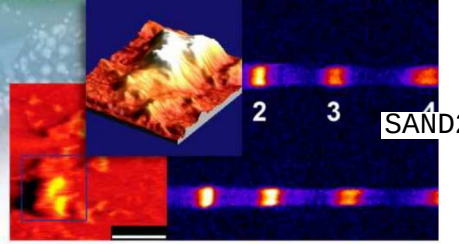
SANDIA BIOSCIENCE



SAND2019-1884C

More with Less: Novel Approaches to LAMP Assay Design for Better Performance with Fewer Resources

Robert Meagher
Sandia National Laboratories
Biotechnology and Bioengineering Department
Livermore, CA, USA

Molecular Medicine Tri-Conference 2019
San Francisco, CA

Sandia National Laboratories is a multimission laboratory managed and operated by National Technology and Engineering Solutions of Sandia, LLC., a wholly owned subsidiary of Honeywell International, Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA-0003525!

Acknowledgements

Sandia (and ex-Sandia) team

- Yooli Light – Assay characterization
- Aashish Priye – Smart phone platform & modeling
- Cameron Ball – Portable devices, Assay characterization
- Sara Bird – Virology
- Oscar Negrete - Virology

Collaborators

- Lark Coffey (UC Davis)
- Michael Busch (UCSF/BSRI)
- Scott Weaver (UTMB)
- Slobodon Paessler (UTMB)
- Barbara Johnson (CDC)
- Brandy Russell (CDC)

Funding

- Sandia Laboratory-Directed Research and Development (LDRD)
- NIH NIAID (R21-R33)

Disclaimers

- This presentation describes objective technical results and analysis. Any subjective views or opinions that might be expressed in the presentation do not necessarily represent the views of the U.S. Department of Energy or the United States Government.

- 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.

Challenges in Deployed Diagnostics

- Emerging, re-emerging, and neglected diseases like Zika and Ebola occur in parts of the world where medical infrastructure is lacking.
- Safety, security, and speed of sample transport to reference labs is a major concern in areas with poor infrastructure.
- Point-of-Need assays requires robust assay chemistry, cheap consumables, and simple instrumentation.
- WHO **ASSURED** criteria: **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free and **D**eliverable to end-users.

Photo/Justin Williams

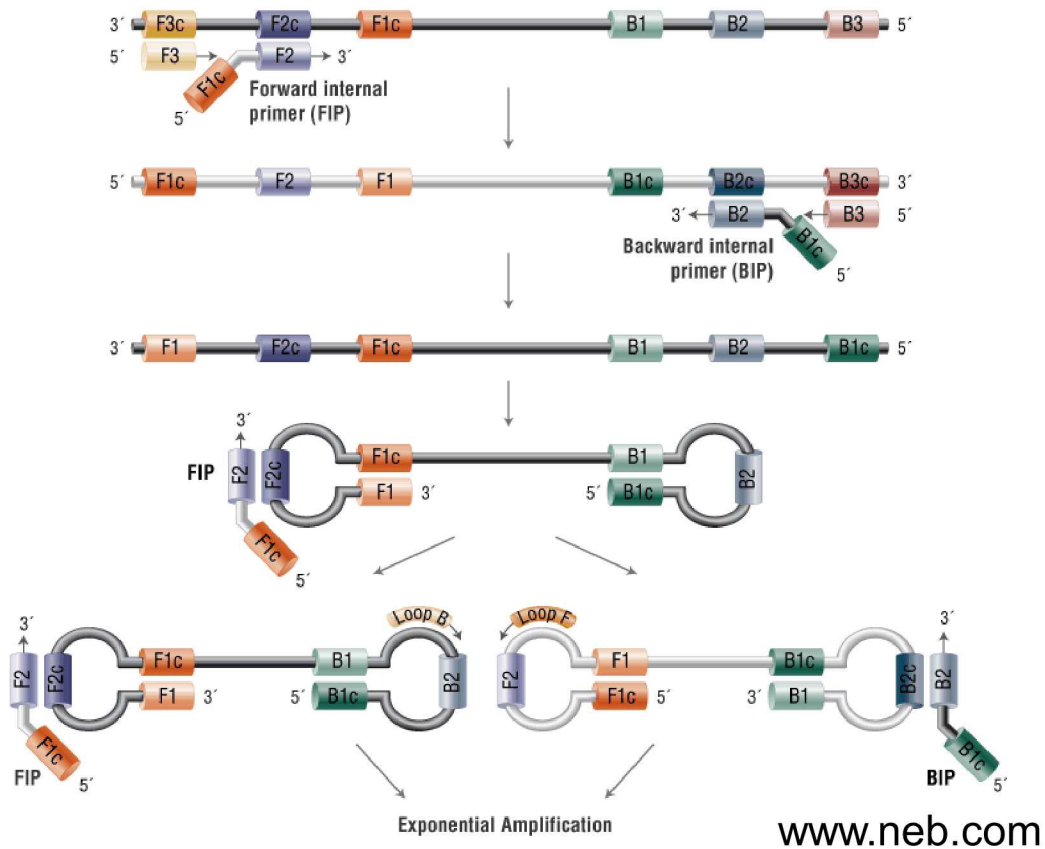
Photo/Sampson Dolo

Sample transport: safety, stability, security, and speed?

MMWR 2014 / Vol. 63 / No. 50 (Liberia / Ebola outbreak)
Photos: Justin Williams, Sampson Dolo

LAMP is a PCR alternative well suited to low resource settings

- Loop Mediated Isothermal Amplification (LAMP): primer-based amplification of DNA/RNA targets
- Fast (5-30 min), robust, simple, sensitive
- Low capital expense/Low power
- Can work with minimal/no sample pretreatment
- Can't easily multiplex
- Most detection techniques are non-specific (turbidity, colorimetric, etc)
- Prone to false positives
- Less quantitative than qPCR
- LAMP is just one of many isothermal amplification schemes (also including NASBA, TMA, NEAR, RPA, HDA, CRISPR-based diagnostics *etc*) that have been proposed for simplified or POC NAATs



Complex reaction scheme of LAMP involves strand displacement instead of thermal denaturation

Why is LAMP prone to false positives?
(exponential amplification in absence of a target)

- Historical perspective
  - The original detection methods were often “open-tube” which inevitably involved a risk of amplicon contamination
  - Newer closed-tube techniques are mostly immune to this problem, but the reputation has stuck!
- Chemistry perspective
  - LAMP involves high concentrations of 6-8 primers, resulting in a high probability of primer-primer interaction
  - LAMP typically involves a high concentration of Mg^{++} further promoting primer-primer interaction
  - *Bst* DNA polymerase and other strand-displacing polymerases used for LAMP have some ability to extend from 3’ mismatches
- Author’s Personal Perspective
  - Investment in PCR >> LAMP (or any other isothermal technique)
  - “crowdsourced” experience with design rules for PCR >> >> >> LAMP *etc*

Analyzing LAMP false-positives

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2004, p. 257–263 Vol. 42, No. 1
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.1.257–263.2004

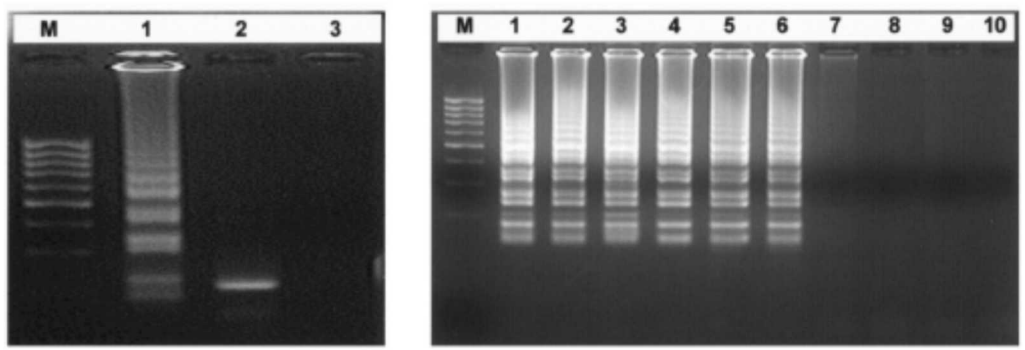
Real-Time Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of West Nile Virus

Manmohan Parida, Guillermo Posadas, Shingo Inoue, Futoshi Hasebe, and Kouichi Morita*

TABLE 1. Details of oligonucleotide primers used for RT-LAMP amplification of E gene of WN virus

Primer name	Type	Length(s)	Genome position[a]	Sequence (5′–3′)
F3	Forward outer	19-mer	1028–1046	TGGATTTGGTTCTCGAAGG
B3	Reverse outer	19-mer	1228–1210	GGTCAGCACGTTTGTCATT
FIP	Forward inner (F1C + TTTT + F2)	46-mer; F1C, 22-mer; F2, 20-mer	F1C, 1121–1100; F2, 1050–1069	TTGGCCGCCTCCATATTCATCATTTTCAGCTGCGTGA CTATCATGT
BIP	Reverse inner (B1C + TTTT + B2)	45-mer (B1C, 22-mer; B2, 19-mer)	B1C, 1144–1165; B2, 1208–1190	TGCTATTTGGCTACCGTCAGCGTTTTTGAGCTTCTCC CATGGTCG
Loop F	Forward loop	19-mer	1093–1075	CATCGATGGTAGGCTTGTC
Loop B	Reverse loop	18-mer	1169–1186	TCTCCACCAAAGCTGCGT

[a] Genome position according to the WN virus strain NY99 (flamingo 382-99) complete genome sequence (GenBank accession number AF196835).



- In our hands, this WNV primer set generates a lot of “false positives” at long incubation times
- Both “true positives” and “false positives” give a ladder-like banding pattern on a gel
- We excised bands and sequenced some of the products of true & false positives

Sequencing LAMP false-positives

True positive amplicon structure (*n* = 9 samples)	
[+LB, -BIP, -inner region, +FIP, -LF]	3/9
[+LF, -FIP, +inner region, +BIP]	4/9
[+LF, -FIP, +inner region, +BIP(partial), +LB, -BIP]	1/9
[+LF, -BIP, -FIP]	1/9

False-positive amplicon structure (*n* = 6 samples)	
[+FIP, -BIP, -LF]	1/6
[+LF, -BIP, -FIP]	3/6
[+ LF, +BIP, -FIP(partial)]	1/6
[+BIP, -LF]	1/6

- True positives (except one) have the “inner region” between the 3’ ends of FIP and BIP
- 7 of 9 true positives have same structure (or reverse complement)
- False positives lack the “inner region” and are comprised of a subset of primers in various configurations
- False positives also have “filler sequence” that doesn’t seem to correspond to primer sequences
  - One of the “true positives” looked like a false positive (?)
- False positives don’t seem to involve the LB primer

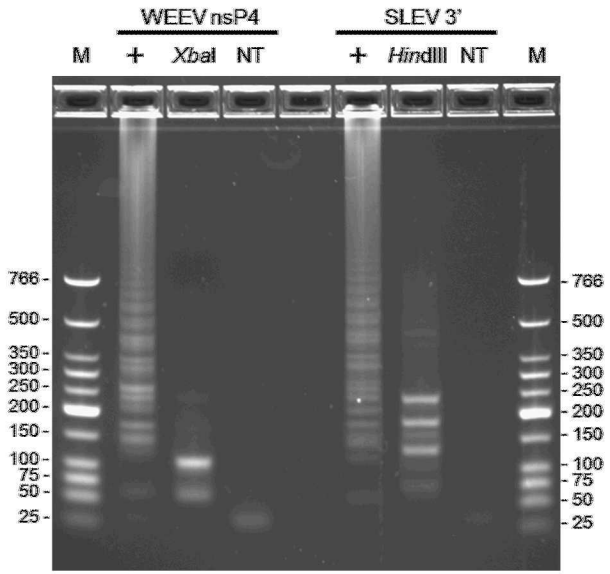
Suppressing LAMP false-positives

- Primer design! (more on this later)
- Tinker with the chemistry
  - Reaction conditions
  - Various additives
  - Other polymerases

  } **Author's Anecdotal Observation**: additives and polymerases that amplify faster, also tend to make false-positives sooner
- Monitor in real-time (non-specific amplification happens late)
- Move away from non-specific detection techniques
  - False-positive amplification may still occur, but if you don't detect them, it's not as much of a problem

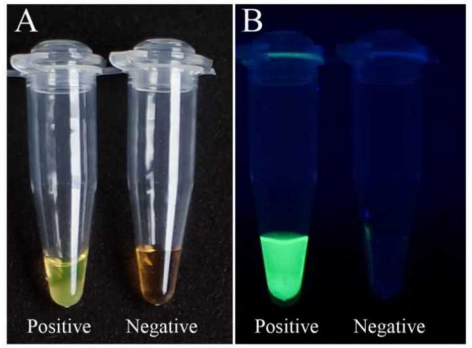
How to know if LAMP worked? (nonspecific)

A. Run product on a gel



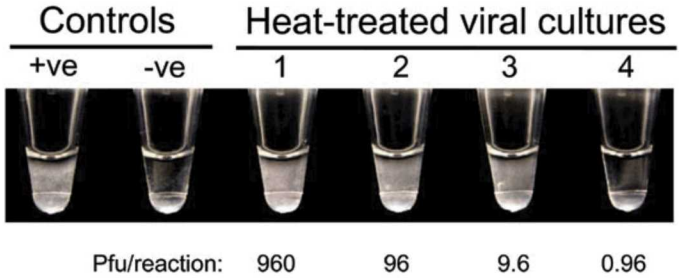
Wheeler *PLoS One* 2016

B. Post-reaction, add SYBR Green



Nie *PLoS One* 2012

C. **Turbidity** (precipitation of Mg^{++} pyrophosphate)



Jayawardena, *Emerg. Inf. Dis.* 2007

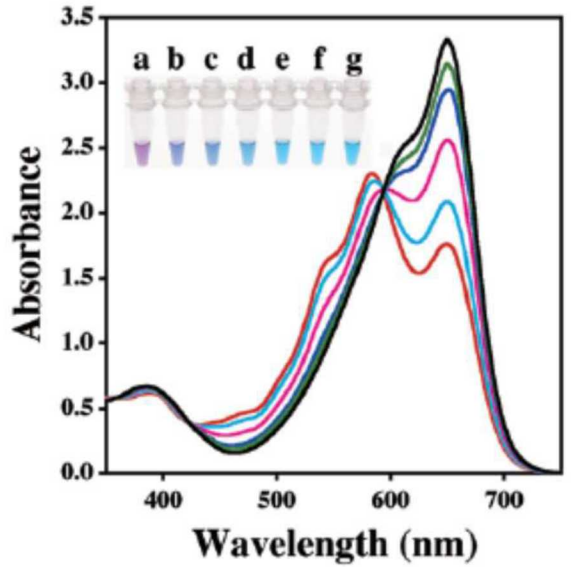
D. Color-change indicators

e.g. hydroxynaphthol blue color change from violet to blue upon complexation of Mg^{++} as reaction proceeds

E. Mn^{++}-quenched calcein

"unquenches" as reaction proceeds (Mn^{++}-PPi forms)

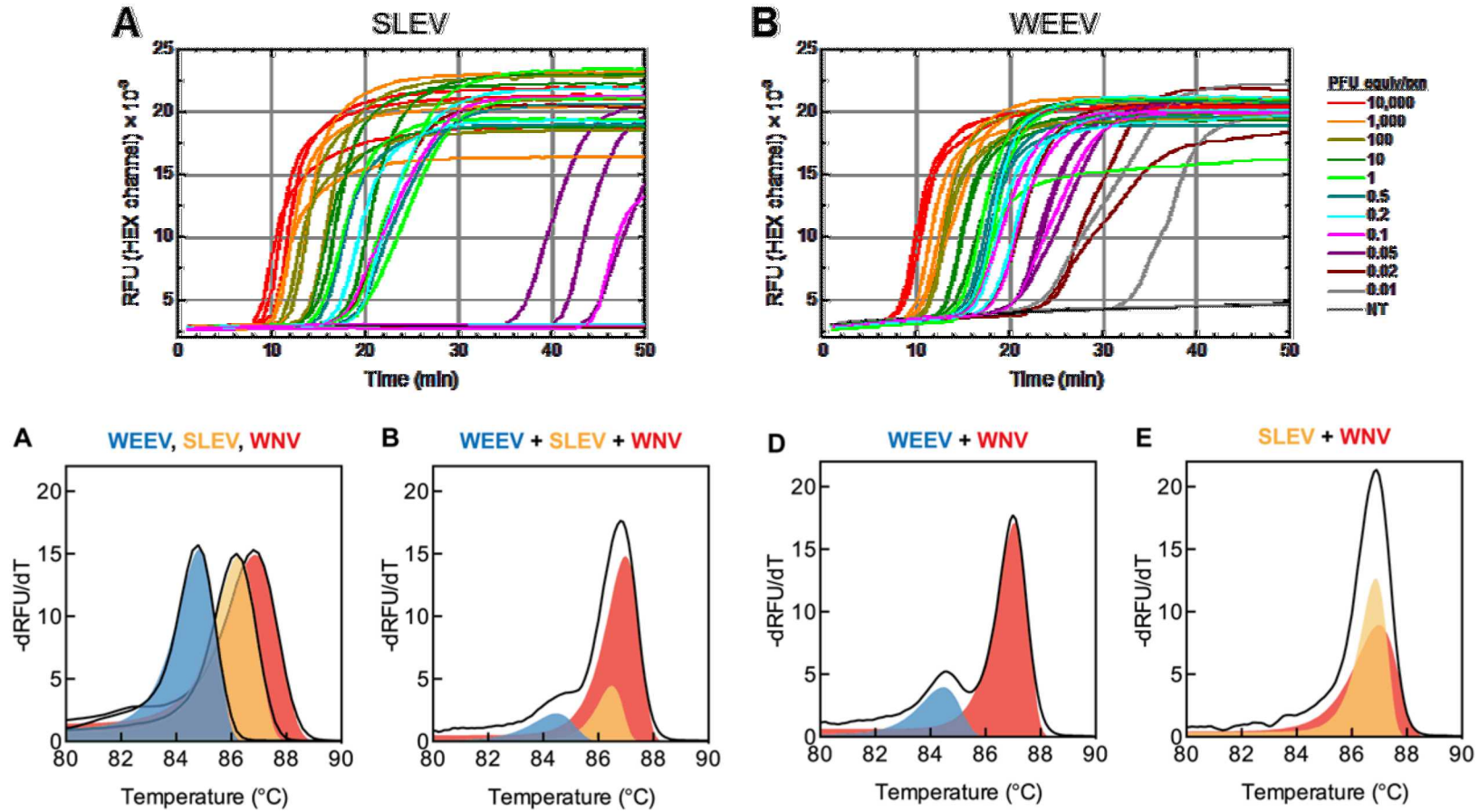
F. pH indicators in weakly buffered LAMP (bright color change as pH drops)



Goto, *Biotechniques* 2009

Real-Time monitoring of RT-LAMP

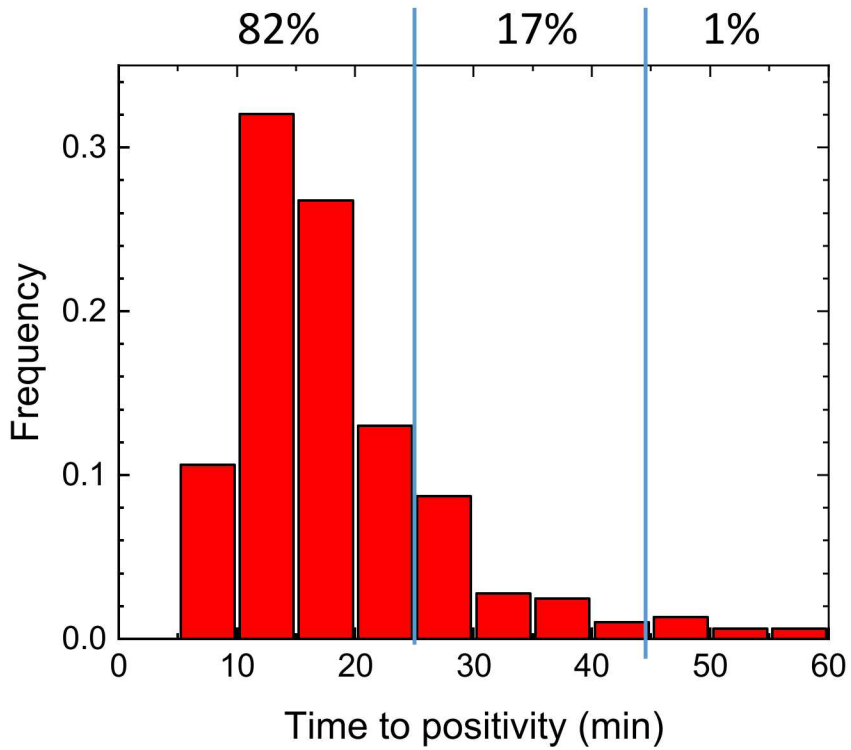
- Real-time monitoring with LAMP-compatible intercalating dye (SYTO 9, SYTO 82, SYTO 62)
- High resolution melt curves (capable of multiplexing, “false positives” usually have different melt characteristics)
- But high-resolution melt curves may not be so field-deployable!



Wheeler *et al*, *PLOS One* 2016

Time-thresholding for true vs false positives

- RJM's non-systematic study of >1400 positive control RT-LAMP assays (varying template concentration)
- Most true positives arise < 45 minutes



- Recent work from Ismagilov group on digital LAMP: Rolando *et al, Anal Chem* 2019
- Background amplification usually happens later

analytical chemistry
Article
Cite This: *Anal. Chem.* 2019, 91, 1034−1042
pubs.acs.org/ac

Real-Time, Digital LAMP with Commercial Microfluidic Chips Reveals the Interplay of Efficiency, Speed, and Background Amplification as a Function of Reaction Temperature and Time

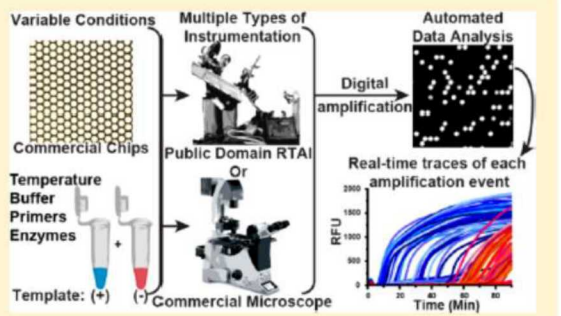
Justin C. Rolando,[†] Erik Jue,[‡] Nathan G. Schoepp,[†] and Rustem F. Ismagilov*[,†,‡]

[†]Division of Chemistry & Chemical Engineering, California Institute of Technology, 1200 East California Boulevard, Mail Code 210-41, Pasadena, California, 91125, United States

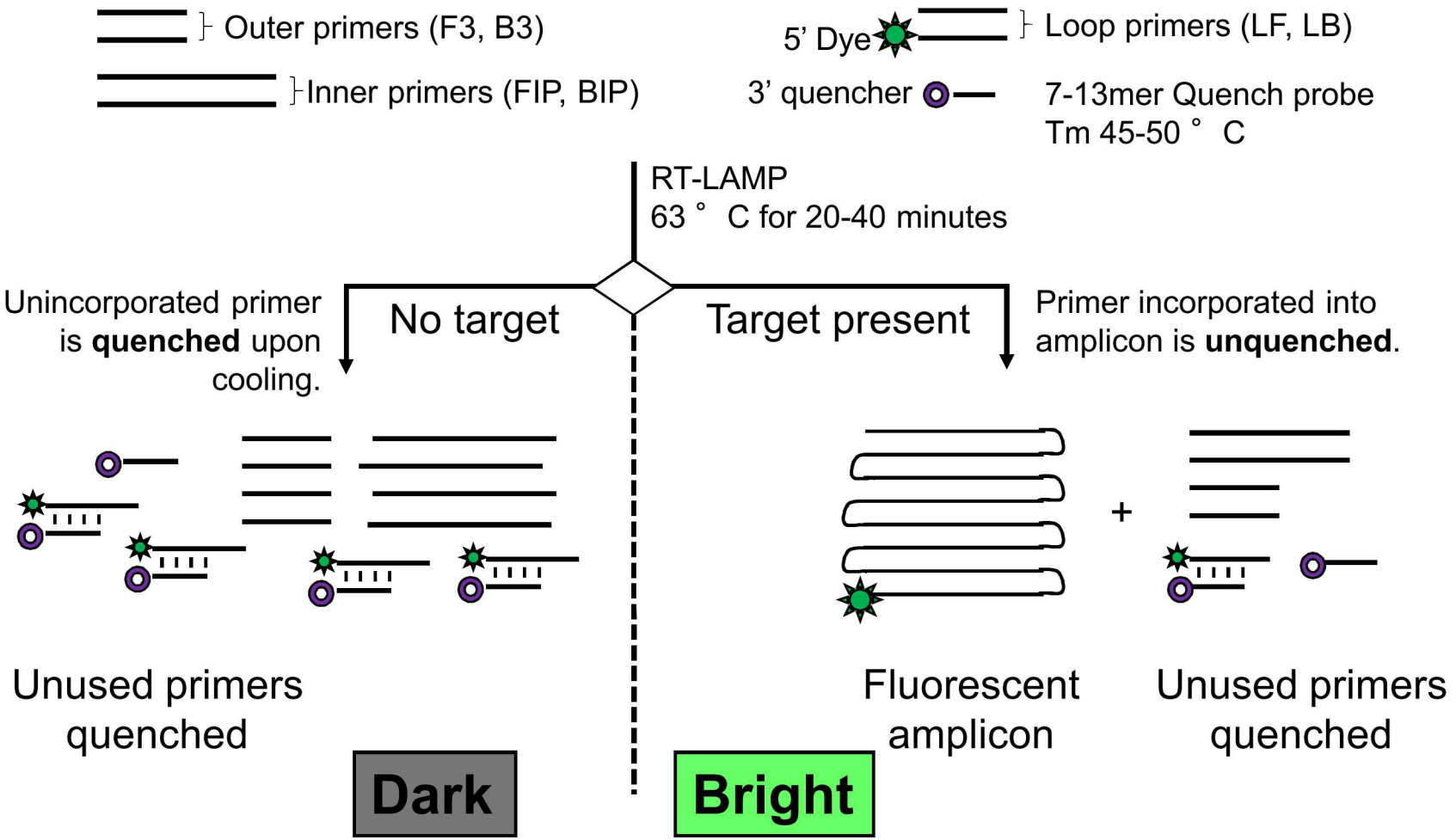
[‡]Division of Biology & Biological Engineering, California Institute of Technology, 1200 East California Boulevard, Mail Code 210-41, Pasadena, California 91125 United States

Supporting Information

ABSTRACT: Real-time, isothermal, digital nucleic acid amplification is emerging as an attractive approach for a multitude of applications including diagnostics, mechanistic studies, and assay optimization. Unfortunately, there is no commercially available and affordable real-time, digital instrument validated for isothermal amplification; thus, most researchers have not been able to apply digital, real-time approaches to isothermal amplification. Here, we generate an approach to real-time digital loop-mediated isothermal amplification (LAMP) using commercially available microfluidic chips and reagents and open-source components. We demonstrate this approach by testing variables that influence LAMP reaction speed and the probability of detection. By analyzing the interplay of

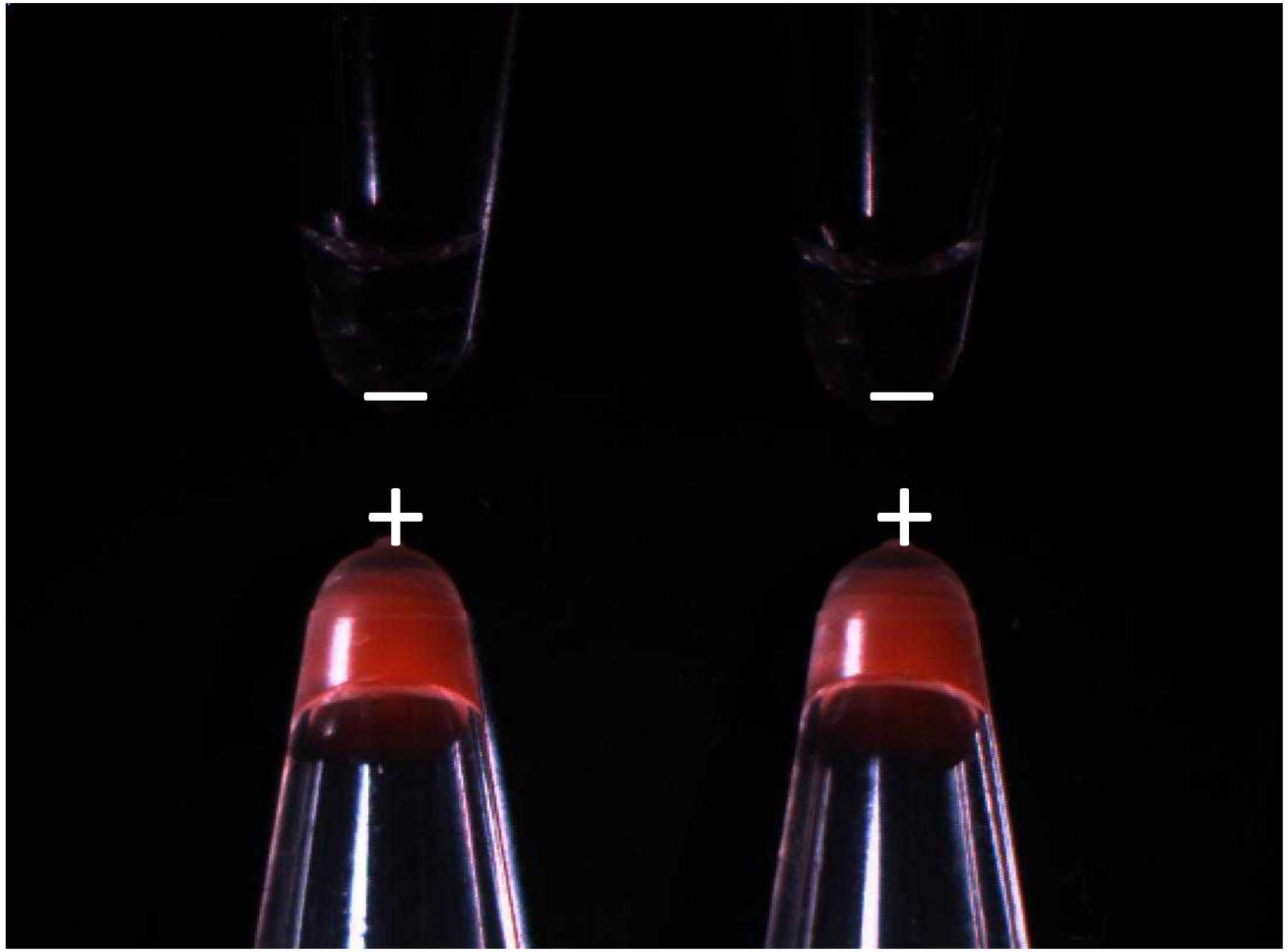


QUASR: Quenching of Unincorporated Amplification Signal Reporters



Ball *et al*, *Analytical Chemistry* 2016

WNV QUASR assay (ROX label)



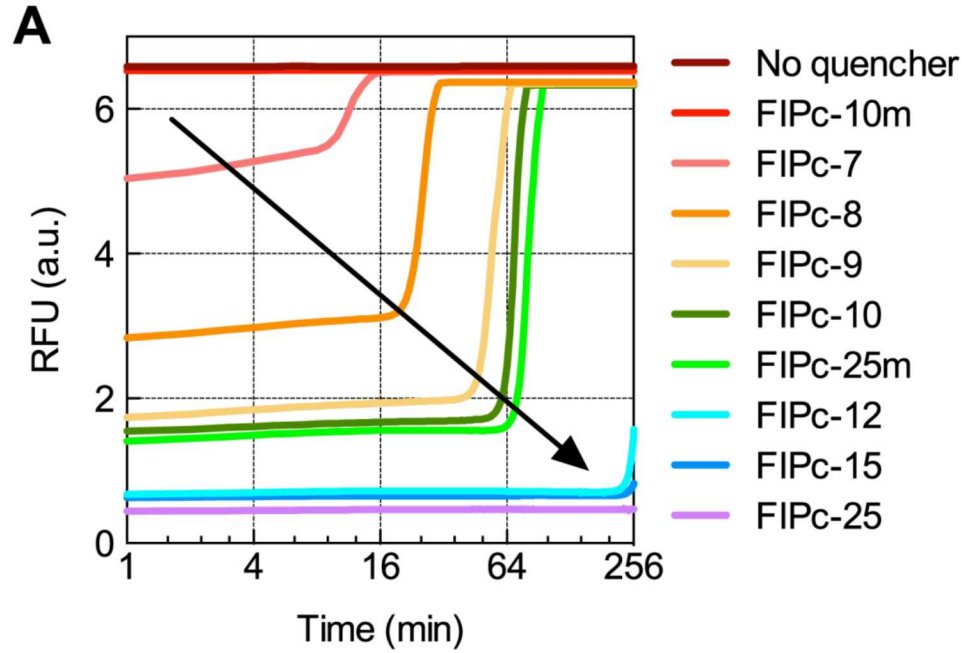
Published WNV RT-LAMP primer set from Parida *et al* adapted to QUASR detection

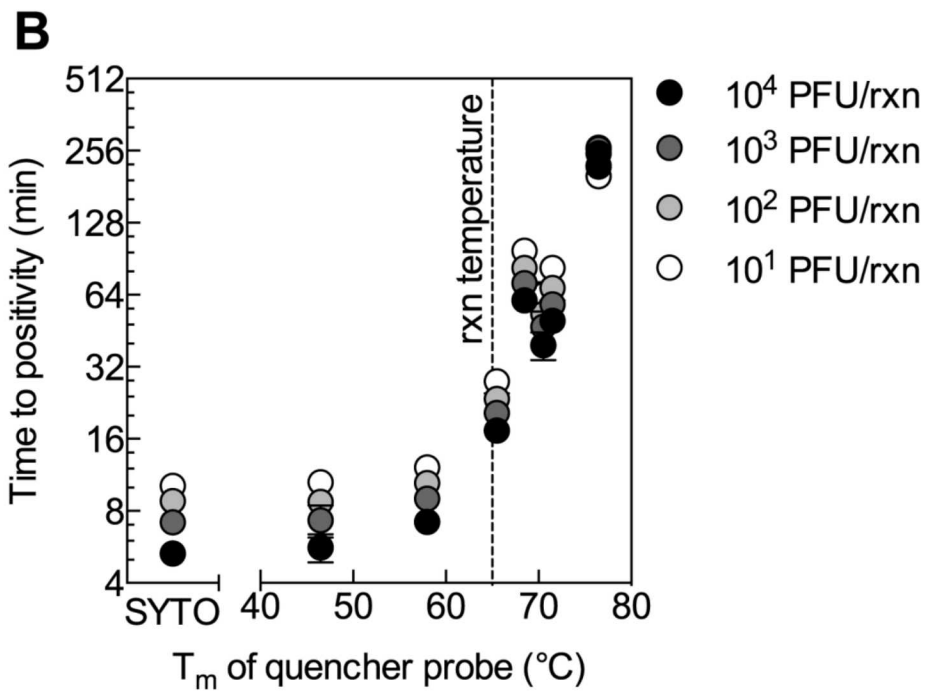
Color photo taken with green LED flashlight and magenta theatre lighting gel as a filter

- ☑ **Closed-tube detection**
- ☑ **Bright endpoint signal**
- ☑ **Large difference between positive and negative**

Optimized QUASR quenchers results in no inhibition

- As T_m of quencher approaches reaction temperature, reaction is inhibited
- If probe T_m is > 50-55 ° C, we can observe probe being displaced in real time, similar to Tanner *et al* "DARQ" *Biotechniques* 2012 but reaction is slower
- Constraints on lower limit for T_m:
  - Ambient temperature for performing detection
  - Temperature at which labeled primer forms a stable hairpin structure

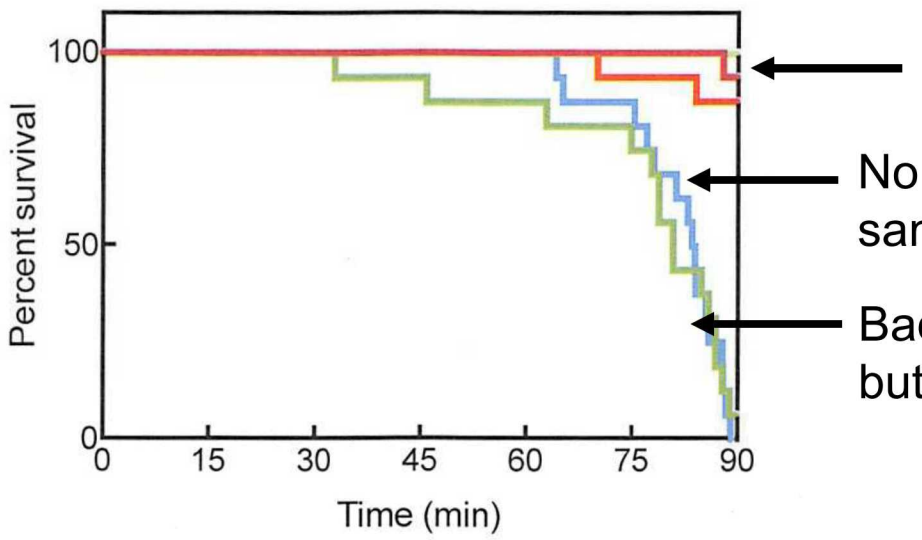




Ball *et al*, *Analytical Chemistry* 2016

QUASR can suppress detection of LAMP "false positive" amplification

"Survival" of WNV no-template controls
(time to appearance of non-specific SYTO 62 signal)



FIP-ROX/FIPc-7+3 mm IBRQ, with SYTO 62
FIP-ROX/FIPc-10+1 internal mm IBRQ, with SYTO 62
LB-Cy3/LBc-12 IBFQ, with SYTO 62 –
LB-ROX/LBc-12 IBRQ, with SYTO 62
LB-ROX/LBc-11 IBRQ, with SYTO 62
SYTO 62 only, no QUIP

LB was absent from "false positives"

C. Ball, *Analytical Chemistry* 2016

Good QUASR: Spontaneous amplification is suppressed; and not evident in endpoint signal

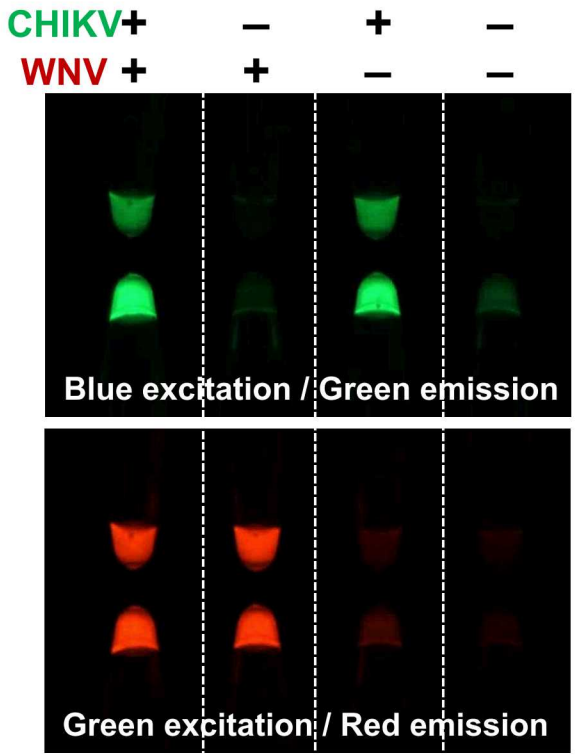
No QUASR: Spontaneous amplification in all samples, and visible with non-specific DNA dye

Bad QUASR? Spontaneous amplification happens, but is not evident in endpoint probe signal (still ok)

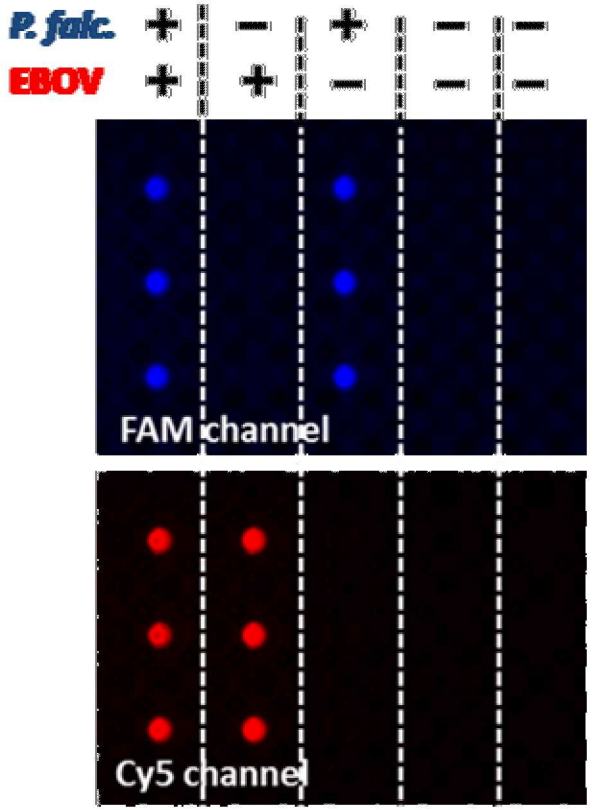
- "False positive" amplification usually occurs >40 minutes, so we define a cutoff of 30-40 minutes
- Even if a sample shows false positive with the SYTO dye, the QUASR signal is usually still negative
- *e.g.* 1/197 FP for QUASR, *vs* 67/145 FP for SYTO, in 90-minute extended rxns)
- The choice of dye might matter?
  - LB-Cy3 bad / LB-ROX good?

Multiplexing RT-LAMP with QUASR

(A) Chikungunya virus + West Nile virus



(B) *Plasmodium falciparum* + Ebola virus



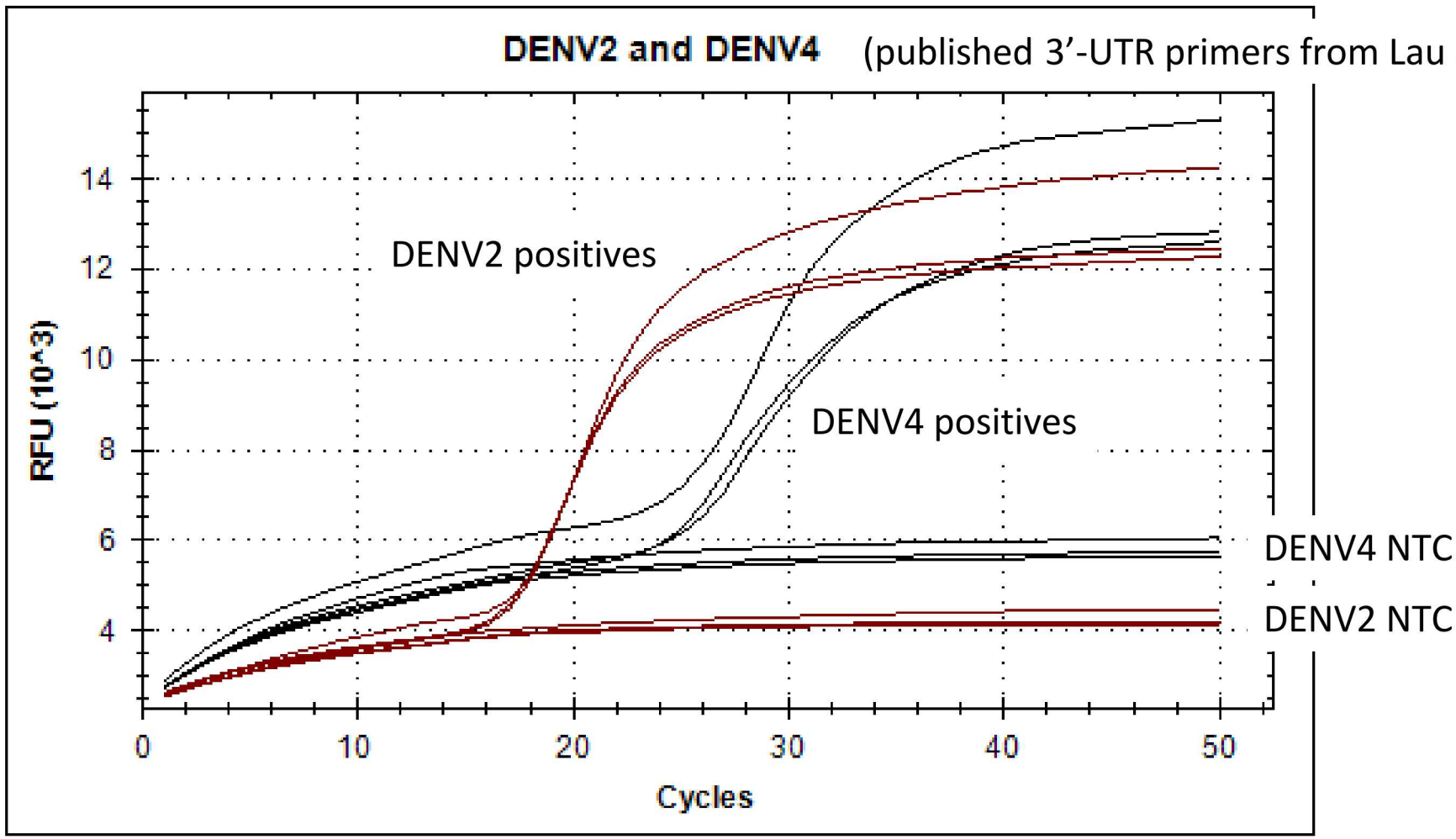
Yooli Light

☑ **Target specific** ☑ **Muliplexable**

LAMP historically was hard to multiplex

LAMP Background Issue #2: Rising Baselines

- Non-exponential DNA synthesis occurring independently of the LAMP reaction?
- Not discussed in most published LAMP assays because endpoint techniques only are used.



Multiple primer analyzer indicates dimers involving 3’ end of FIP

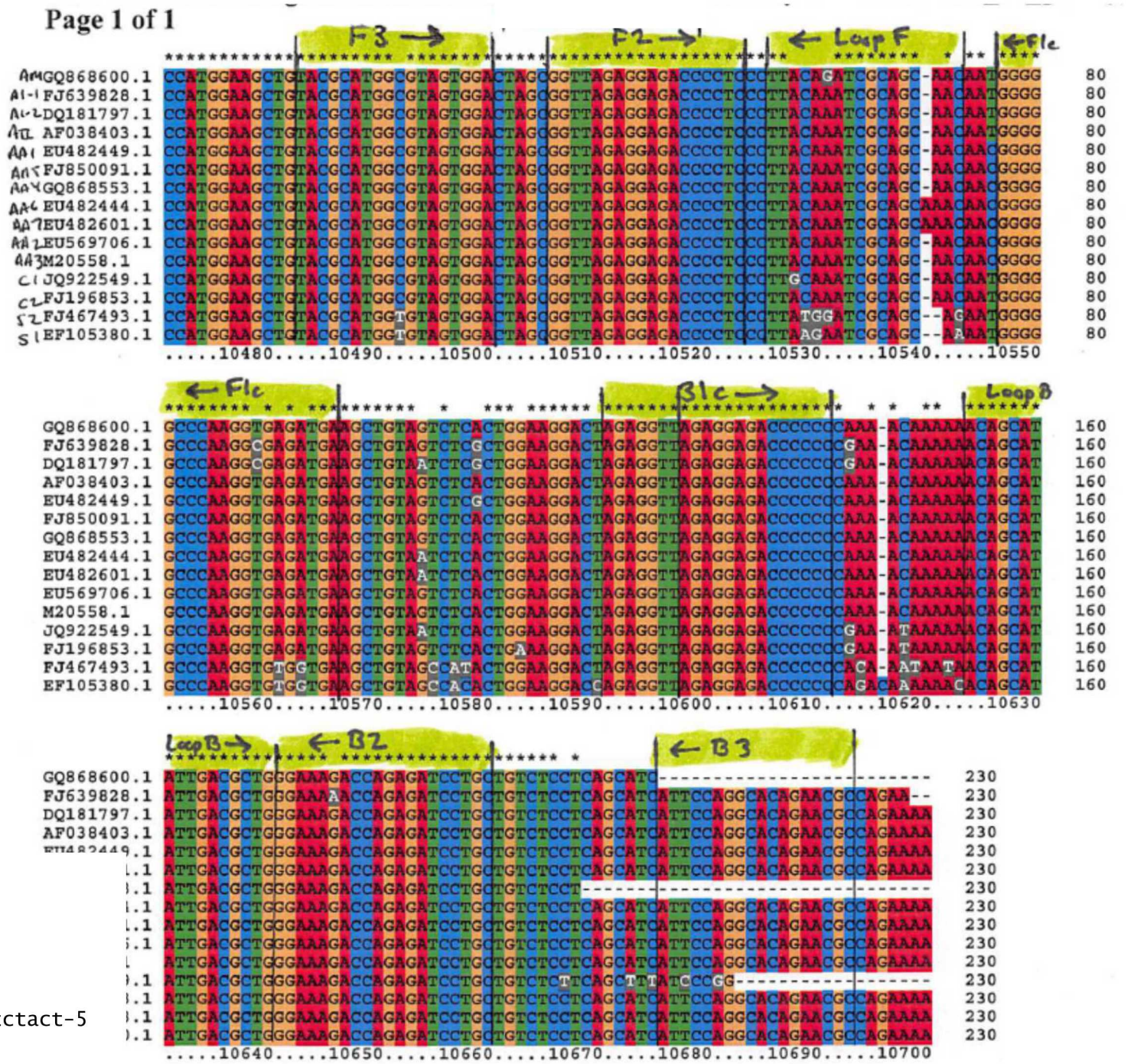
Primer dimers identified for DENV-2 (primers for DENV-4 are very similar)

```
 Self-Dimers:

1 dimer for: FIP
5-tcatctcaccttgggccccccggttagaggagacccctc->
                            |||| | | ||||
                          <-ctccccagaggagattggccccccgggttccactctact-5


               Cross Primer Dimers:

FIP with BIP
FIP
5-agaggttagaggagacccccccgcaggatctctggtctttcc->
          |||| | | || |  |
        <-ctccccagaggagattggccccccgggttccactctact-5
```

Would you expect this to be stable enough to extend at 63 °C?

Consult sequence alignment for target region

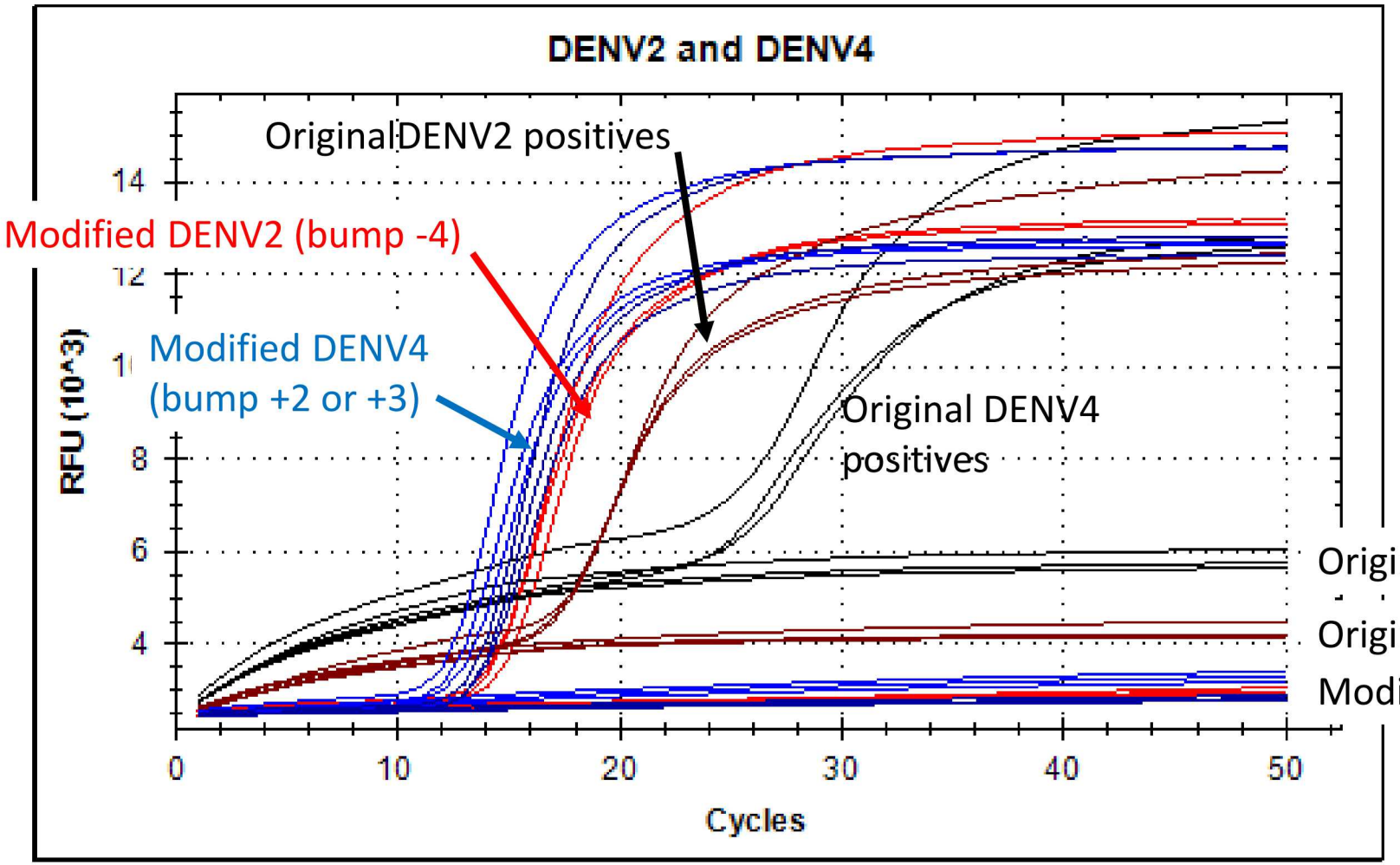
- Sequence alignment showing representatives of 15 DENV-2 genotypes
- There is limited room to “adjust” F2 without having to adjust other primers
- In this case: all adjustments to F2 are allowed by the sequence alignment



- “Bump” F2 4 bases to left

```
5-tcatctcaccttgggccccctagcggttagaggagacc->
              || | || ||| | | ||| || | ||
           <-ccagaggagattggcgatccccccgggttccactctact-5
versus
5-tcatctcaccttgggccccccggttagaggagacccctc->
                          |||| | | ||||
                      <-ctccccagaggagattggccccccgggttccactctact-5
```

Bumping F2 reduces background and speeds amplification in both cases



Thermodynamic basis for rising baseline?

- nearest-neighbor model for primer hybridization

$$\Delta G^{o}_{hyb} = \Delta G^{o}_{initiation} + \sum_{i,j=1}^{4} n_{i,j} \Delta G \left(\frac{N_i N_j}{N_i^{'} N_j^{'}} \right)$$

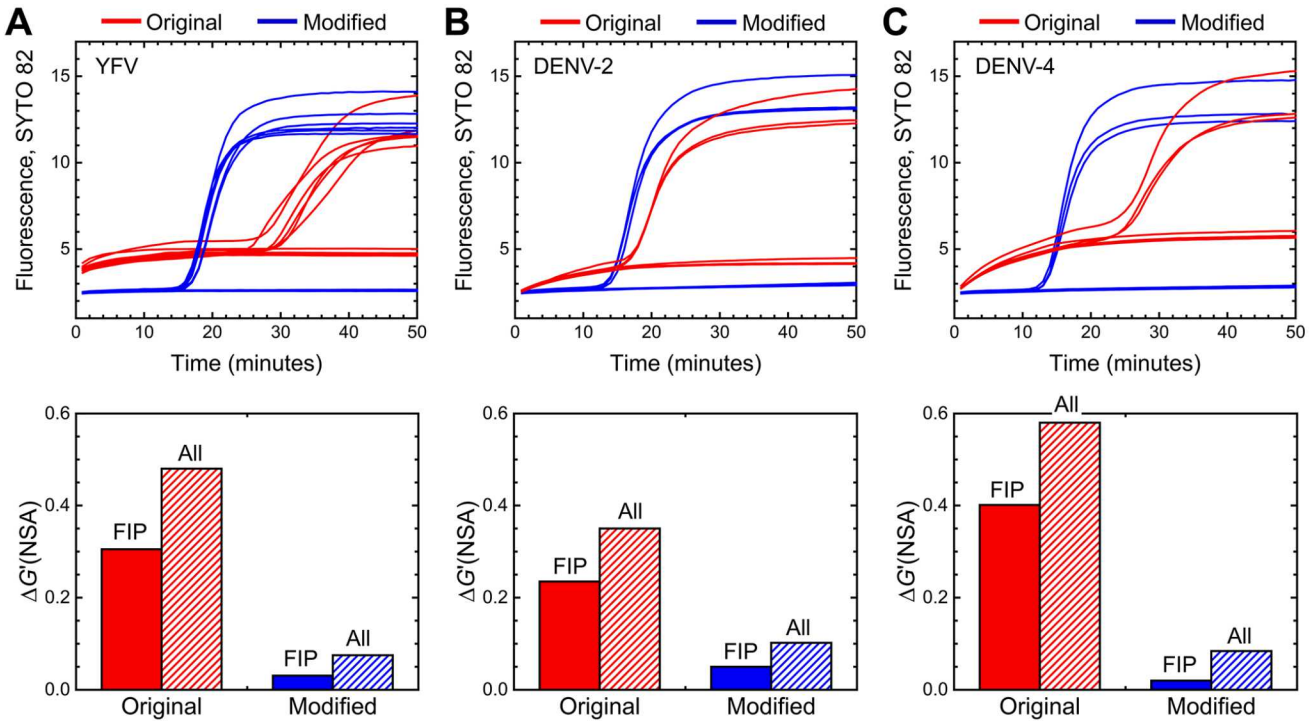
- Normalized $\Delta G'$ for all possible primer-primer interactions

$$\Delta G^{'}(NSA) = \left(\frac{1}{\Delta G^{perfect-binding}_{hyb}} \right) \left(\sum_{Hairpins} \Delta G_{hyb} + \sum_{Dimers} \Delta G_{hyb} \right)$$

- Probability of non-specific amplification is related to $\Delta G'$ of primer interactions

$$\ln(P_{NSA}) \propto \Delta G^{'}(NSA)$$

Small adjustments to primers to minimize $\Delta G'$(NSA) eliminate baseline rise and improve rate of reaction

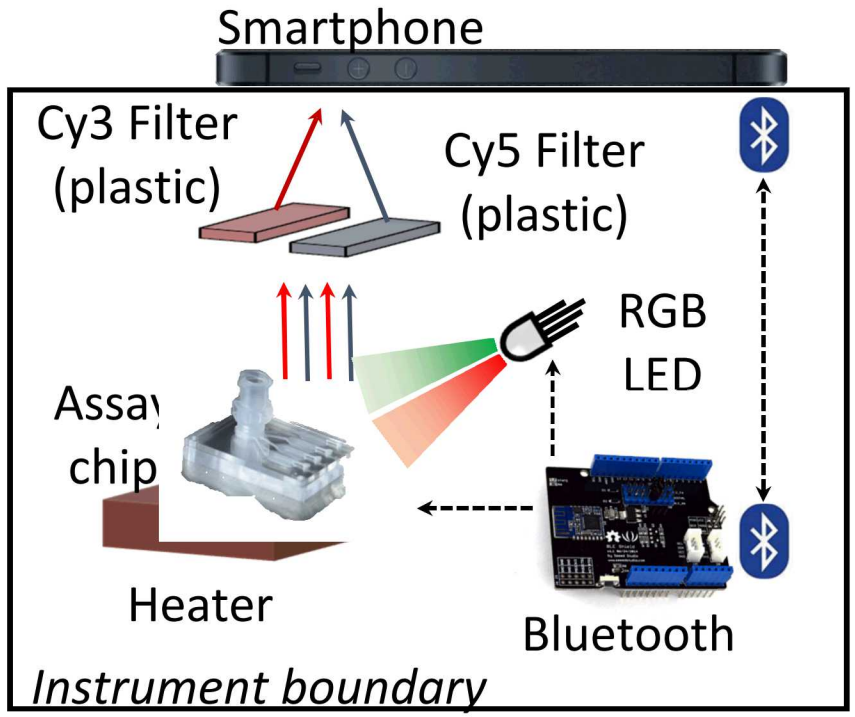


Meagher *et al*, *Analyst* 2018

- Anecdotally, $\Delta G'$(NSA) may also be predictive of primer sets prone to "false positives"

Smartphone instrument for QUASR LAMP

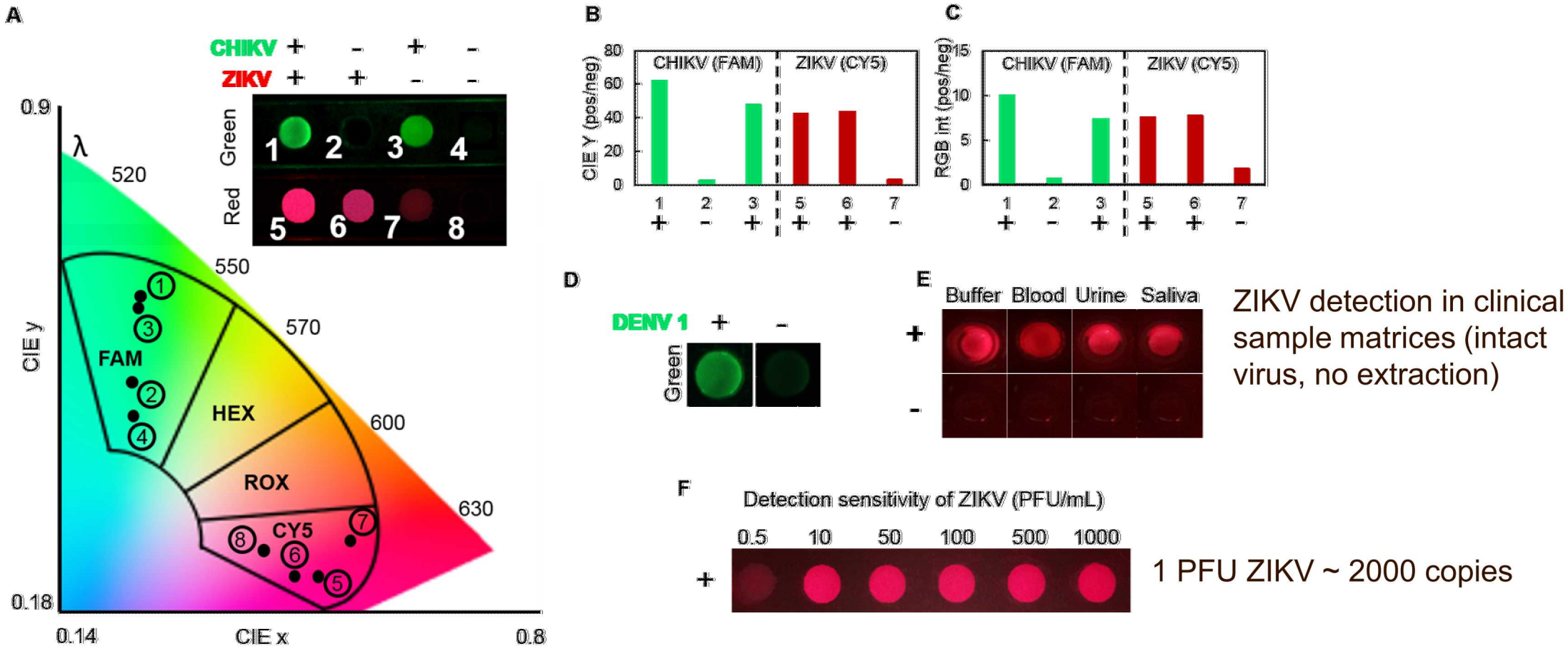
- Handheld box contains heater, optics, and Bluetooth enabled microcontroller
- Compatible with iPhone and Android phones
- Smart phone app “front end” controls heater, timing, fluorescence image acquisition, and scoring
- Real-time or endpoint measurements
- Accepts inexpensive consumables (PCR tubes, microwells)
- Raw material costs: about $50 with plastic filters; $500 with high quality coated glass filters, plus phone



Priye *et al*, *Scientific Reports* 2017

Smartphone assay detects CHIKV/ZIKV duplex and pan-DENV assays

Phone app maps multicolor fluorescence images onto chromaticity-luminance (CIE xyY) color space to allow automated assay scoring with high signal-to-background



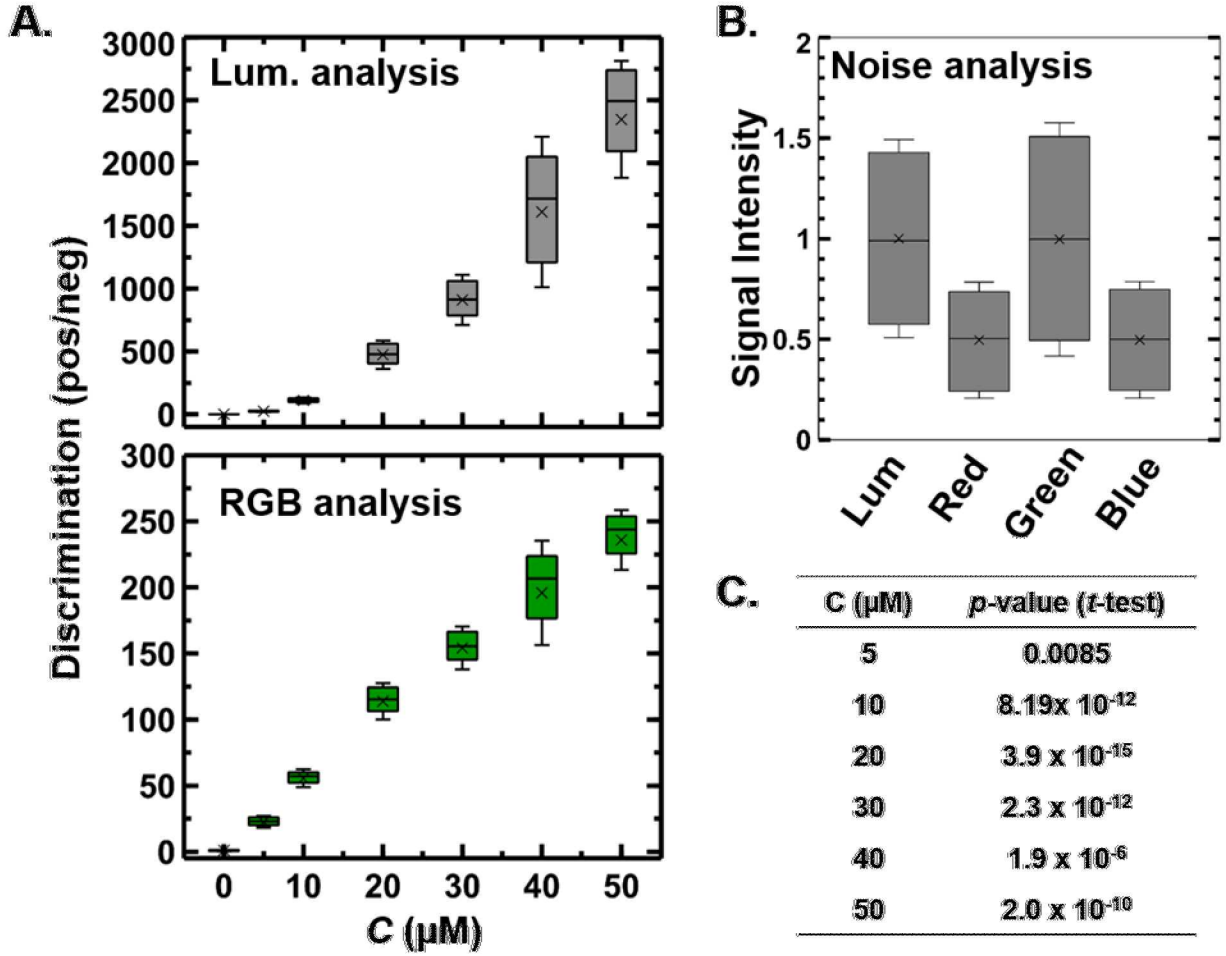
ZIKV detection in clinical sample matrices (intact virus, no extraction)

1 PFU ZIKV ~ 2000 copies

Priye *et al*, *Scientific Reports* 2017

Chromaticity transformation improves LAMP signal-to-noise with smartphone

Comparison of signal strength and noise for QUASR images indicates luminance enhances positive/negative discrimination

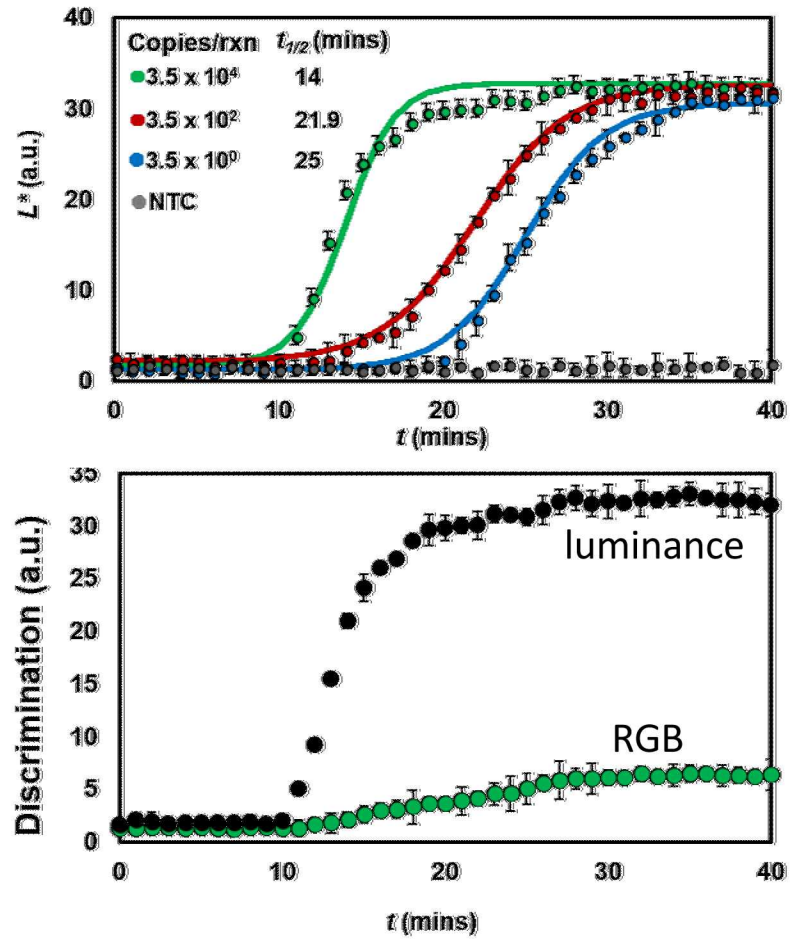


C.

C (μM)	*p*-value (*t*-test)
5	0.0085
10	8.19×10^{-12}
20	3.9×10^{-15}
30	2.3×10^{-12}
40	1.9×10^{-6}
50	2.0×10^{-10}

Priye *et al*, *Anal Chem* 2018

Real-time LAMP on smart phone (*N. ghonorrhea* DNA)



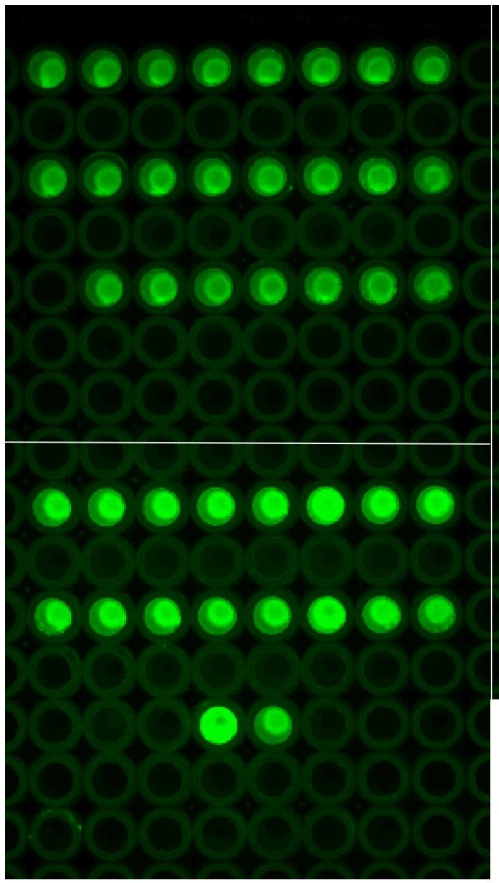
Sample prep is a major challenge (or opportunity for simplification)

- For PCR, usually sample prep = DNA/RNA extraction
  - Serves to release DNA/RNA from the confines of cell or virus particles, remove inhibitory substances, and (in some cases) concentrate DNA/RNA
  - Usually some variation upon lyse / bind / wash / elute
  - Losses of DNA/RNA can occur at each of these steps!
  - Silica or charge-based; spin columns, vacuum columns/plates, magnetic beads
  - Usually requires multiple reagent additions
  - Can be a major source of variabilty, particularly when performed ”manually”
- Several demonstrations of adapting the lyse / bind / wash / elute paradigm to easier, field-deployable format

Sample prep without bind/wash/elute

- Boiling or detergent lysis
- Or no sample prep at all (dilution)
- Many isothermal amplification techniques, and even PCR with novel variants of enzymes, can tolerate inhibitory substances such as blood
  - *Bst* DNA polymerase used in LAMP is known for its tolerance of crude samples
- Many pathogens spontaneously “lyse” at temperature of LAMP, meaning a separate lysis step is not always required.
- We have tried to leverage these characteristics to try to “eliminate” sample prep
  - Tradeoff is simplicity, *vs.* limited sample input (typically max 10% of sample matrix is tolerated, with some reduced performance), and no concentration of DNA/RNA
  - Will not be applicable to the most dilute pathogens!

“No Sample Prep” for *P. falciparum* parasites in blood



Blood diluted 1:20 in RBC lysis buffer

Parasite concentration in blood

10^3/µL

10^2

10^1

1

10^3 /µL

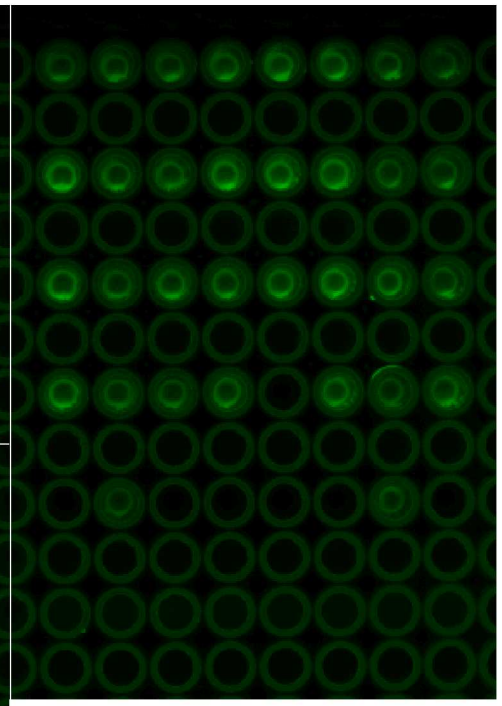
10^2

10^1

1

4 µL lysis buffer per 10 µL reaction (total of 0.2 µL blood per 10 µL reaction) Positive/Negative signal ratio ~ 10 ± 1

2 µL lysis buffer per 10 µL reaction (total of 0.1 µL blood per 10 µL reaction) Positive/Negative signal ratio ~ 11 ± 3



No Treatment

Parasite concentration in blood

10^4/µl

10^3

10^2

10^1

1

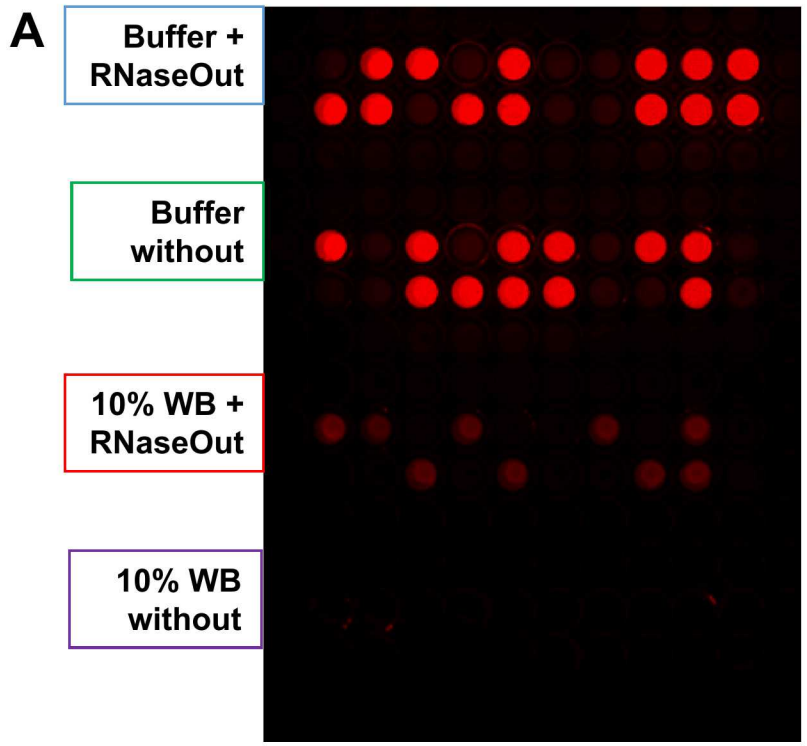
NTC (lysis buffer)

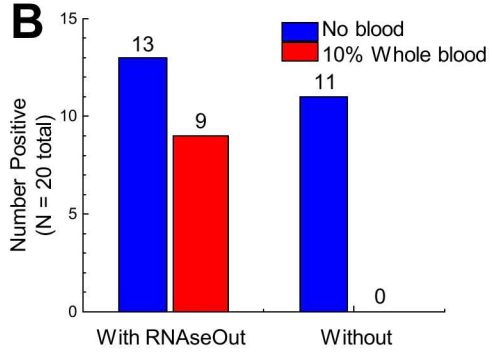
1 µL whole blood per 10 µL reaction

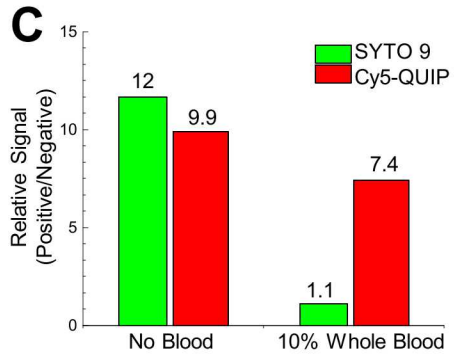
Positive/Negative signal ratio ~ 7 ± 1

No Treatment: 1ul of whole blood sample
RBC lysis buffer : 1:20 diluted, 2 or 4ul added to rxn (so sampling 0.1 or 0.2 uL per reaction)
BIP-Cy3, 500ms exposure

Addition of RNAse Inhibitor enables detection of Ebola RNA from whole blood



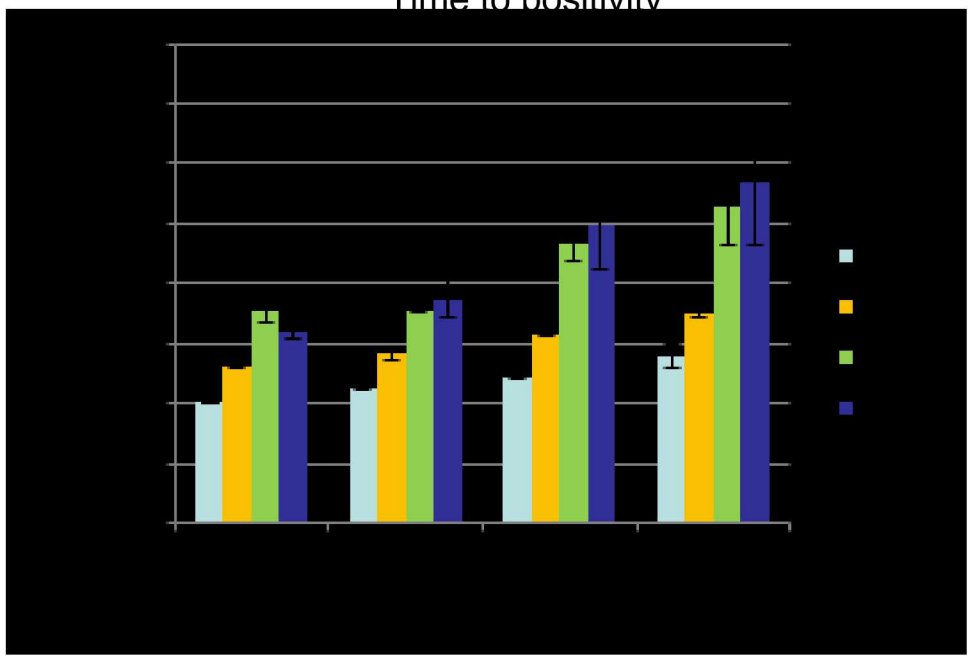




Zika RT-LAMP in clinical matrices

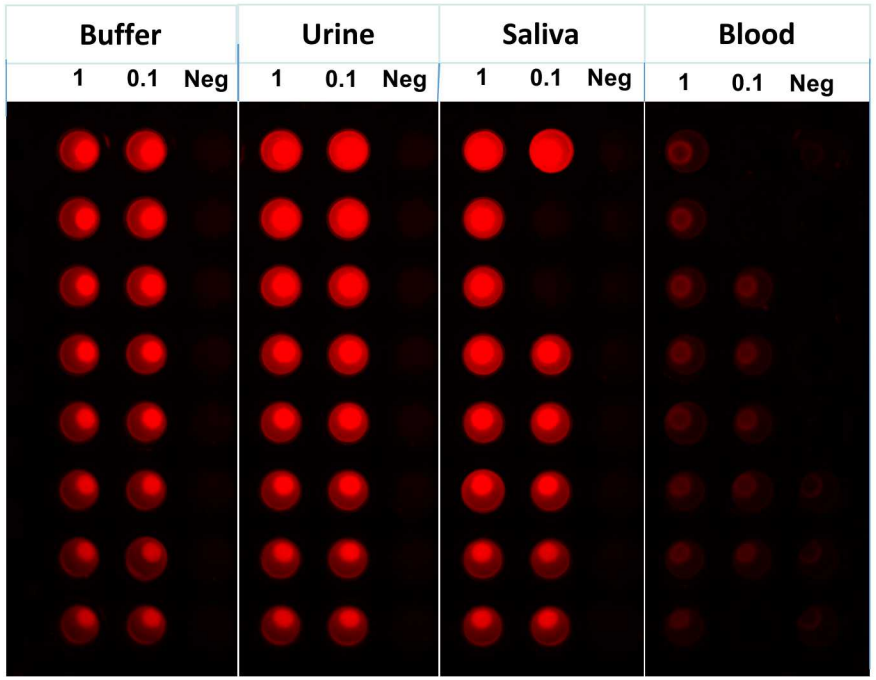
- Intact Zika virus spiked directly into human blood, saliva, or urine
- Spiked samples added (1/10 dilution) into dry LAMP mix (no lysis or RNA extraction)
- Performance in urine most similar to buffer.
- Reactions slow down somewhat in saliva and blood, slight dropoff in rate of positive detection at 0.1 PFU

Time to positivity



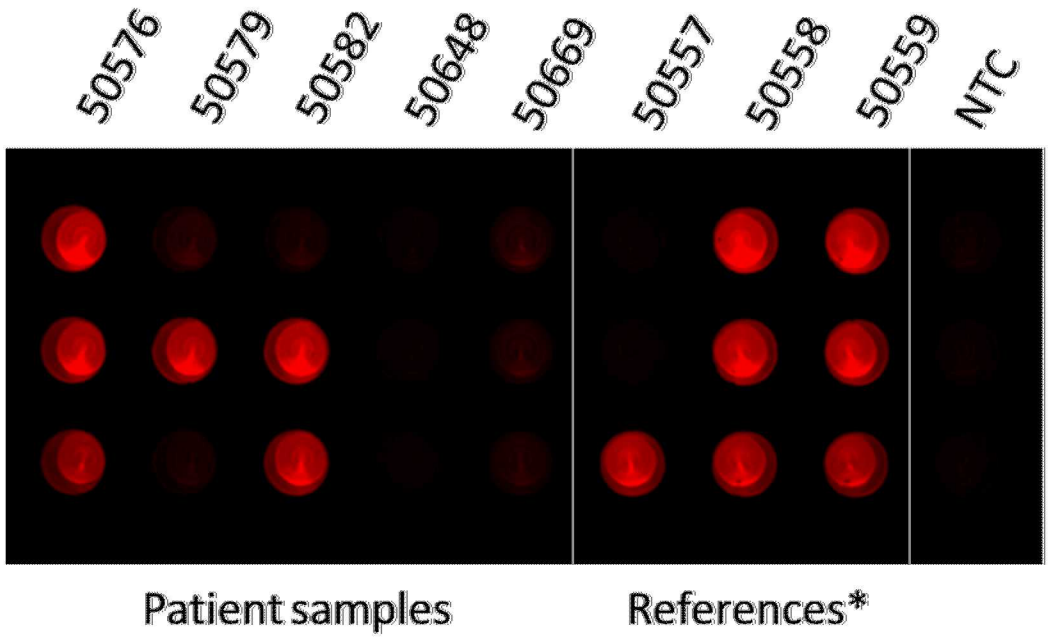
Priye *et al*, *Scientific Reports* 2017

QUASR End-point

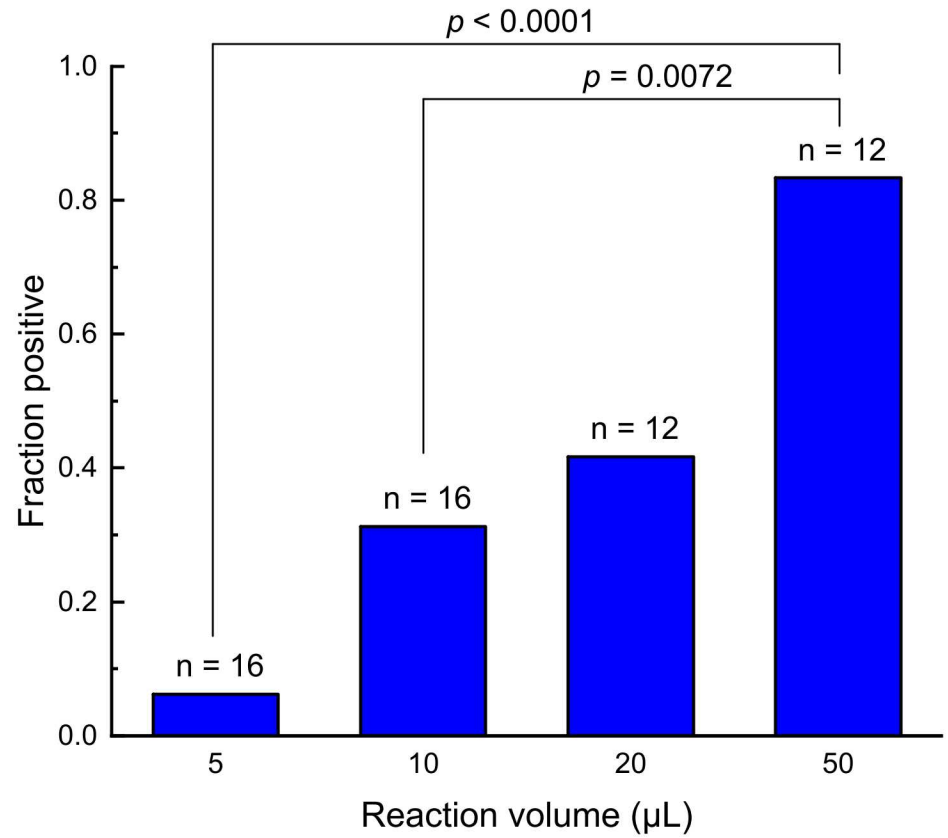


When target concentration is low, scale up reactions.

A. ZIKV patient sera analyzed by QUASR. Only one positive when using 10 µL reactions (1 µL serum), but better success using 50 µL reactions (5 µL serum per reaction)



B. Scaling QUASR reaction volume at constant *ratio* of sample to total volume increases probability of detection (target = WNV)



Conclusions & Future Work

- “False positives” and background amplification are an issue with LAMP
  - Potential to mitigate through improved assay chemistry and better understanding of LAMP chemistry and primer design
- QUASR modification to LAMP helps satisfy requirements for a point-of-care diagnostic
  - Many published LAMP primer sets can be improved with simple changes
- Simplified approaches to sample prep and instrumentation combined with improvements to chemistry may enable LAMP to move to a field-deployable technique
- LAMP is one of several isothermal techniques with desirable characteristics
- Meanwhile the limitations of qPCR continue to be addressed, and untargeted approaches including sequencing continue to become easier too.

Contact: Robert Meagher, rmeaghe@sandia.gov