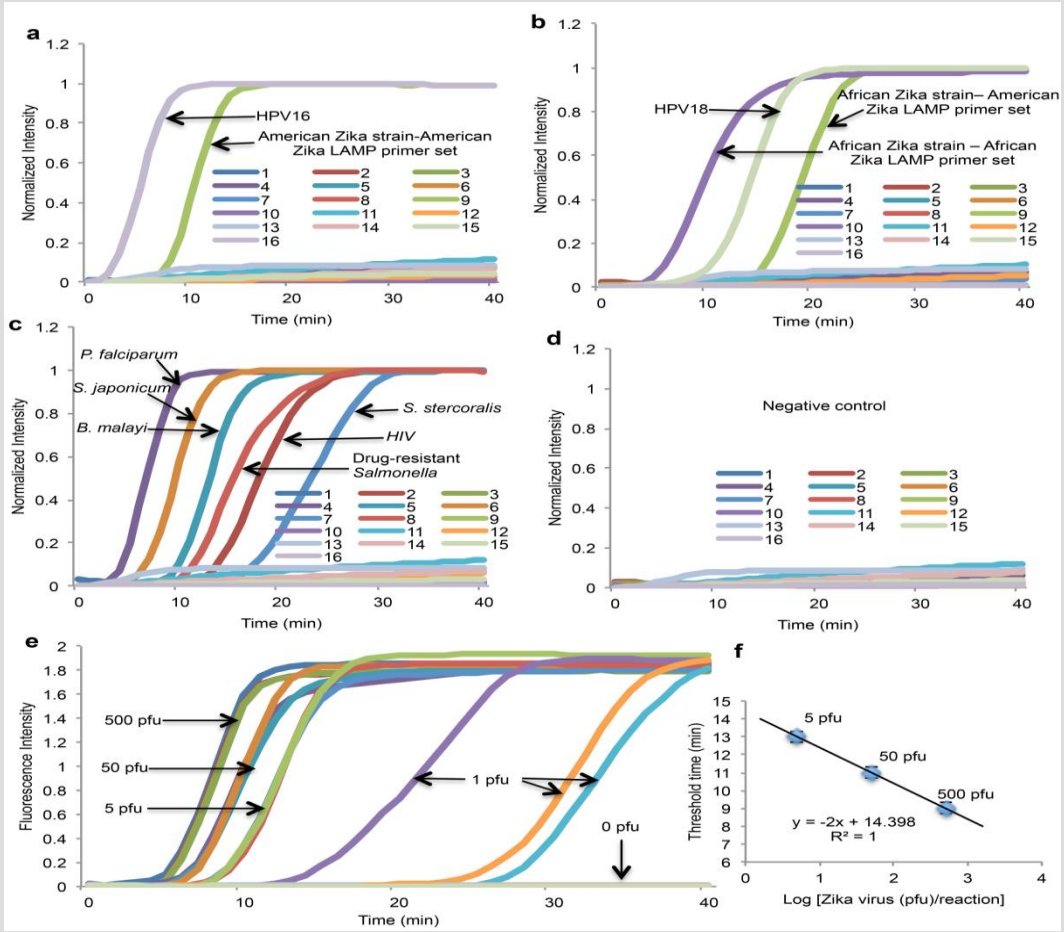


Specific Loop-Mediated Isothermal Amplification (LAMP) ~65°C

Example: 16-plex RAMP

RAMP assay to detect: (1) *S. mansoni*, (2) HIV-1 B, (3) *S. haematobium, (4) P. falciparum*, (5) *S. japonicum,* (6) *Brugia malayi,* (7) *Strongyloides stercoralis,* (8) Drug-resistant *Salmonella,* (9) ZIKV (mex 2-81), (10) ZIKV (*MR 766, Uganda*), (11) HPV-58, (12) HPV-52, (13) HPV-35, (14) HPV-45, (15) HPV-18, and (16) HPV-16.

Samples: (**a**) HPV-16 (100 copies) **+** ZIKV (American, 50 PFU); (**b**) HPV-18 (100 copies) **+** ZIKV (African, 50 PFU); (**c**) HIV-1 clade B (100 copies), *P. falciparum* (300 fg), *S. japonicum* (1 pg), *B. malayi* (13 pg), *S. stercoralis* (1 pg) **+** drug-resistant *Salmonella* (100 copies); and (**d**) no target (negative control) (**e**) 1, 5, 50, & 500 ZIKV PFU.

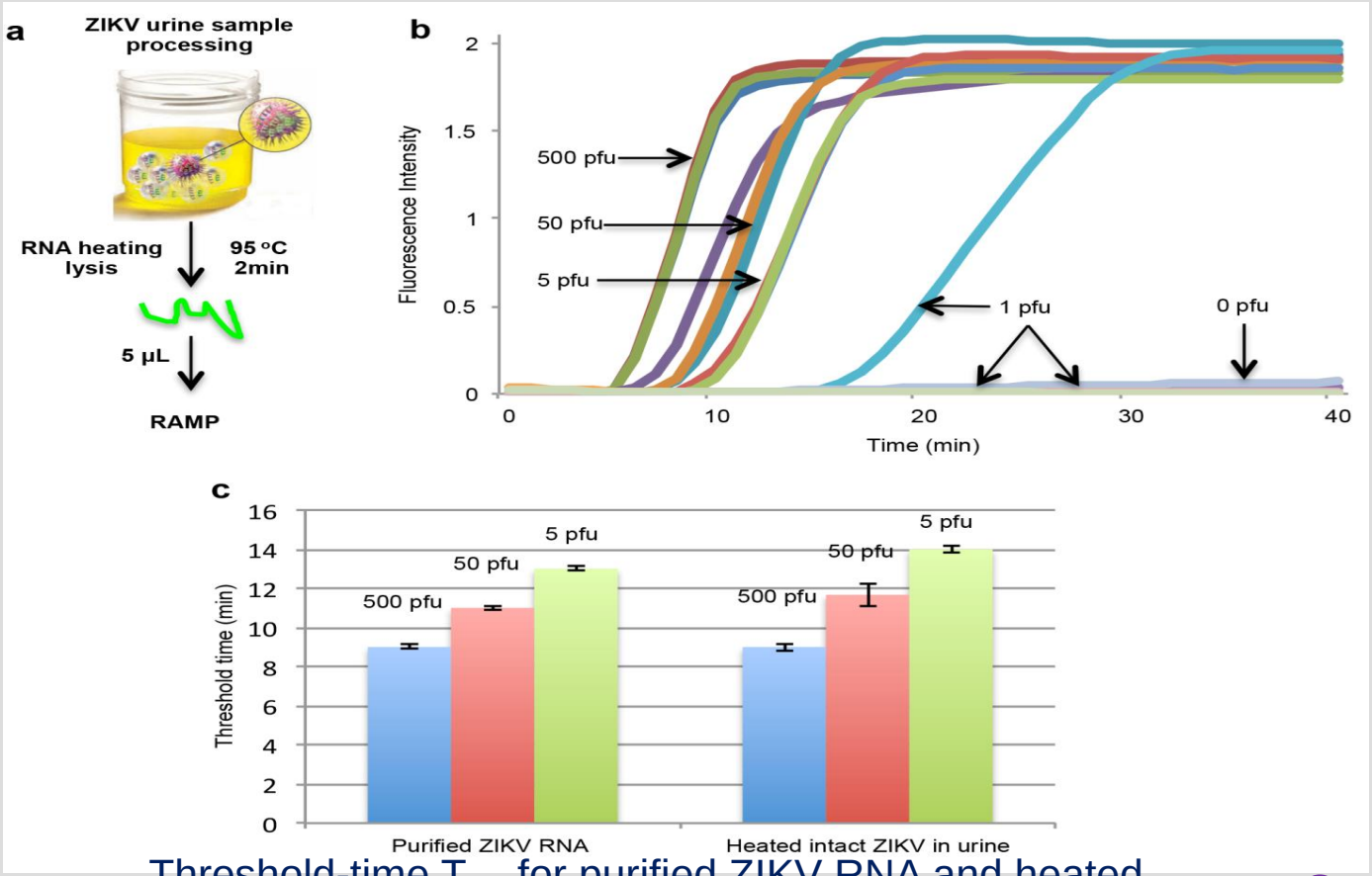


No false positives!

Minimally-Processed Samples

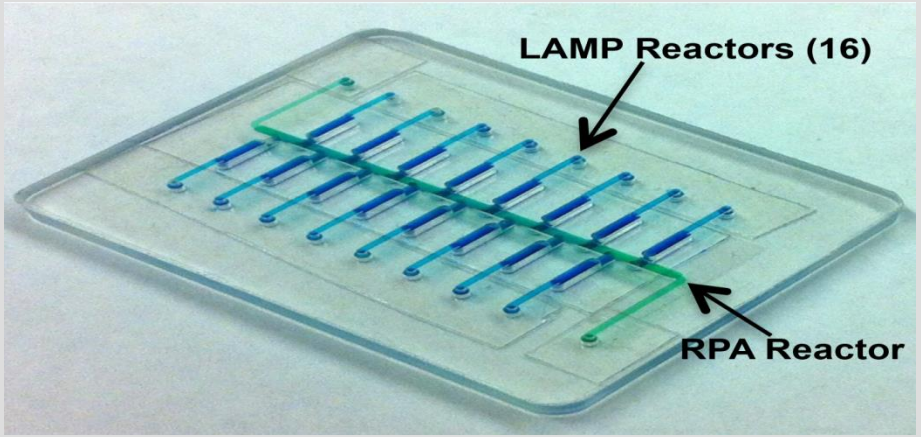
16-plex RAMP with urine sample laden with ZIKV.

The assay is tolerant to inhibitors



Threshold-time $T_{1/2}$ for purified ZIKV RNA and heated urine laden with ZIKV as functions of the number of PFU.

RAMP – Microfluidic Implementation

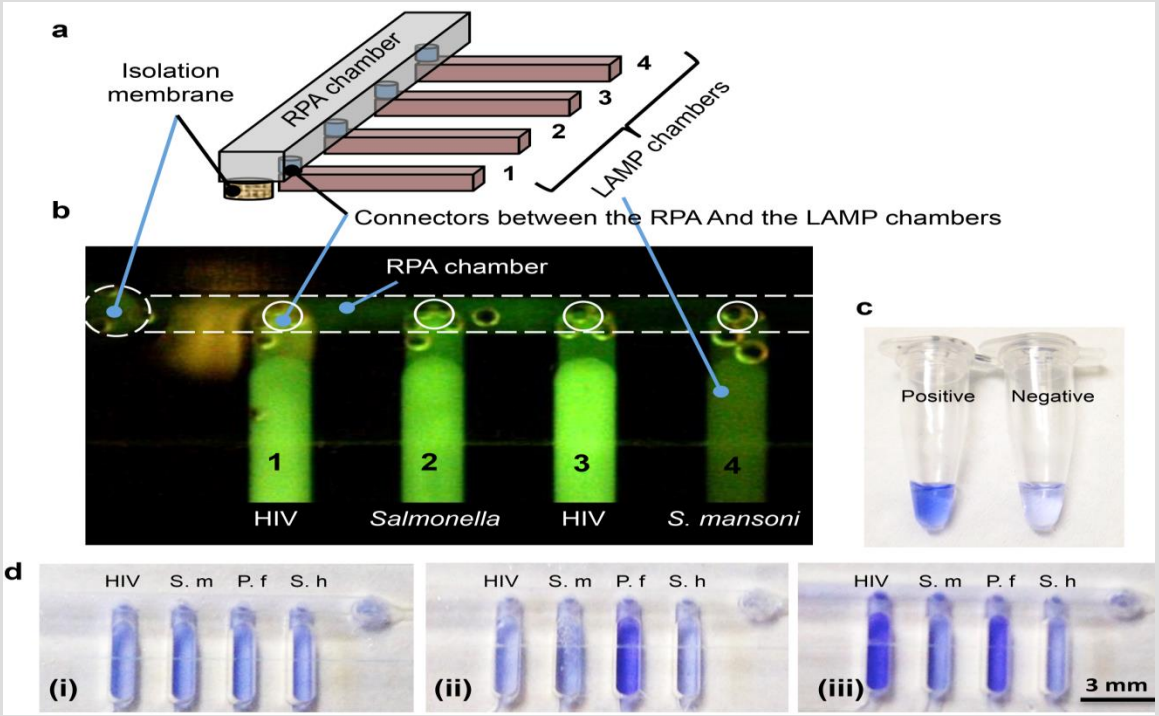


* Plastic chip with a central RPA chamber for amplification of ***all*** targets, and multiple branching LAMP chambers for *specific* amplification of individual targets.
* A sample mixed with binding / lysis agent is introduced into the central RPA reactor, nucleic acids (NAs) are captured on a NA-isolation membrane. Following washes, the reactor is filled with water and heated to ~40^{o}C for RPA step.
* RPA amplicons diffuse to branching, LAMP reactors, each storing LAMP reagents for a *specific* target. When the chip is heated to ~65^{o}C, RPA is quenched and LAMP proceeds. Optionally, the central chamber and branching chambers can be separated, e.g., with paraffin “valves” that melt, just-in-time.
* Amplicons can be detected with fluorescent or colorimetric dye.
* The entire process takes place in a *closed* system, avoiding the need for manually transferring first-stage amplicons and risking contamination.

An example: Four-Plex microfluidic implementation

(**b**) Fluorescence image of products. Sample : HIV RNA (1000 copies), drug-resistant *Salmonella* gDNA (~100 copies), and *S. mansoni* gDNA (0 fg) (50 min).

(**c**) Colorimetric detection: 100 copies HIV RNA in tube with Leuco crystal violet (LCV) dye that changes from colorless to violet in the presence of dsDNA.



(**d**) Colorimetric detection of HIV, *S. mansoni*, *P. falciparum*, *S. haematobium* on chip with LCV dye. Samples: (i) no targets, (ii) *P. falciparum* DNA (300 fg), (iii) *P. falciparum* DNA (300 fg) **+** HIV RNA (1000 copies).

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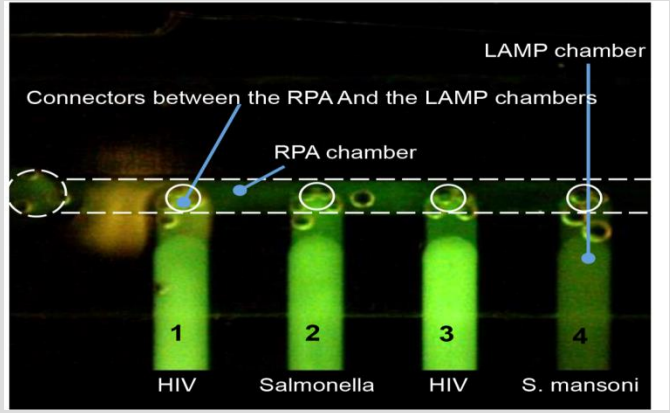
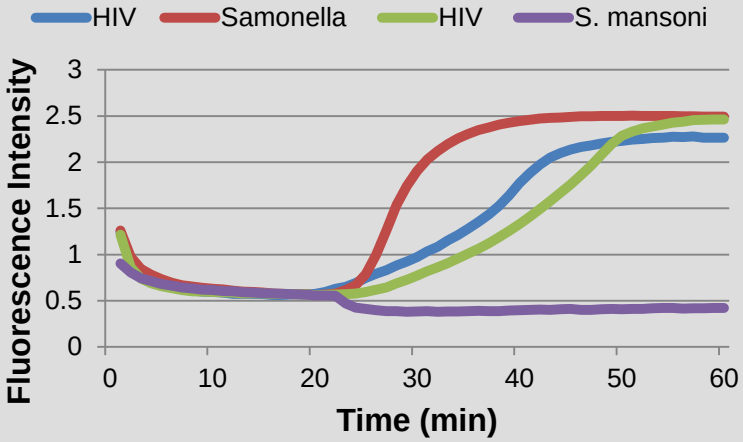
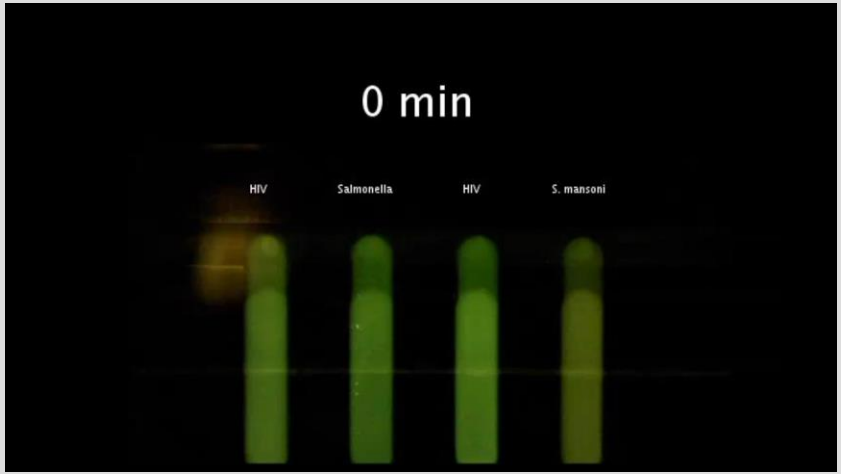
A tri-plex RAMP assay for drug-resistant *Salmonella* gDNA, HIV, and *S. mansoni* gDNA (without dry storage). Sample: HIV RNA (1000 copies) and drug-resistant *Salmonella* gDNA (~100 copies), but no *S. mansoni* gDNA (negative control). Two second-stage reactors are specialized for HIV to demonstrate reproducibility.







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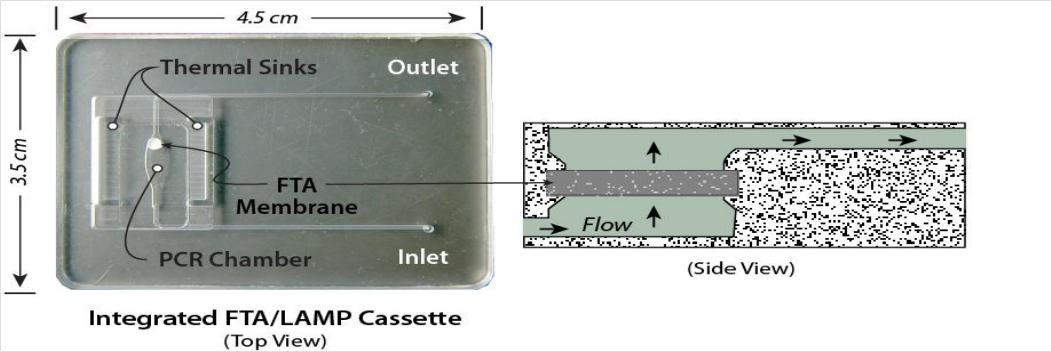
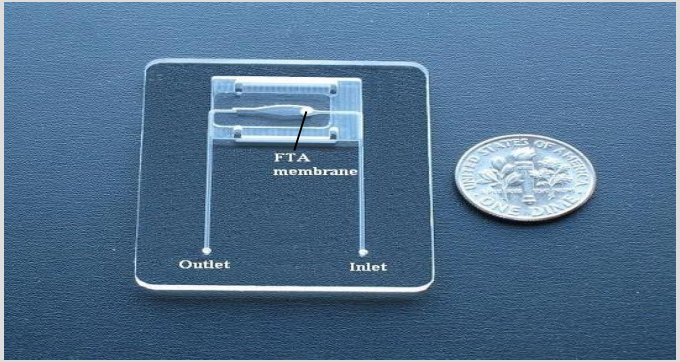
Key to Simplicity: Multi-Function Amplification LAMP Reactor (with nucleic acid isolation membrane and dry reagent storage)



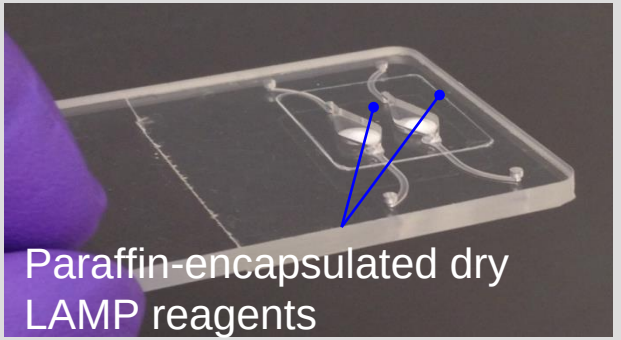


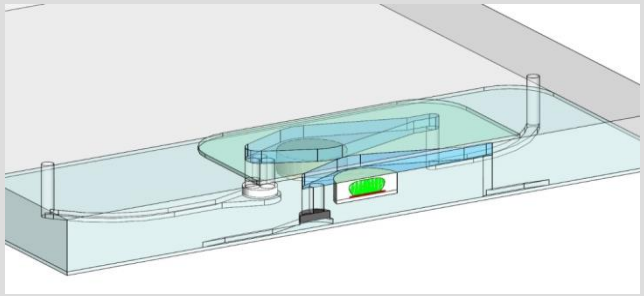
Typically, rapid POC molecular tests do ***not*** include nucleic-acid concentration. When unprocessed sample is added directly to the reaction volume, the sample volume is limited to a few μl. In contrast, our approach **decouples** sample volume from the reaction volume, enabling large sample processing to achieve sensitivity on par with laboratory-based equipment.

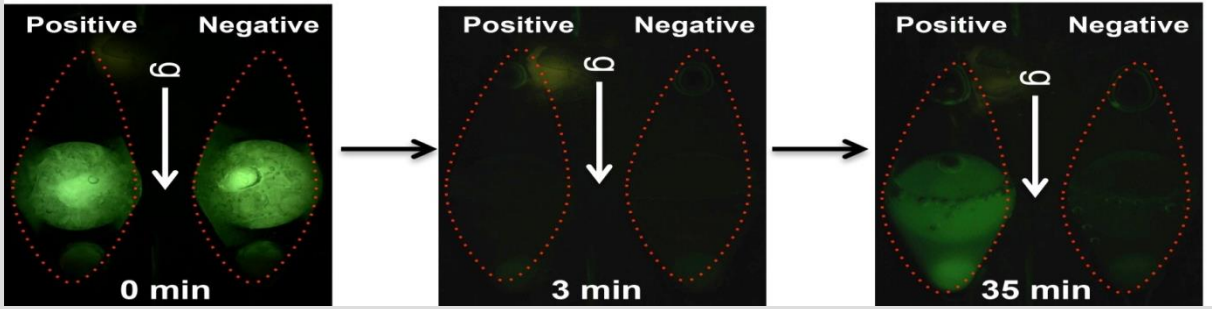
C. Liu, E. Geva, M. Mauk, X. Qiu, W. R. Abrams, D. Malamud, K. Curtis, S. M. Owen, and H. H. Bau, 2011, An Isothermal Amplification Reactor with an Integrated Isolation Membrane for Point-of-Care Detection of Infectious Diseases, *Analyst* 2011, **136**, 2069-2076

In-Situ Dry-Storage of Reaction Mix in the LAMP Reaction Chambers for Just-in-Time Release

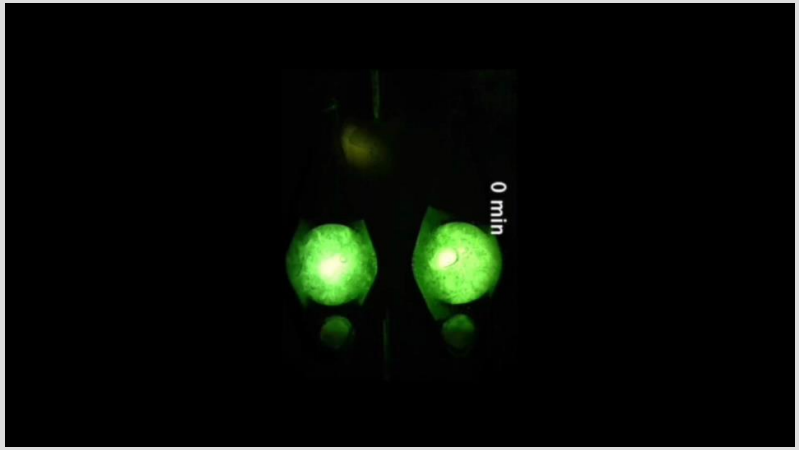
- Dried reaction mix is pre-stored in the reaction chamber, encapsulated with paraffin to protect against liquids transmitted through the reactor.
- When the reactor is heated, the paraffin melts, moves out of the way, and reagents are hydrated
- *Just in time* release upon chamber's heating → ***hot start***
- Prolonged shelf life (12 months) without refrigeration



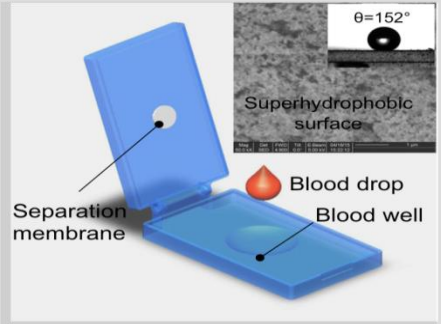


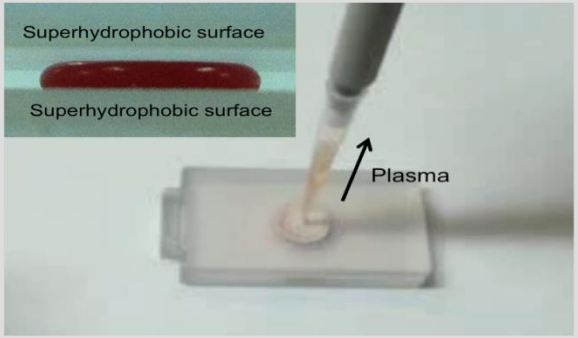


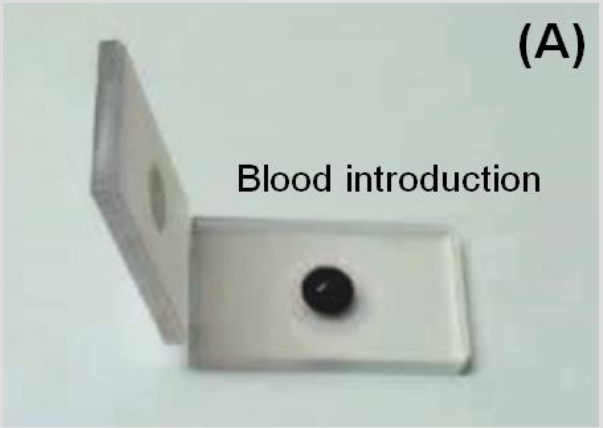
Kim, J., Byun, D., Mauk, M., G., and Bau, H., H., 2009, A Disposable, Self-Contained PCR Chip, Lab on Chip 9, 606-612

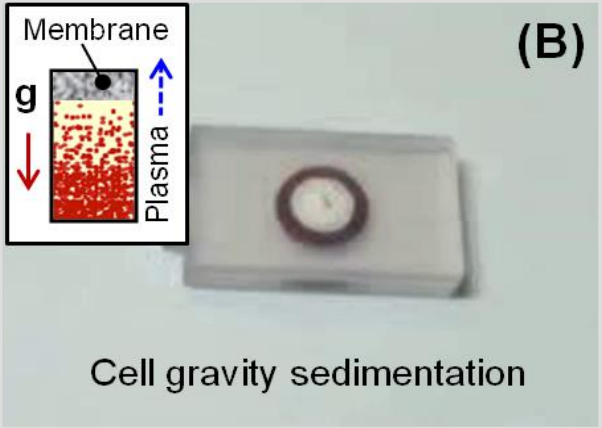

0 min

PLASMA SEPARATOR









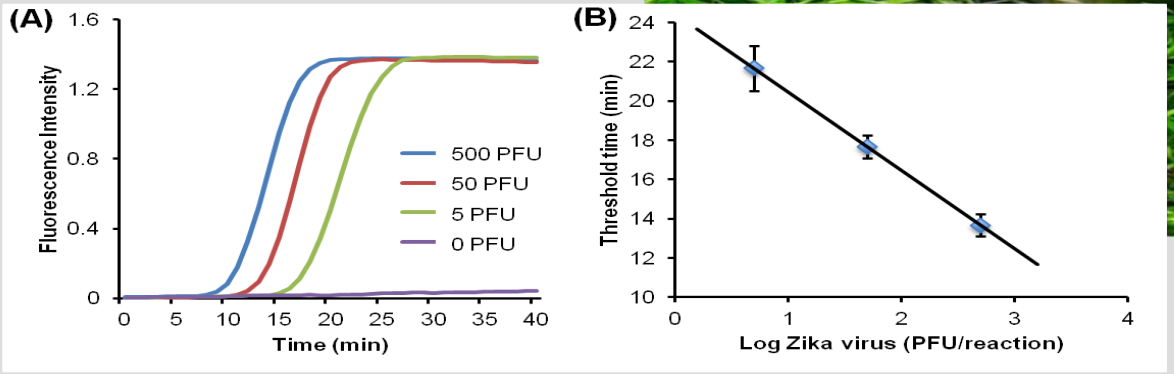
Combination of sedimentation and filtration enables separation of a relatively large volume of cell-free plasma with a small device. Hydrophobic surfaces discourage pathogen binding and keep the blood from spreading when plasma is removed

Liu, et al, 2016, "A High-Efficiency Superhydrophobic Plasma Separator" Lab on Chip 16, 553-560. DOI: 10.1039/c5lc01235j

Conclusions

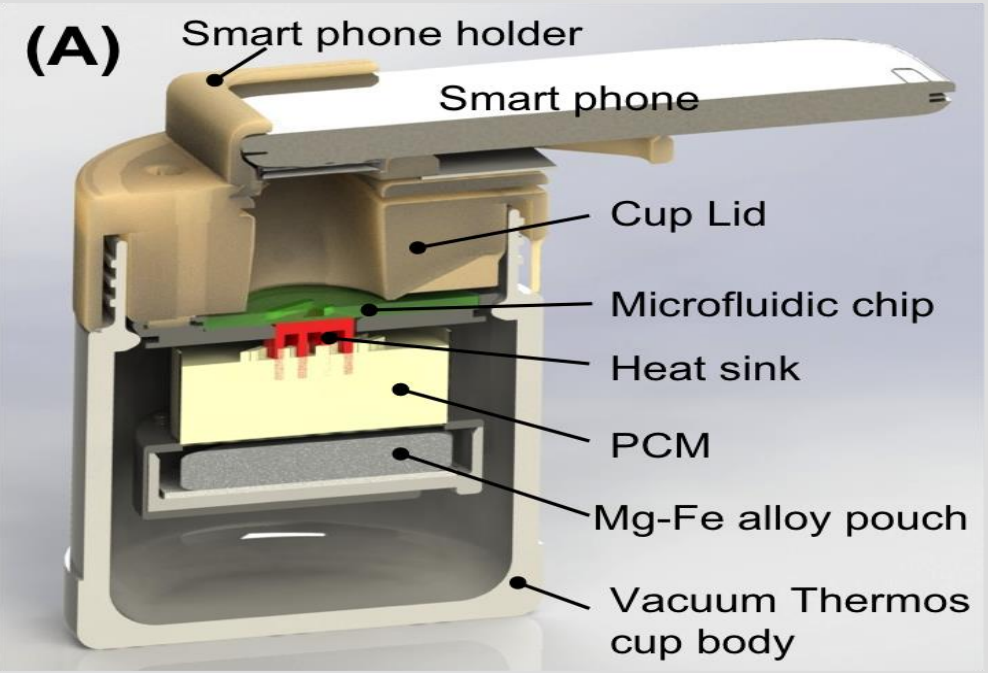
- RAMP is a hybrid, two-stage, rapid, and highly sensitive and specific assay with extensive multiplexing capabilities, combining the advantages of RPA and LAMP, while circumventing their respective shortcomings.
- RAMP can be used in the lab, but its distinct advantages is amenability to simple implementation in a microfluidic format for the point of care, providing health care personnel with an inexpensive, highly sensitive tool to detect multiple pathogens in a single sample, on-site.
- In both the benchtop and microfluidic format, RAMP demonstrated high level of multiplexing capability (≥16); high sensitivity (1 PFU of ZIKV); and specificity (no false positives or negatives); speed (<40 min); ease of use; and ability to cope with minimally-processed samples.
- Future work will focus on developing self-contained cassettes with sample-processing and dry-stored reagents, and various diagnostic panels of medical significance such as vector-borne pathogens and neglected tropical diseases.

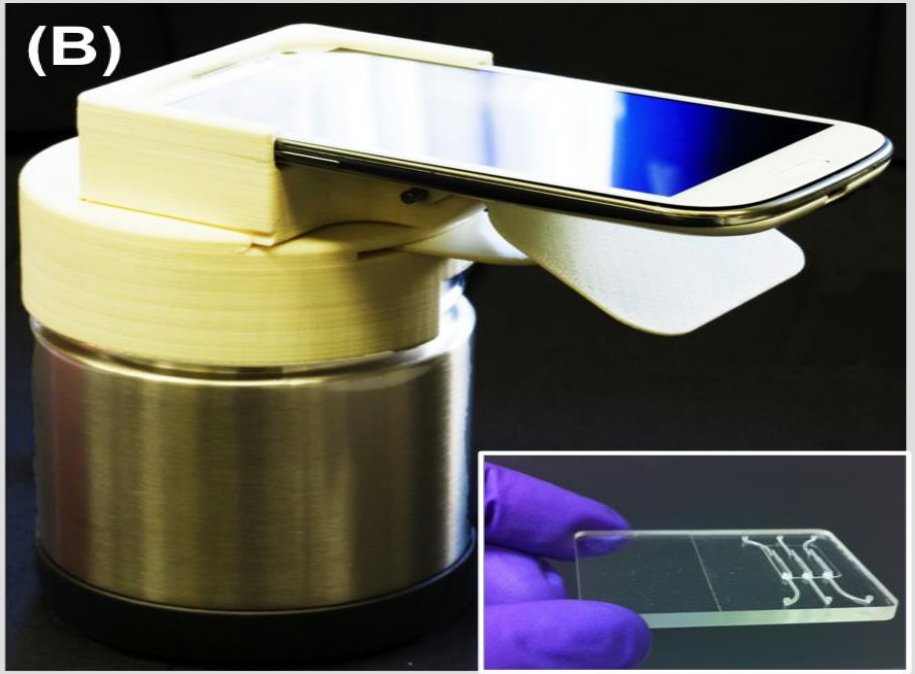
The future is here



Song, J., et al, 2016, Instrument-Free Point-of-Care Molecular Detection of Zika Virus, Analytical Chemistry *88* (14), 7289–7294, DOI:10.1021/acs.analchem.6b01632.

SMARTPHONE-BASED DETECTION AND POWER-FREE OPERATION





Excitation is provided with phone flash. Two optical filters minimize overlap between excitation and emission spectra

Alternatives: bioluminescent label or indicators such as leuco triphenylmethane dyes (remove a need for excitation source and filters)

Liao et al., 2016, Smart Cup: A Minimally-Instrumented, Smartphone-Based Point-of-Care Molecular Diagnostic Device, Sensors and Actuators B, 229, 232-238. doi:10.1016/j.snb.2016.01.073