

Molecular detection of emerging pathogens in low-resource settings

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

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Challenges in Deployed Diagnostics



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



Photo/Justin Williams

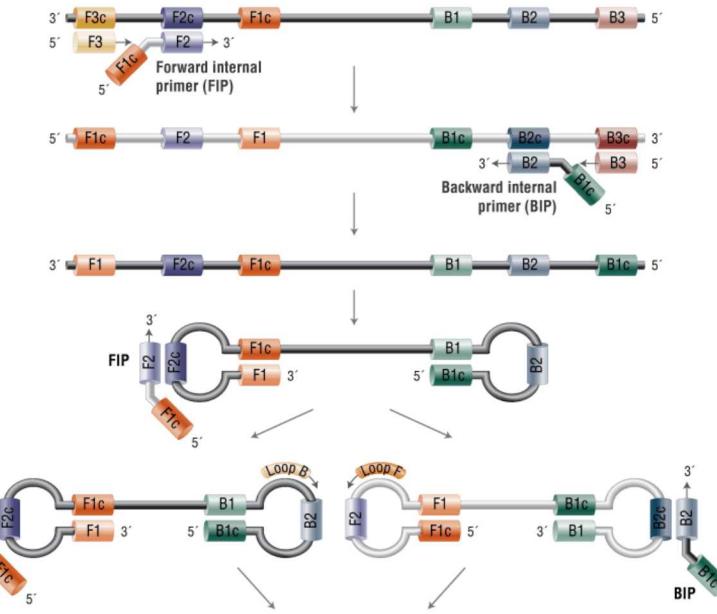
MMWR 2014 / Vol. 63 / No. 50 (Liberia / Ebola outbreak)

Photos: Justin Williams, Sampson Dolo

- 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.
- Can we make a *simple, self-contained* diagnostic assay for use at the site of sample collection?
- Utilize smart phone capabilities for assay control, scoring, data reporting
- Need robust **assay chemistry**, cheap **consumables**, and simple **instrumentation**.

LAMP is a PCR alternative well suited to low resource settings

- Loop Mediated Isothermal Amplification (LAMP): primer-based amplification of DNA/RNA targets
- Fast (5-20 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, etc) that have been proposed for simplified or POC NAATs



www.neb.com

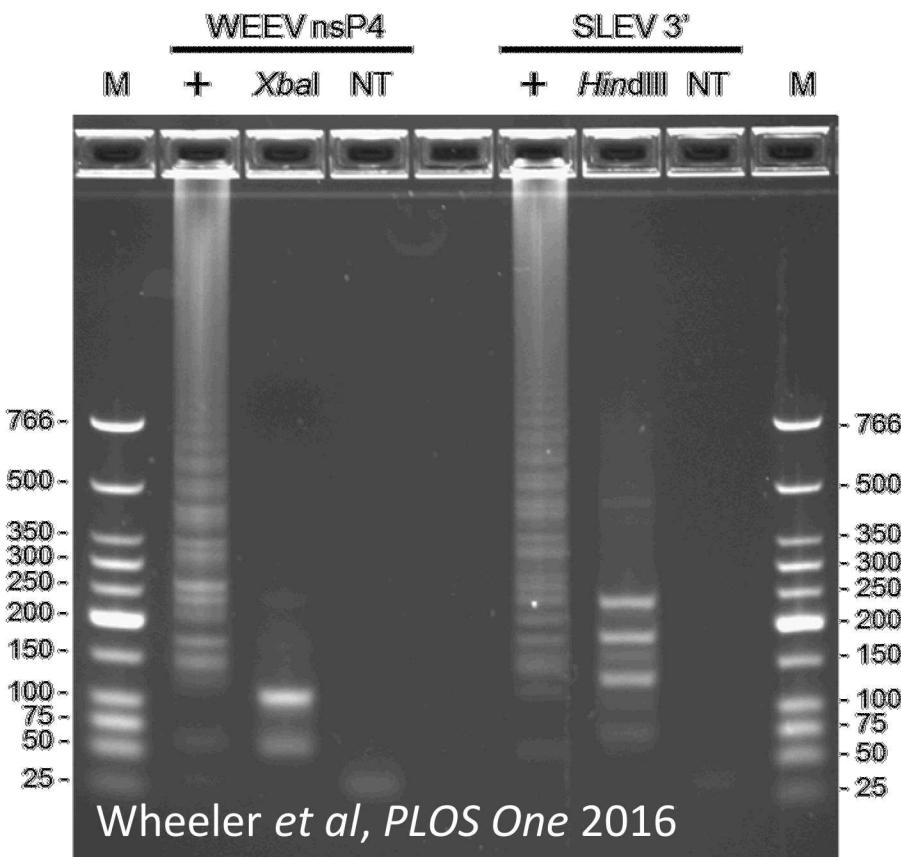
Complex reaction scheme involves strand displacement instead of thermal denaturation

For a deployed LAMP diagnostic:

- *Closed tube* detection – don't want to open the tube after the reaction
- *Large discrimination* between positive and negative samples
- *Bright signals* – naked eye or simple detector
- *Target-specific*, vs detecting total DNA
- *Endpoint* is good enough for yes/no answer (LAMP is semi-quantitative at best anyway!)
- Minimize complex instrumentation or operations

How to know if LAMP worked? (old school)

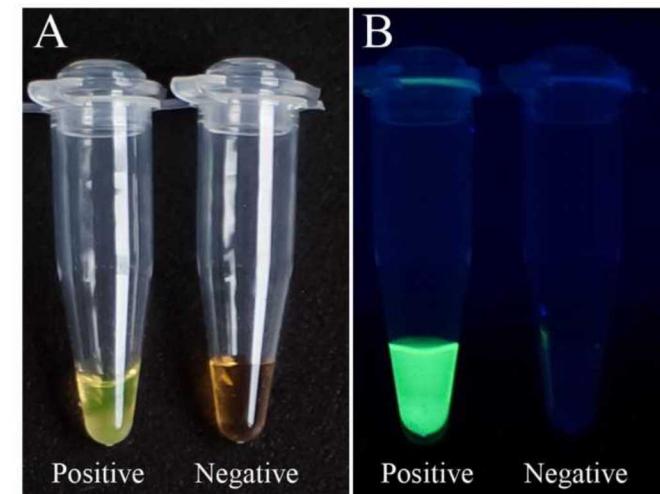
A. Run product on a gel, with optional target-specific restriction digest



B. Turbidity (precipitation of Mg pyrophosphate, from making a ton of DNA)

Jayawardena, *Emerg. Inf. Dis.* 2007

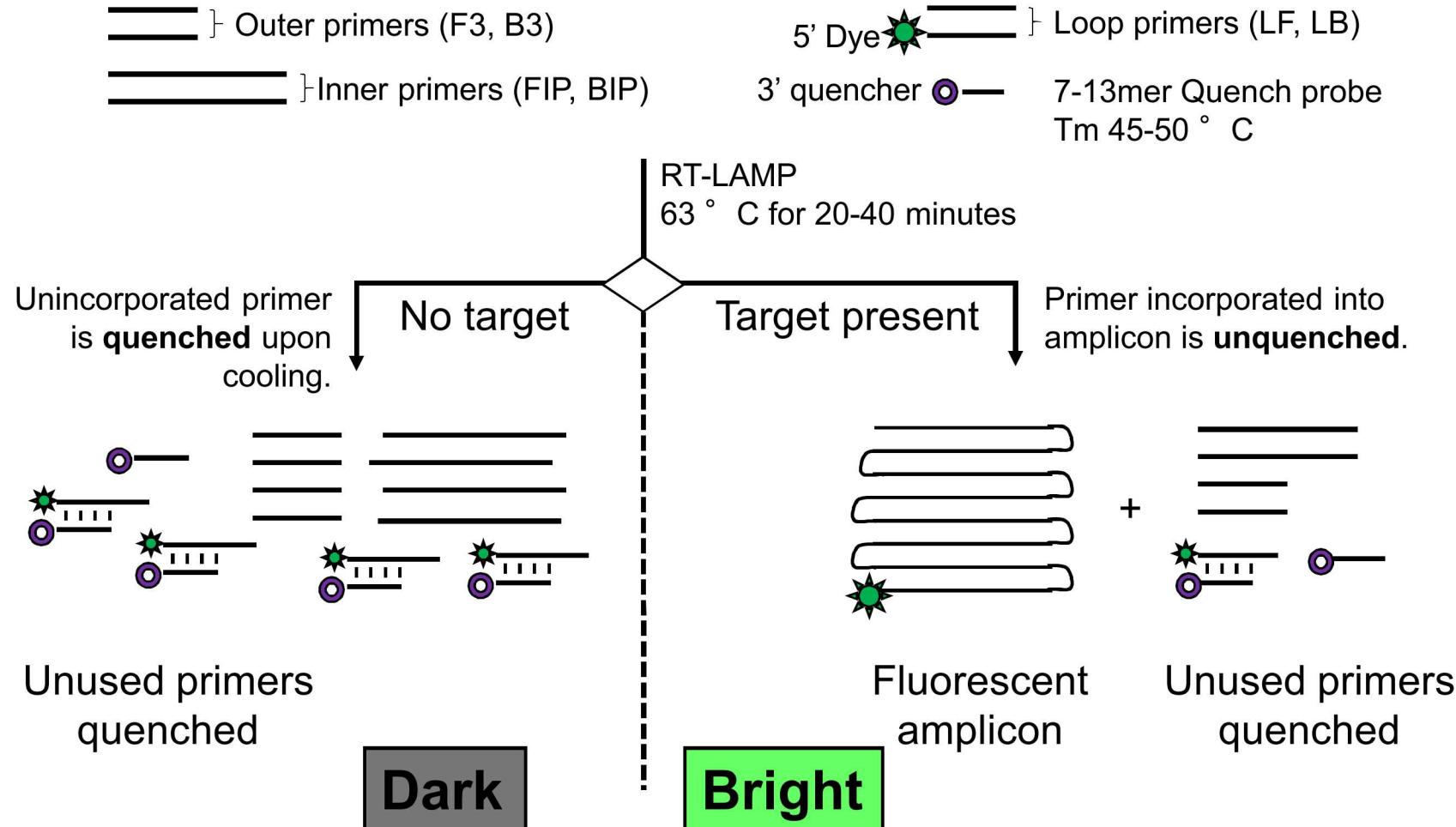
C. Post-reaction, open the tube and add a ton of SYBR Green



Nie *PLoS One* 2012

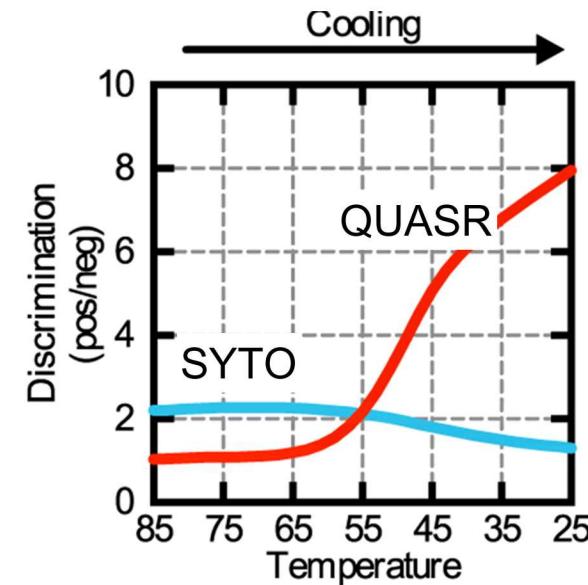
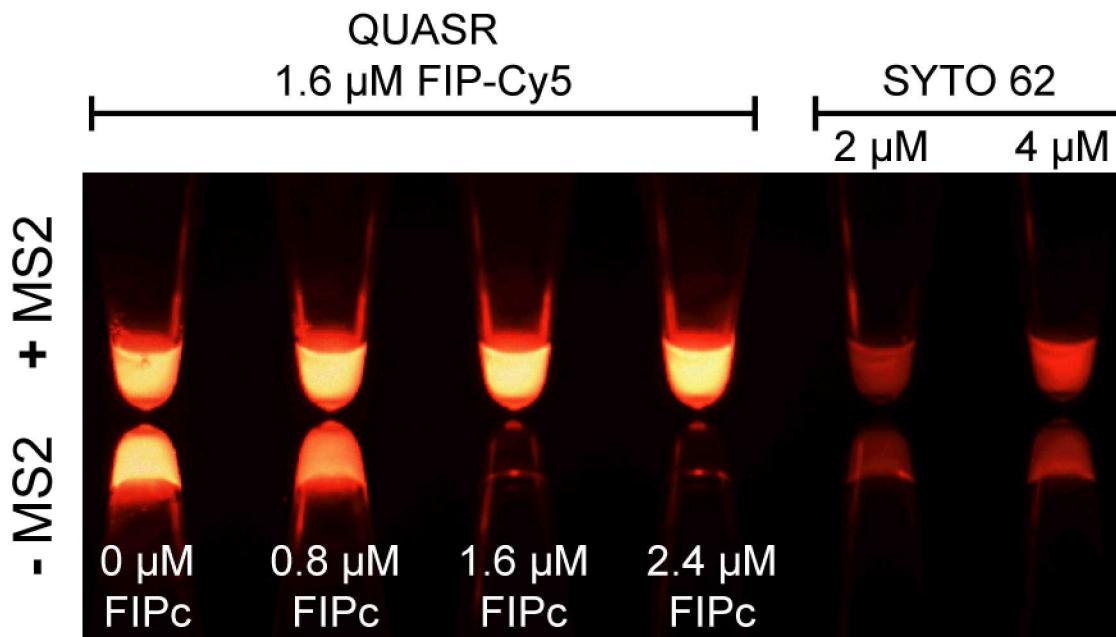
D, E, F, G... Color change and other nonspecific indicators of total DNA synthesis...

QUASR: Quenching of Unincorporated Amplification Signal Reporters

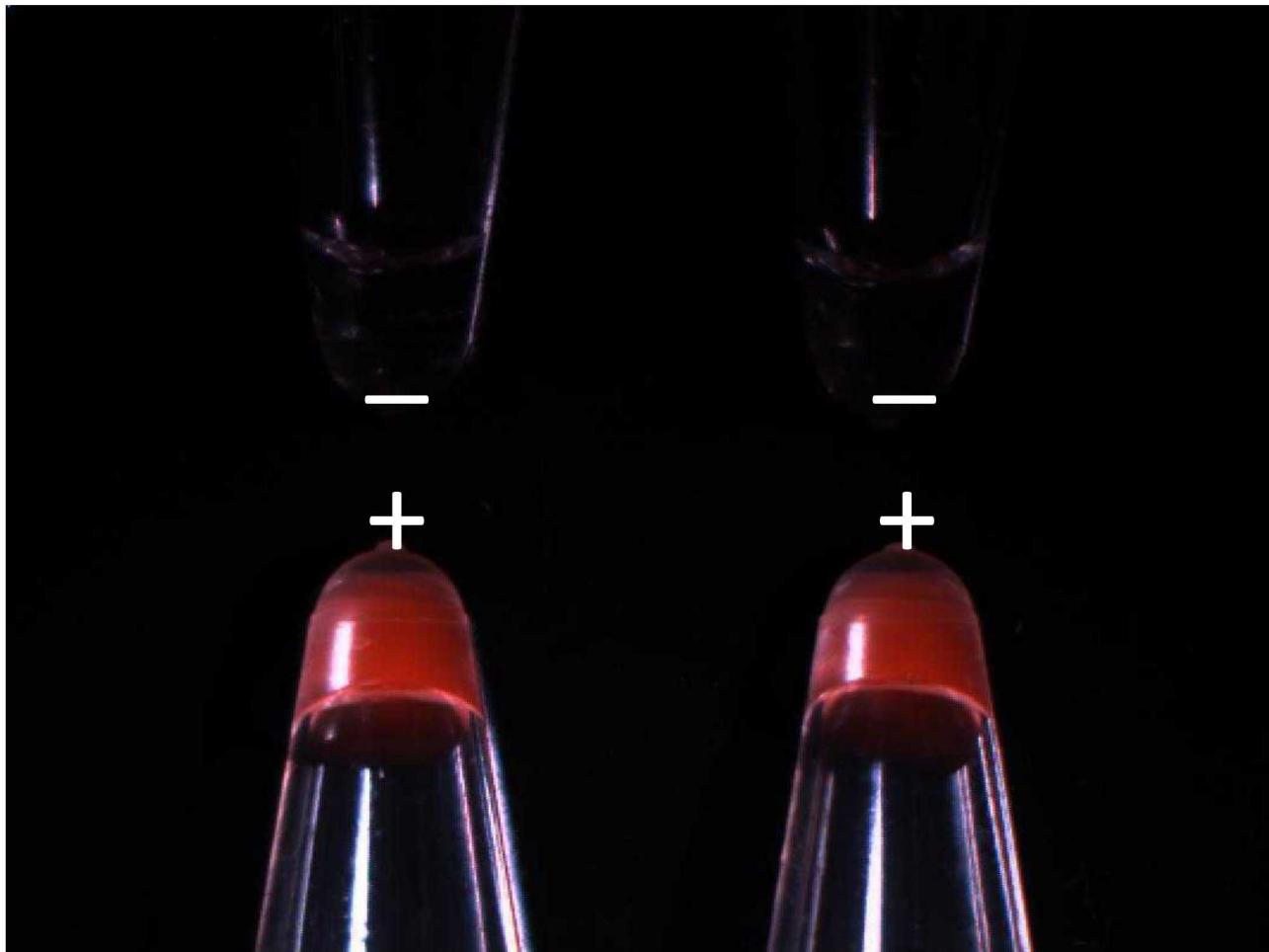


QUASR Proof of Concept (MS2)

- QUASR signal develops as reaction cools below T_m of quench probe
- Contrasts to intercalating dye (e.g. SYTO) where discrimination is highest while hot
- Endpoint only, but closed-tube, very bright signals, and target-specific.



WNV QUASR assay (ROX label)



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

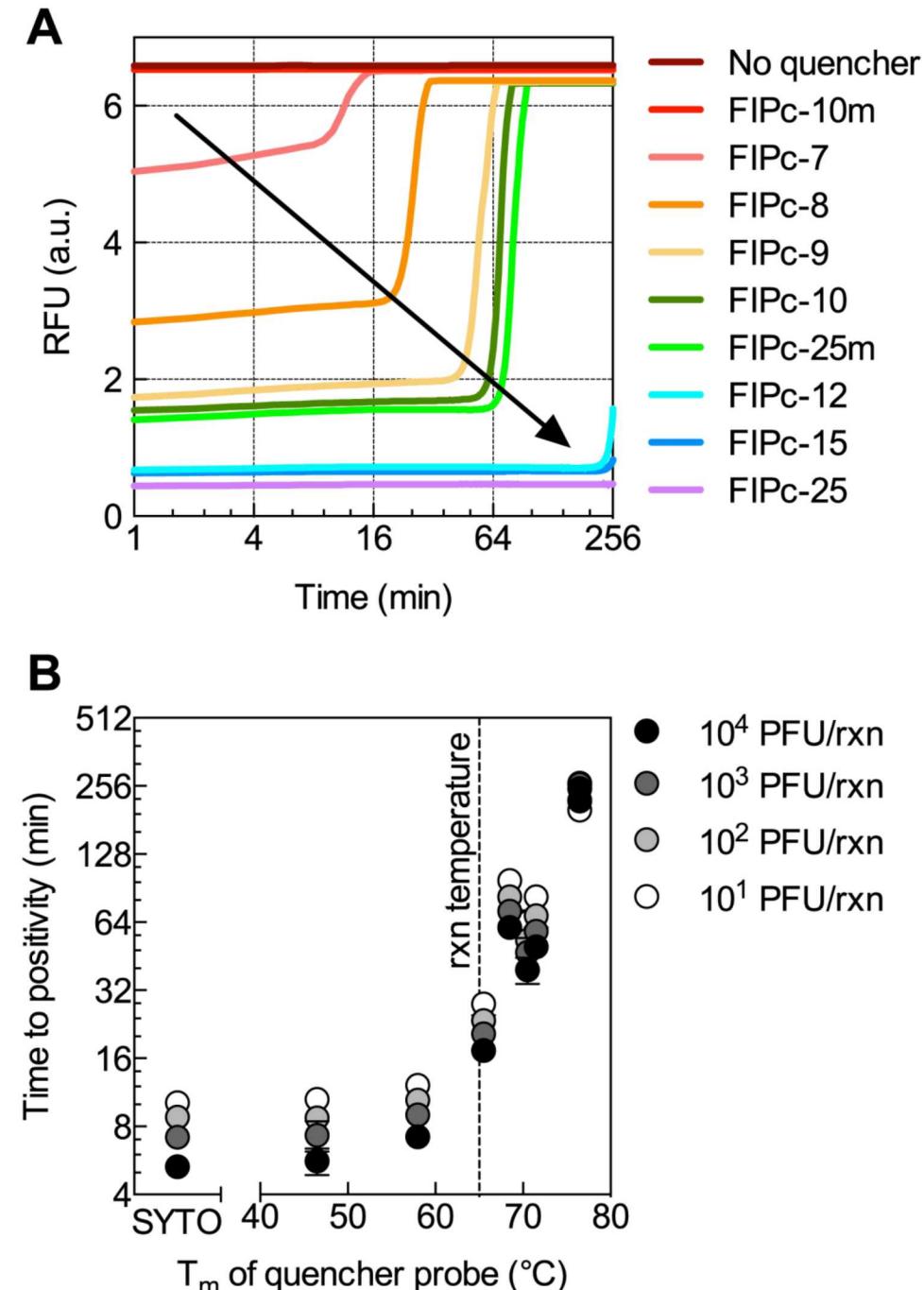
Color photo taken with green LED flashlight and magenta theatre lighting gel as a filter



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

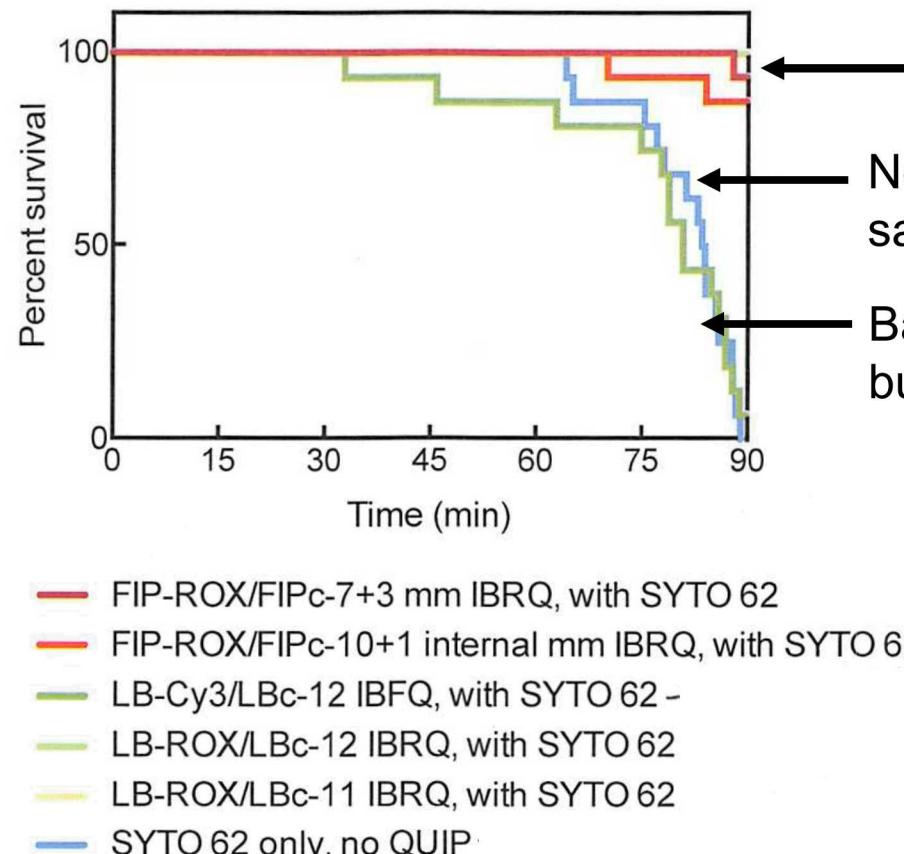
Optimized QUASR quenchers results in no inhibition

- As T_m of quencher approaches reaction temperature, reaction is inhibited
- If probe T_m is $> 50\text{-}55^\circ\text{C}$, 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



QUASR can suppress detection of LAMP “false positive” amplification

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

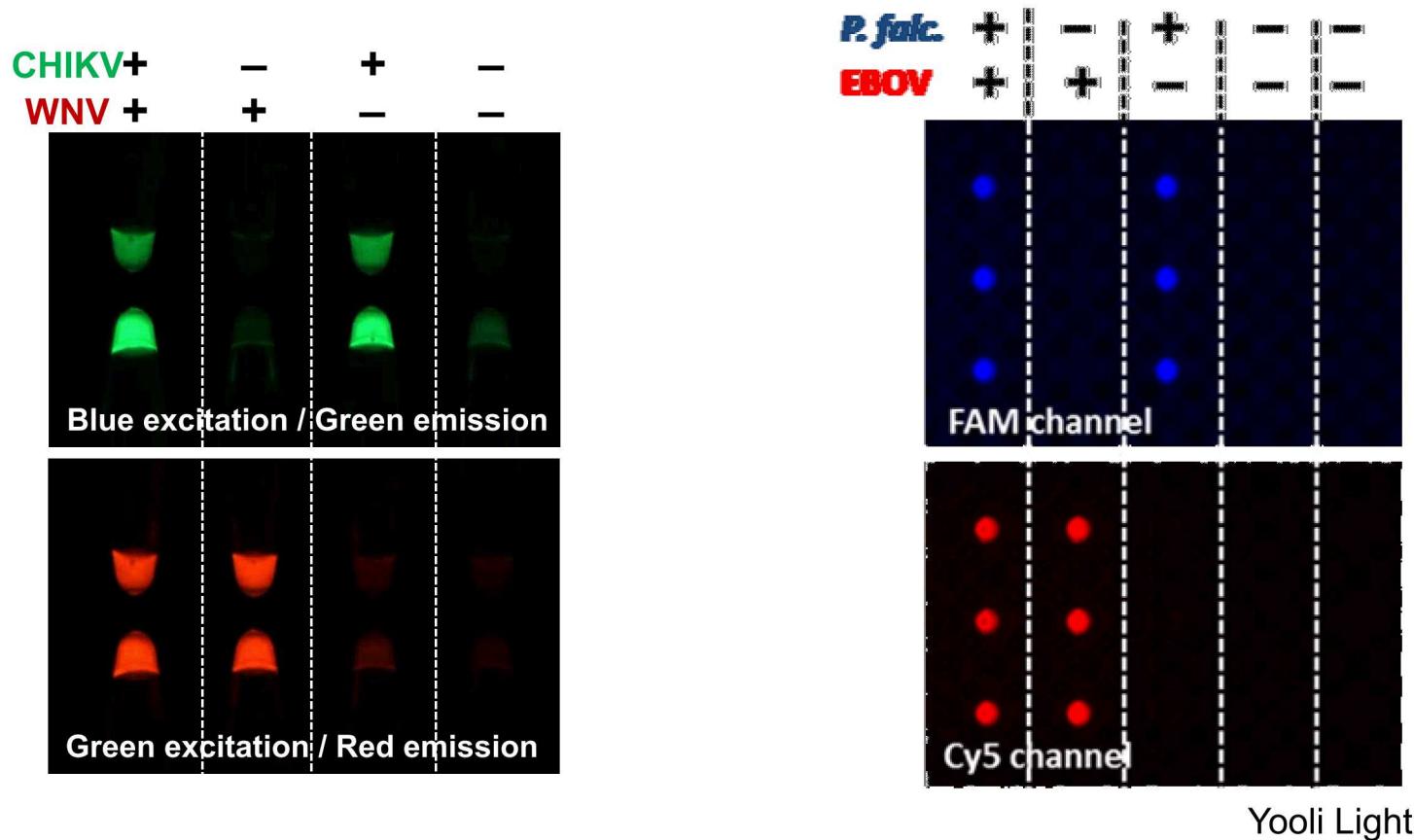


- Good QUASR: Spontaneous amplification is suppressed; and not evident in endpoint signal
- No QUASR: Spontaneous amplification in all samples, and visible with non-specific DNA dye
- Bad QUASR? Spontaneous amplification happens, but is not evident in endpoint probe signal (still ok)

- “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)

Multiplexing RT-LAMP with QUASR

(A) Chikungunya virus + West Nile virus (B) *Plasmodium falciparum* + Ebola virus

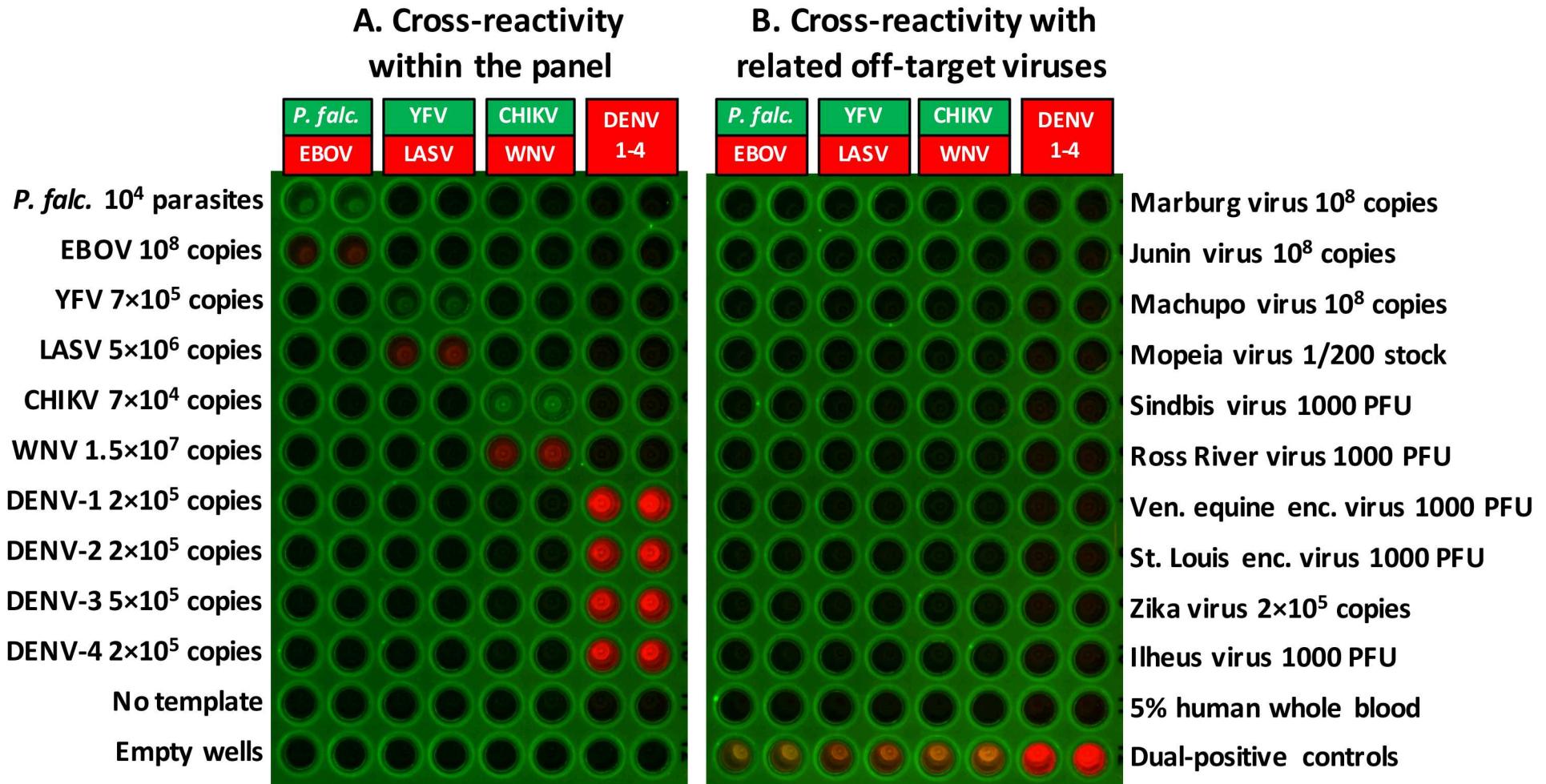


Target specific

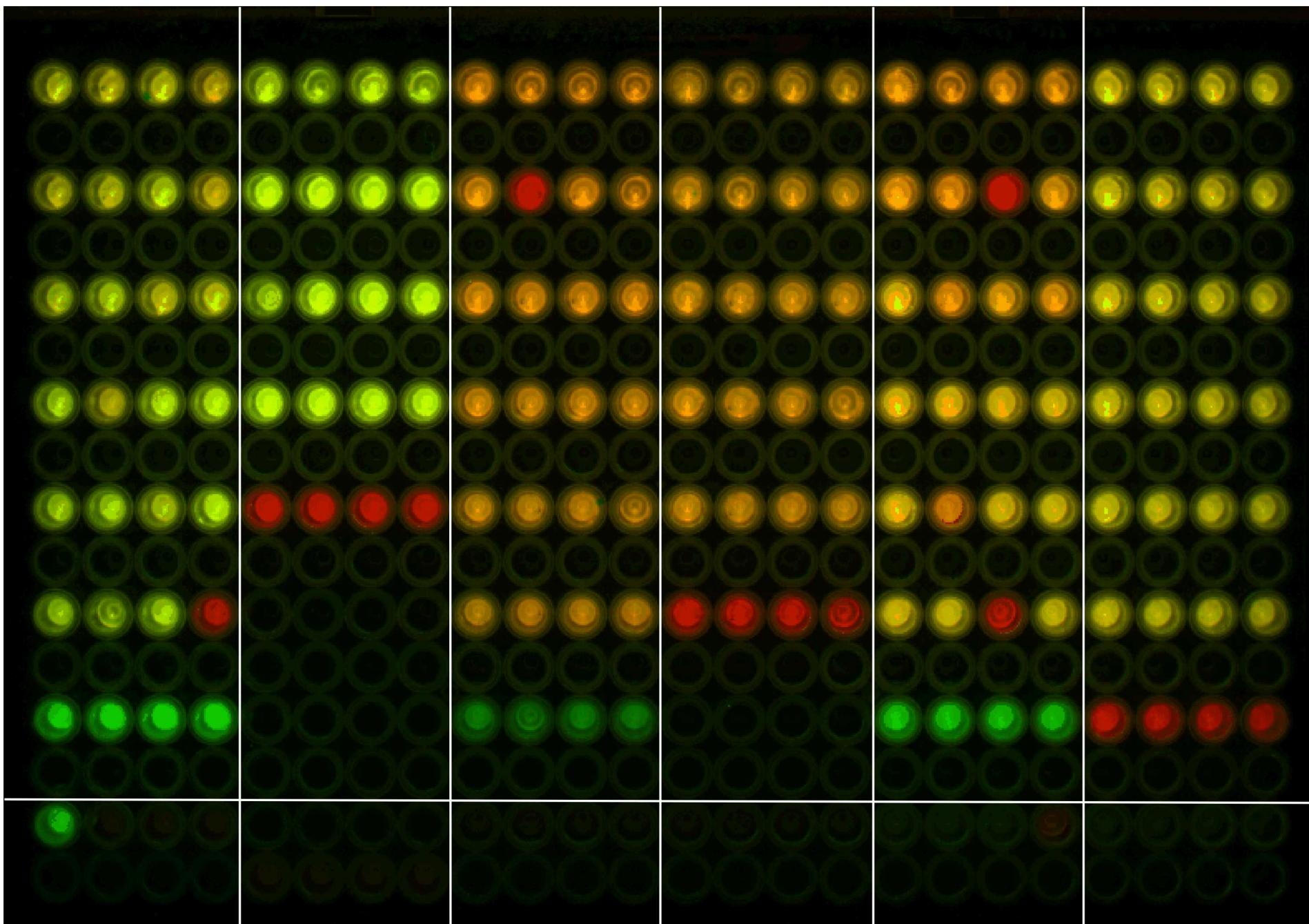
Multiplexable

LAMP historically was hard to multiplex

Multiplexed QUASR panel for febrile pathogens



Decreasing variable target
Bottom row in each group is the fixed target with 0 copies of variable target



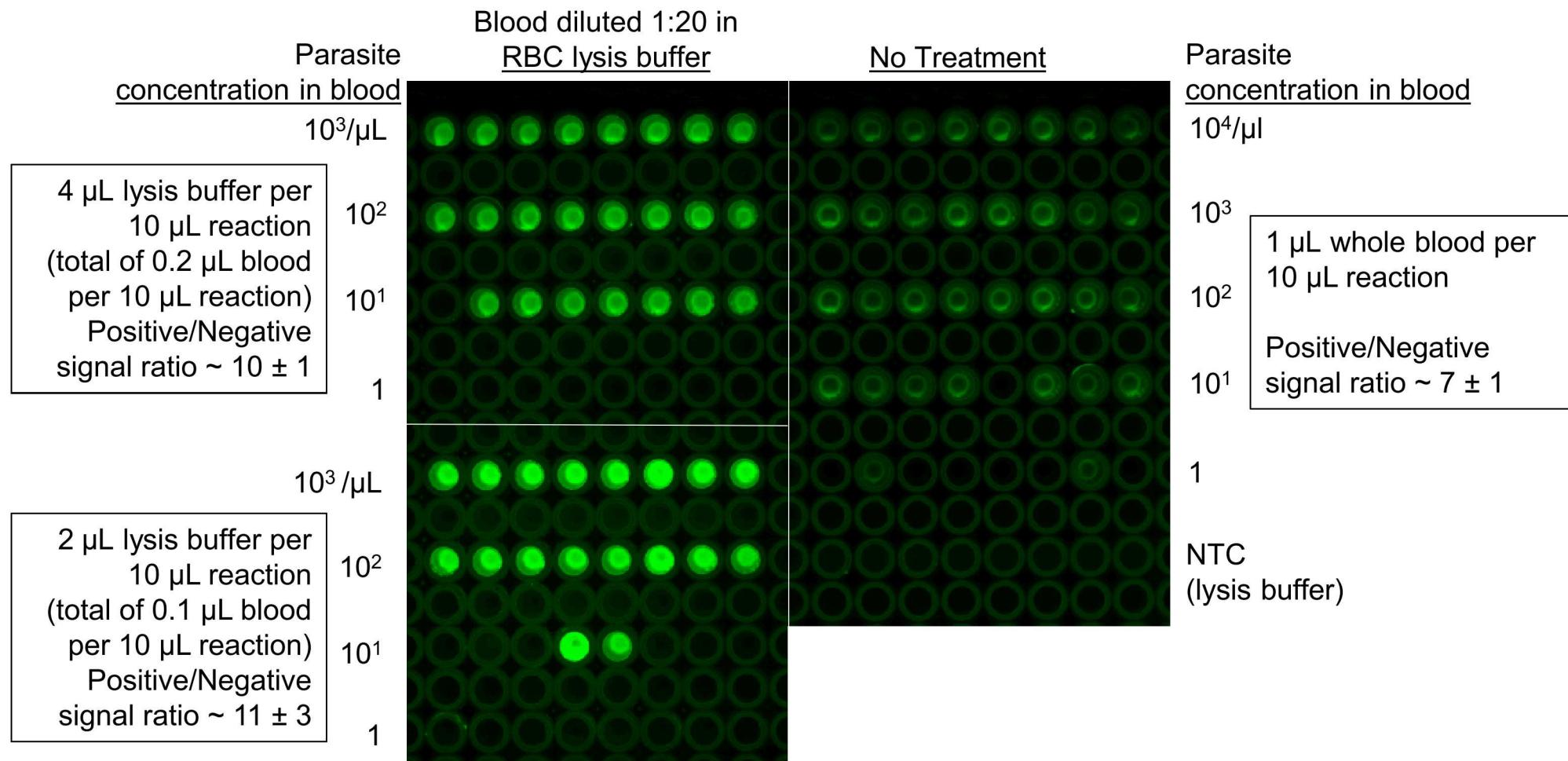
Sample prep is a major challenge (or opportunity for simplification)

- 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 variability, 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

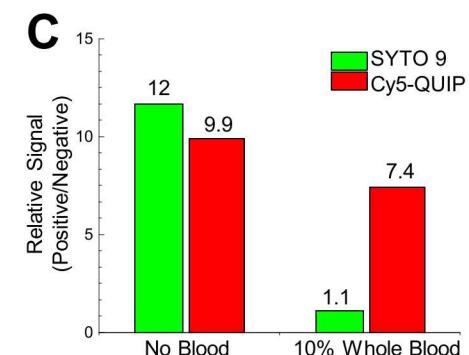
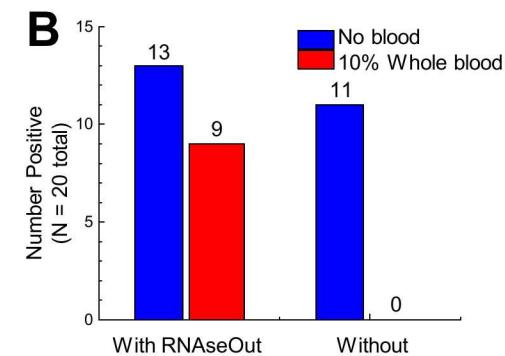
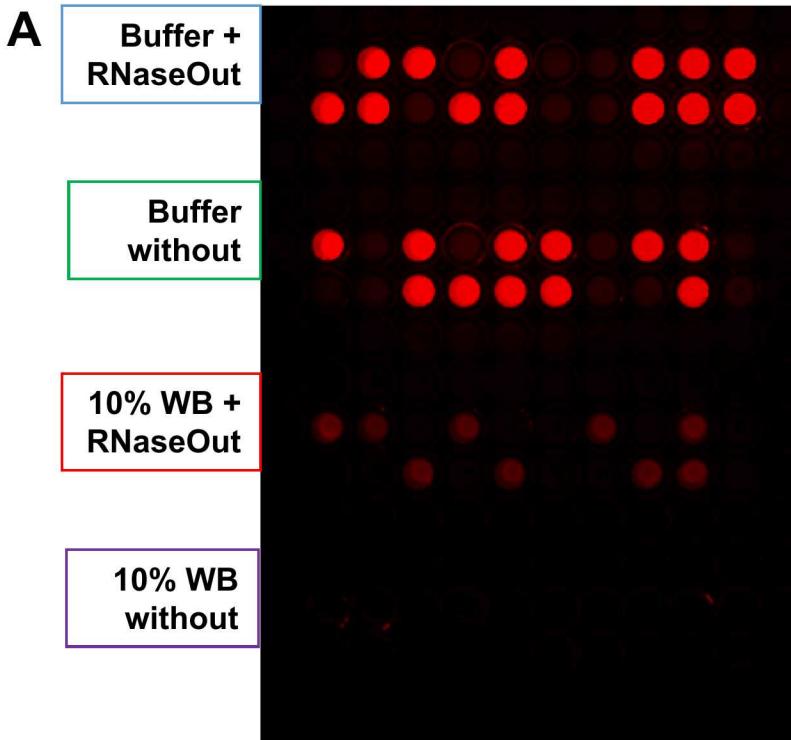


No Treatment: 1ul of whole blood sample

RBC lysis buffer : 1:20 diluted, 2 or 4ul added to rxn (so sampling 0.1 or 0.2 μL per reaction)

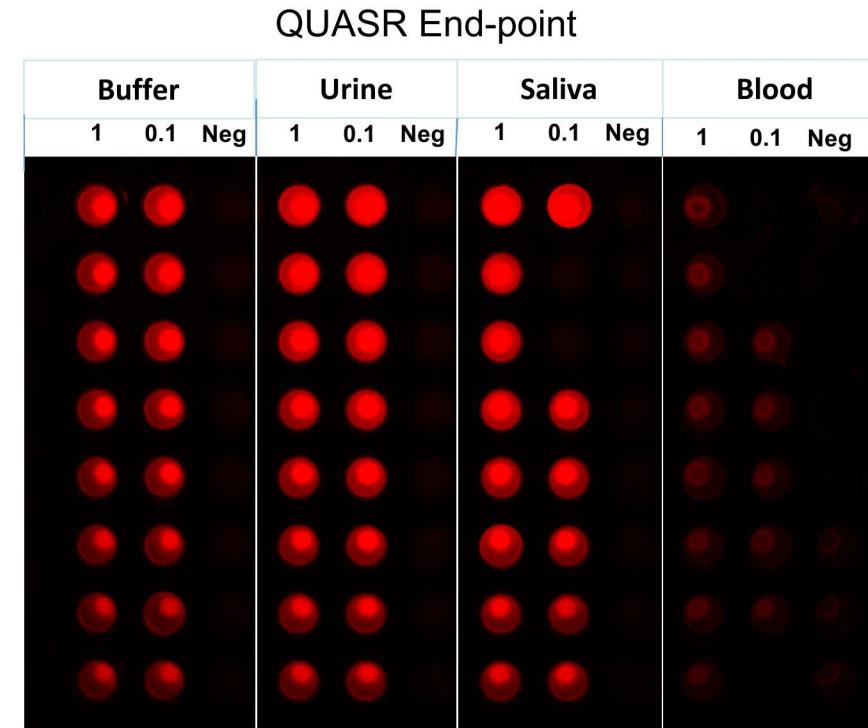
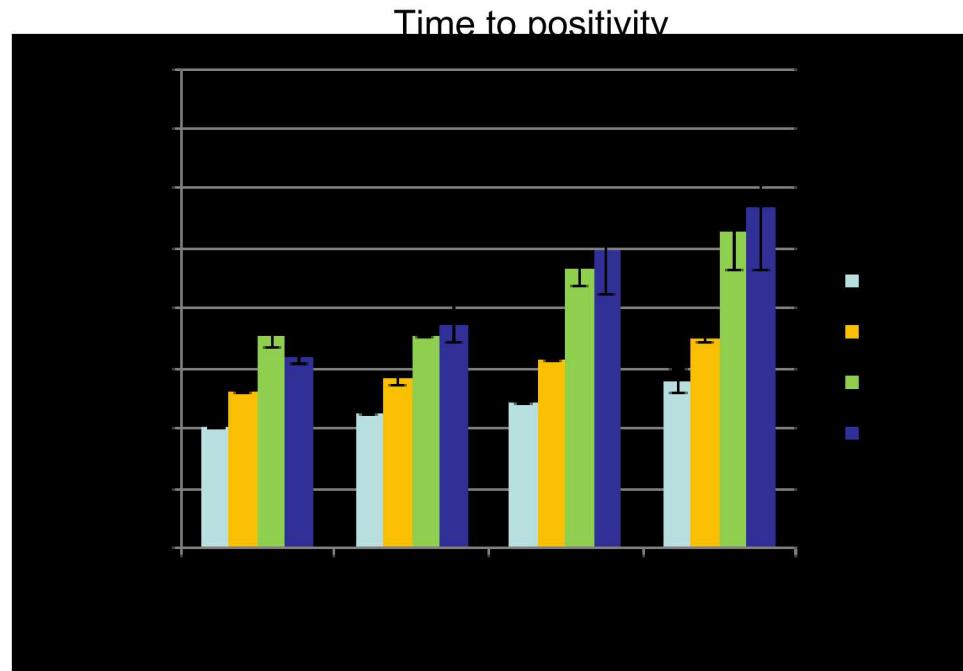
BIP-Cy3, 500ms exposure

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



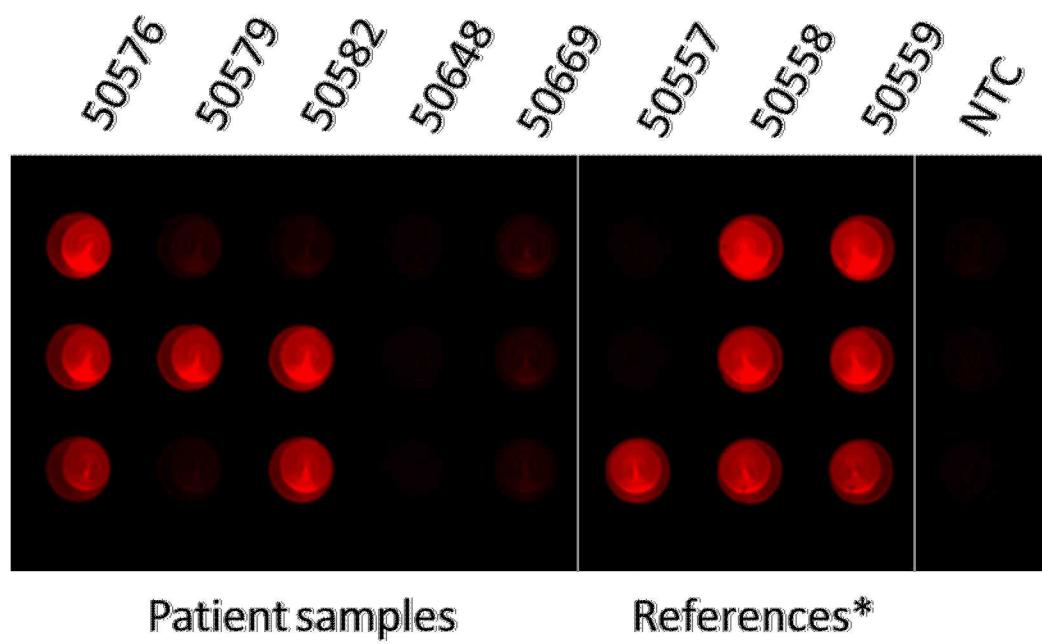
Zika RT-LAMP in clinical matrices

- 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

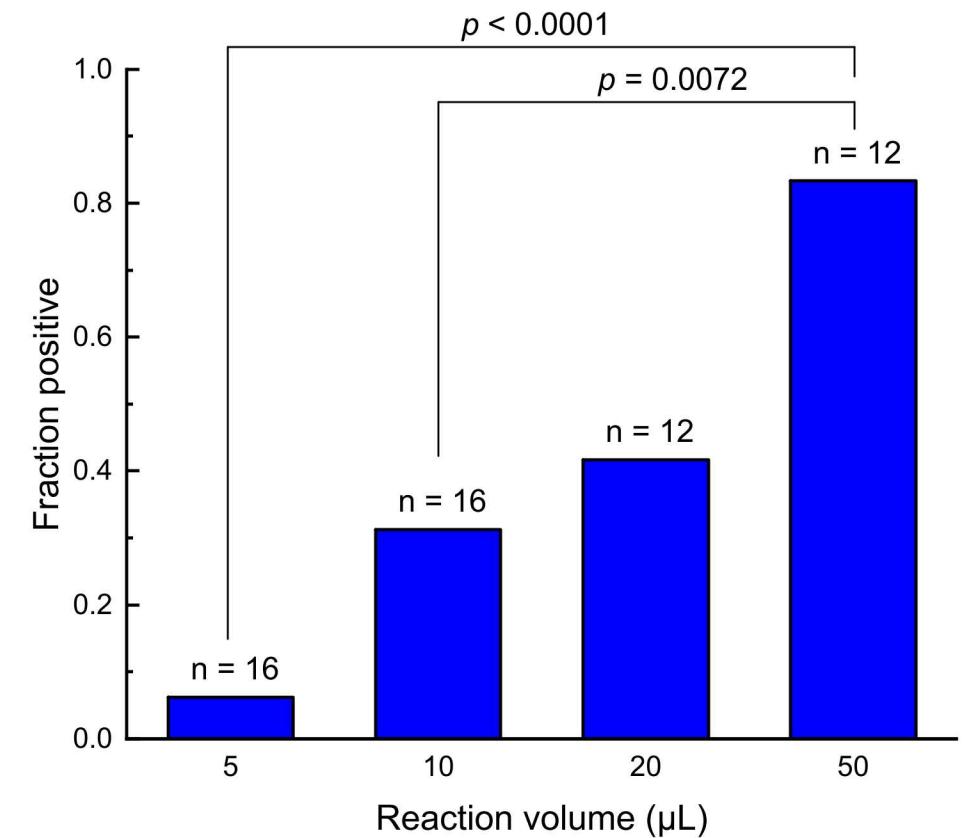


When target concentration is low, scale up reactions.

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

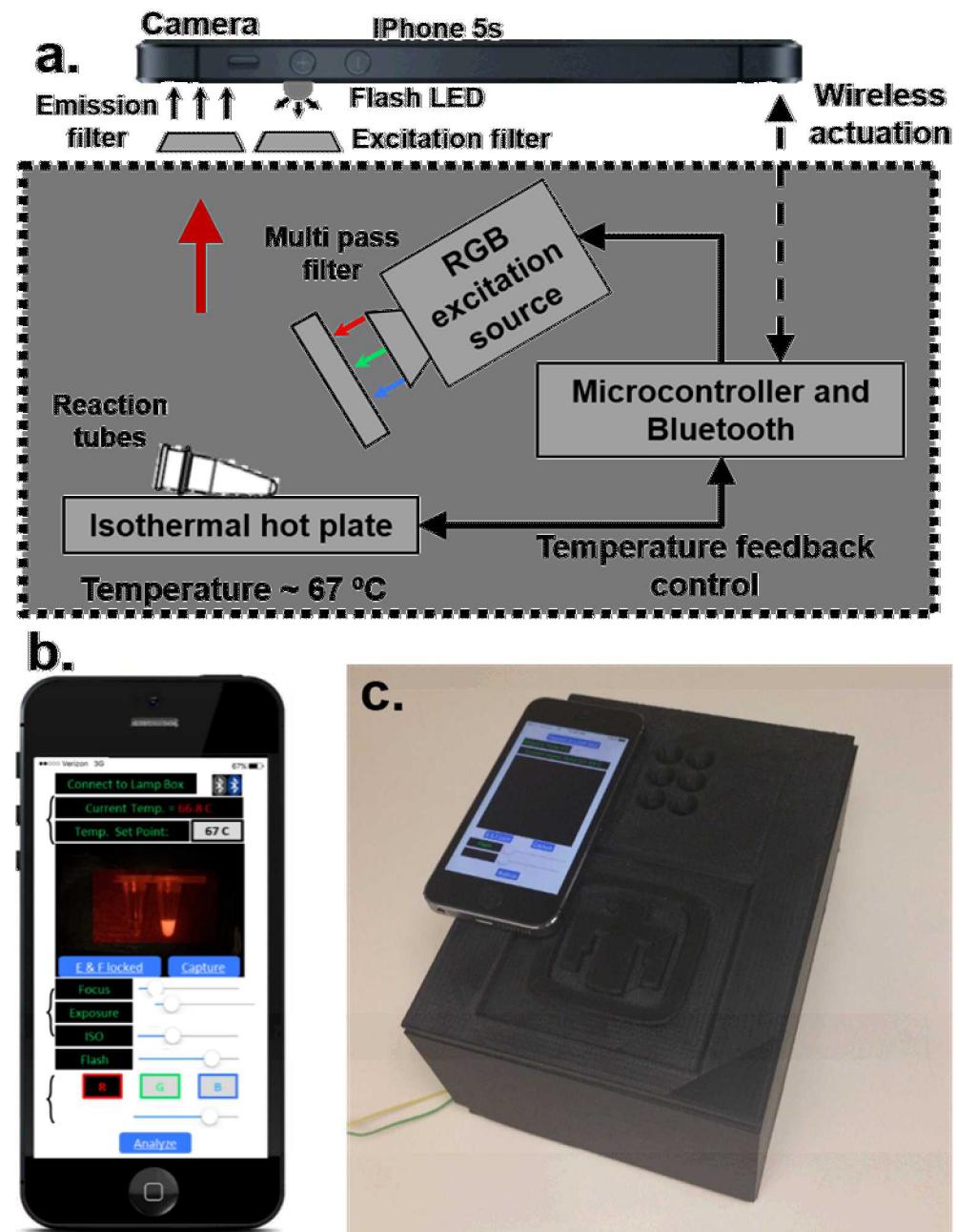


B. Scaling QUASR reaction volume at constant *ratio* of sample to total volume increases probability of detection (target = WNV)

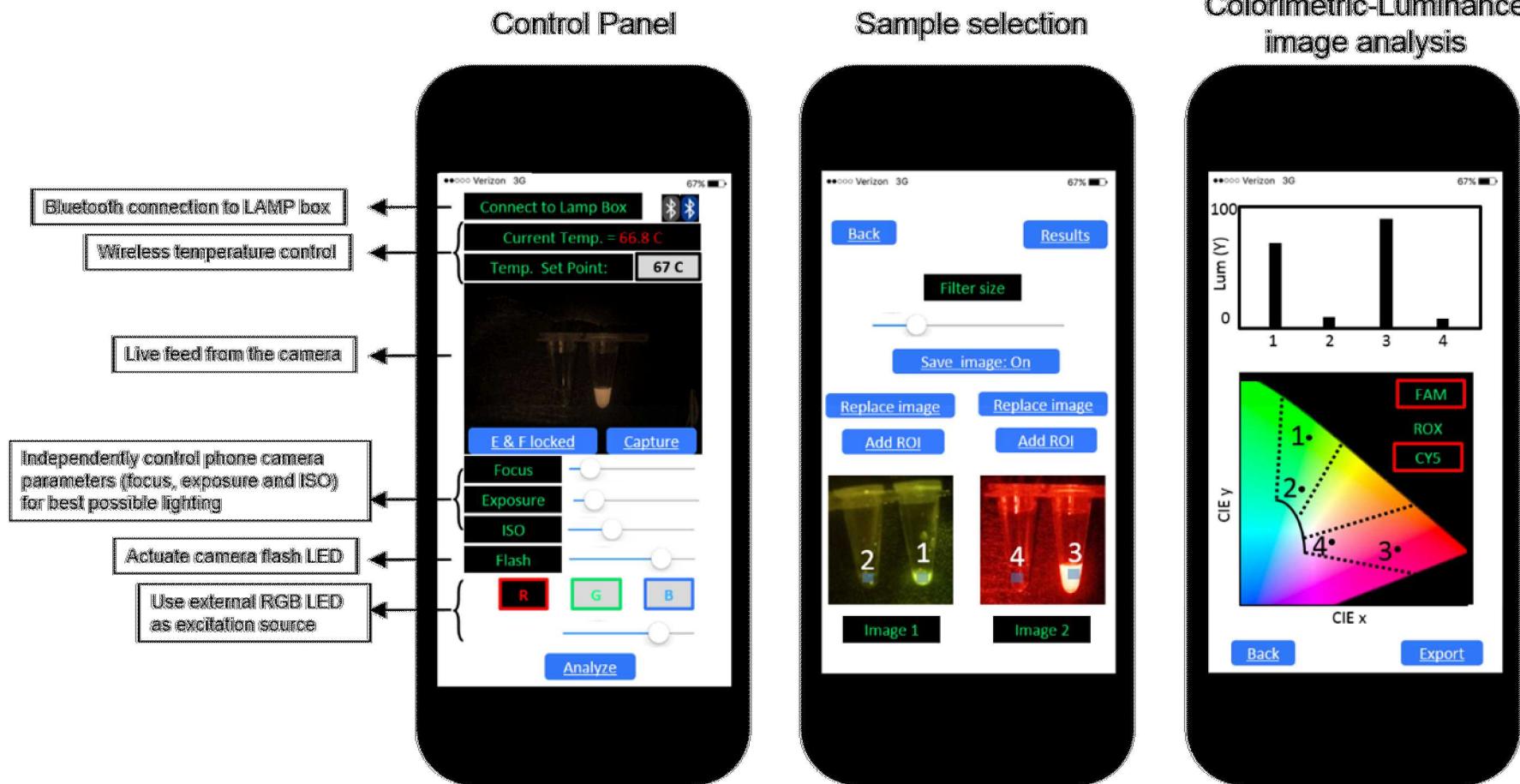


Smartphone-controlled instrument for QUASR LAMP

- Handheld box contains heater, optics, and Bluetooth enabled microcontroller
- Compatible with iPhone and Android phones
- Smart phone app “front end” controls heater, timing, and fluorescence image acquisition
- Heater accepts a variety of formats: PCR tubes, microwells, or planar chips with dry-stabilized reagents.
- Hardware costs: about \$50 with plastic filters; \$500 with high quality coated glass filters, plus phone
 - Compare to \$18-20k for portable isothermal fluorimeter such as Genie III.



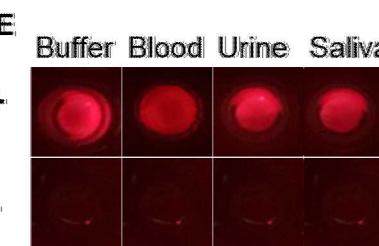
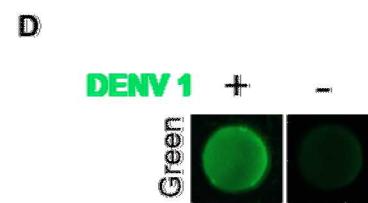
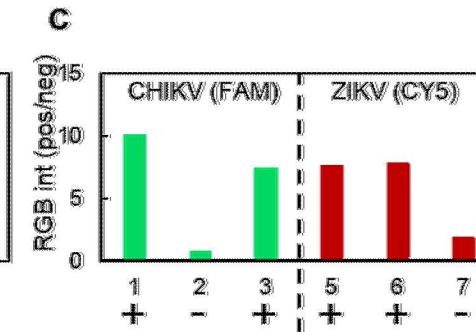
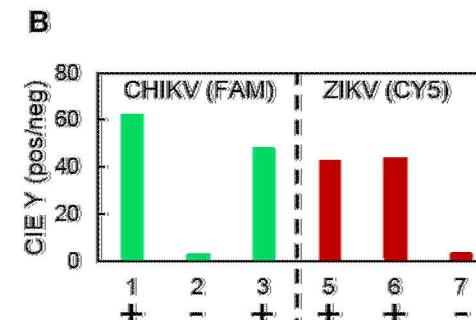
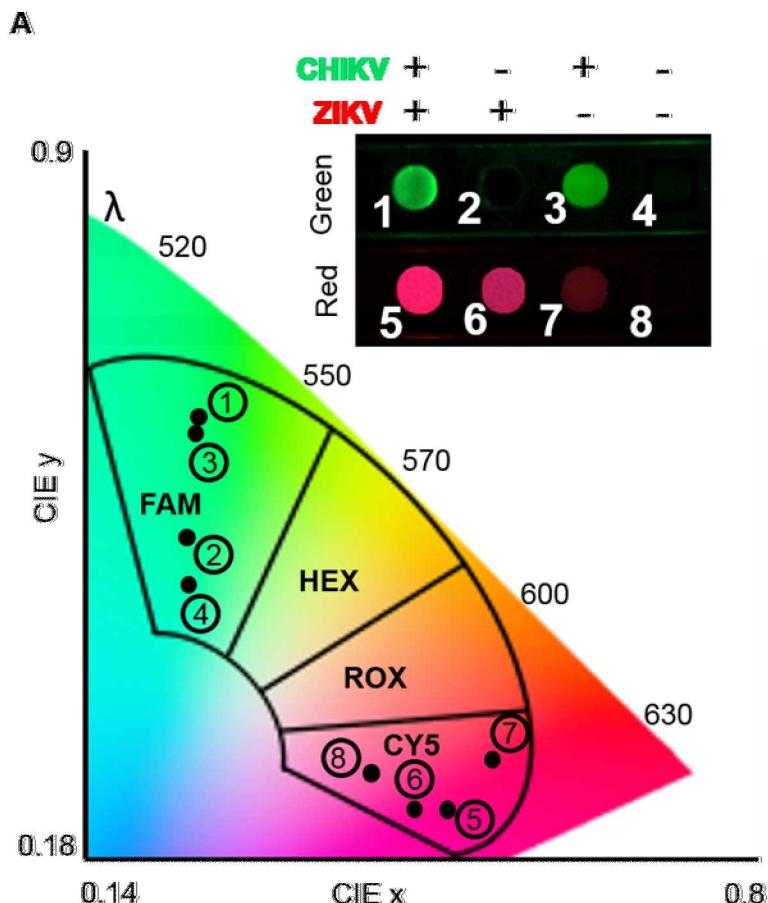
LAMP2Go App



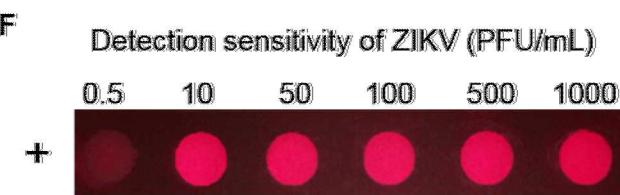
Versions for iPhone and Android
Older model phones ok (e.g. <\$100 HTC Desire)

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

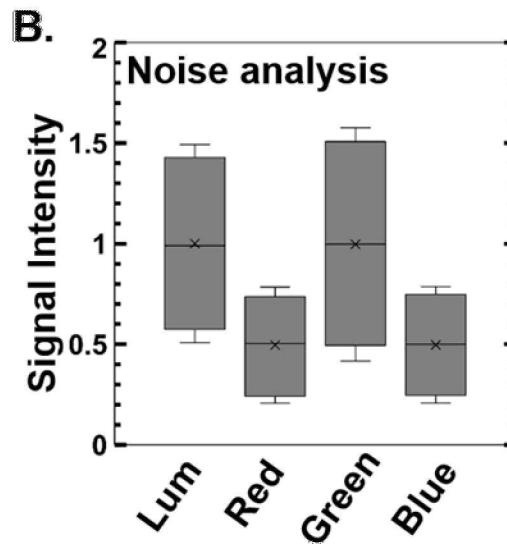
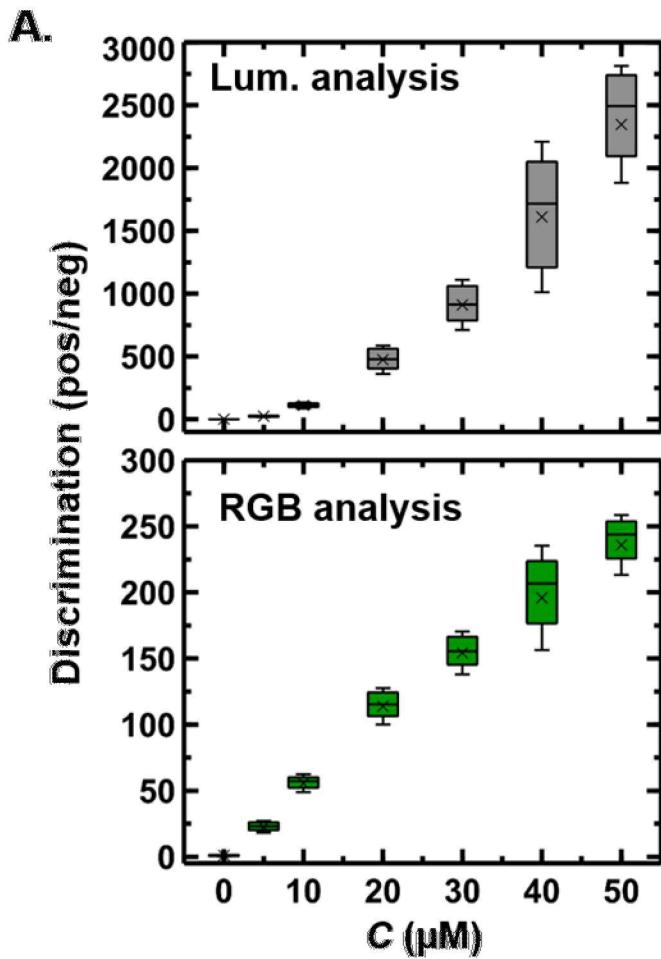


ZIKV detection in clinical sample matrices (intact virus, no extraction)

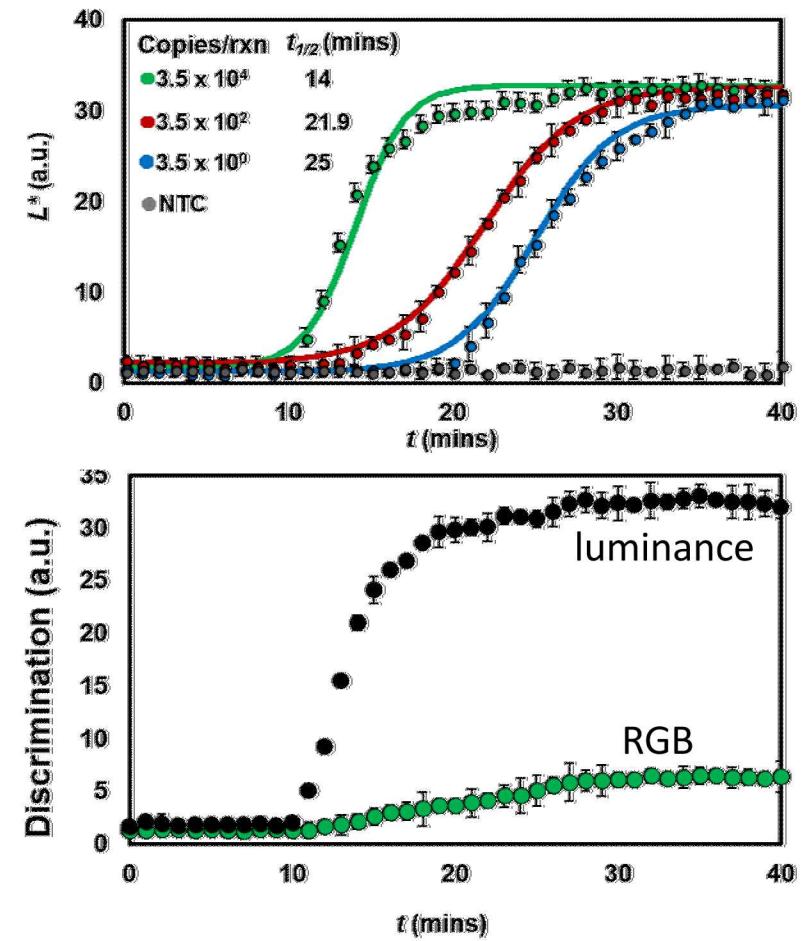


Chromaticity transformation improves LAMP signal-to-noise with smartphone

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



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

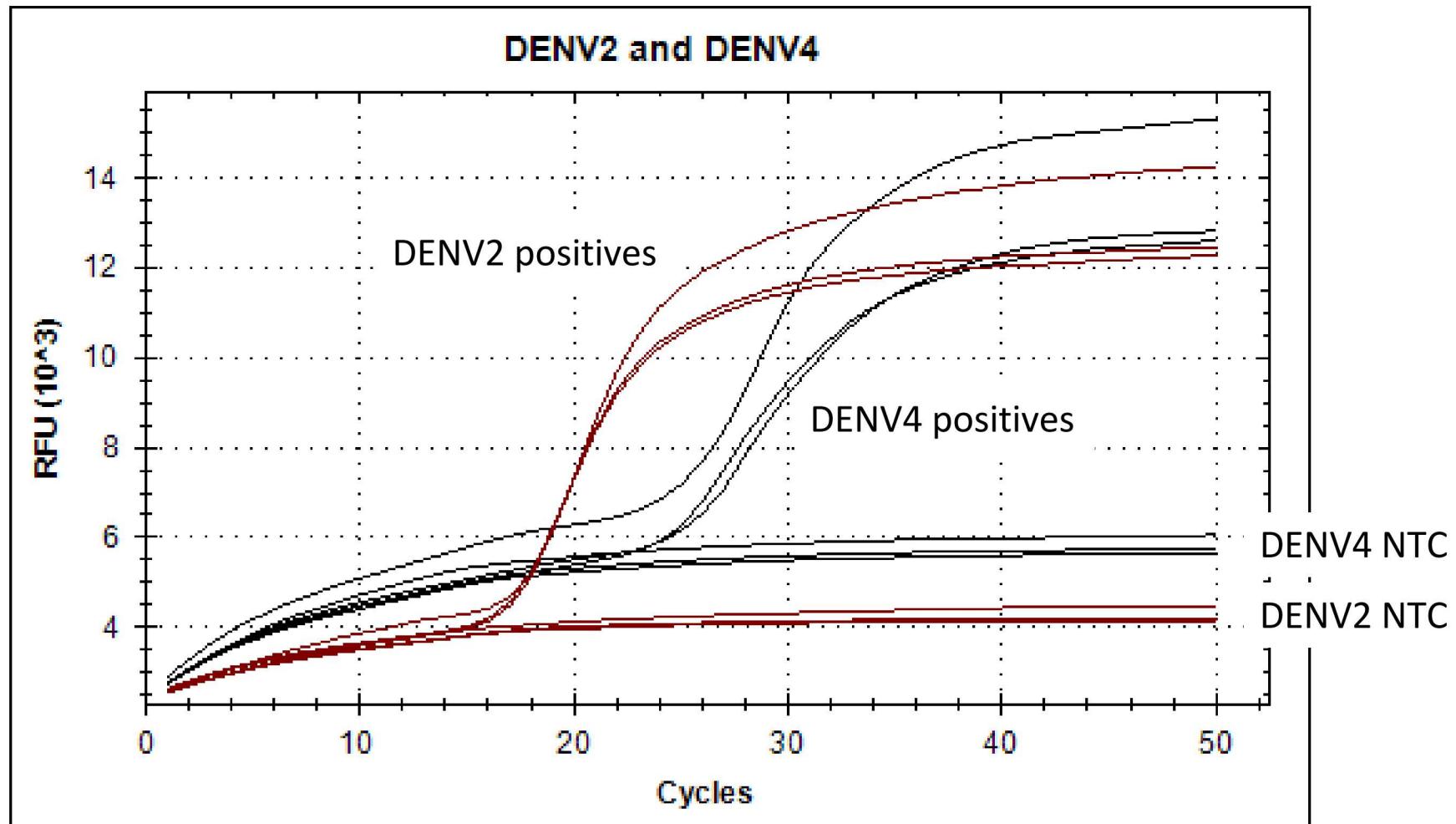


Perspective on LAMP

- Fair comparisons between the many isothermal techniques are difficult because they are coupled to the problem of primer design
- LAMP has a reputation for difficult primer design (requires 6 primers, 8 binding sites), and a reputation for non-specific amplification
- Corollary: requirements for primers are not as well-studied as PCR
- For RNA viruses: juggling the primer design constraints and the inherent divergence of the genome sequence is the hardest part
- For LAMP, the QUASR technique and other probe- or pseudo-probe based methods can reduce the incidence of “false positive” amplification
- If we could develop a mechanistic understanding of LAMP to the same level as has been developed for PCR, perhaps LAMP primer design wouldn’t be so “hard”.

Modeling poor performing LAMP reactions

Example: Rising baseline in published DENV assays



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-tcatctcacccgggggggggttagaggagaccctc->
||||| | | |||||
<-ctccccagaggagattggggggggggttccactctact-5

Cross Primer Dimers:

FIP with BIP

FIP

5-agaggttagaggagaccccccgcaggatctctggtcttcc->
||||| | | | | | |
<-ctccccagaggagattggggggggggttccactctact-5

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

Consult sequence alignment for target region

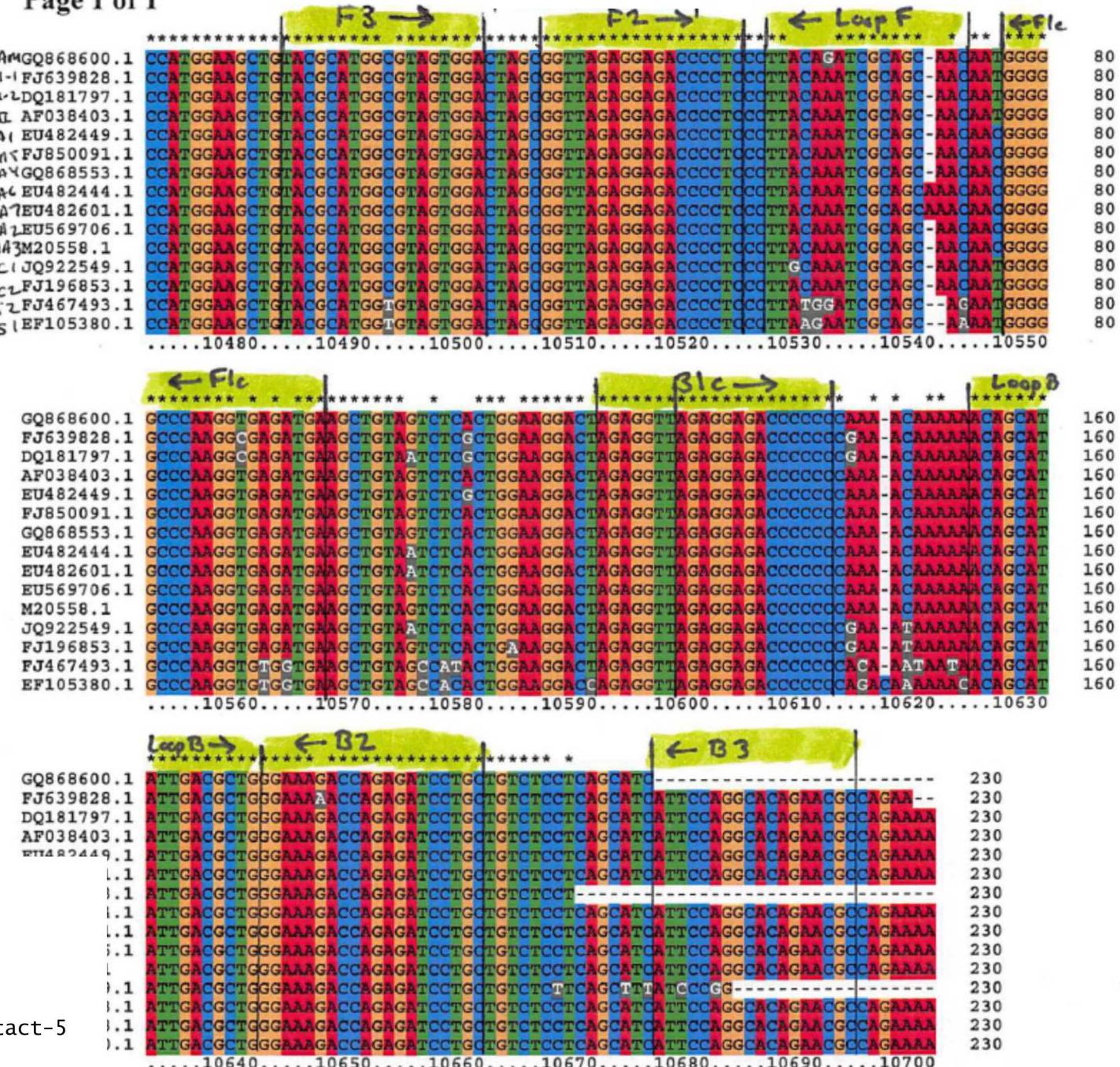
- Sequence alignment showing representatives of 15 DENV-2 genotypes
- There is limited room to “adjust” F2 without having to adjust other primers (up to 2 bases “right” or 5 bases “left”).
- In this case: all adjustments to F2 are allowed by the sequence alignment
- “Bump” F2 4 bases to left

versus

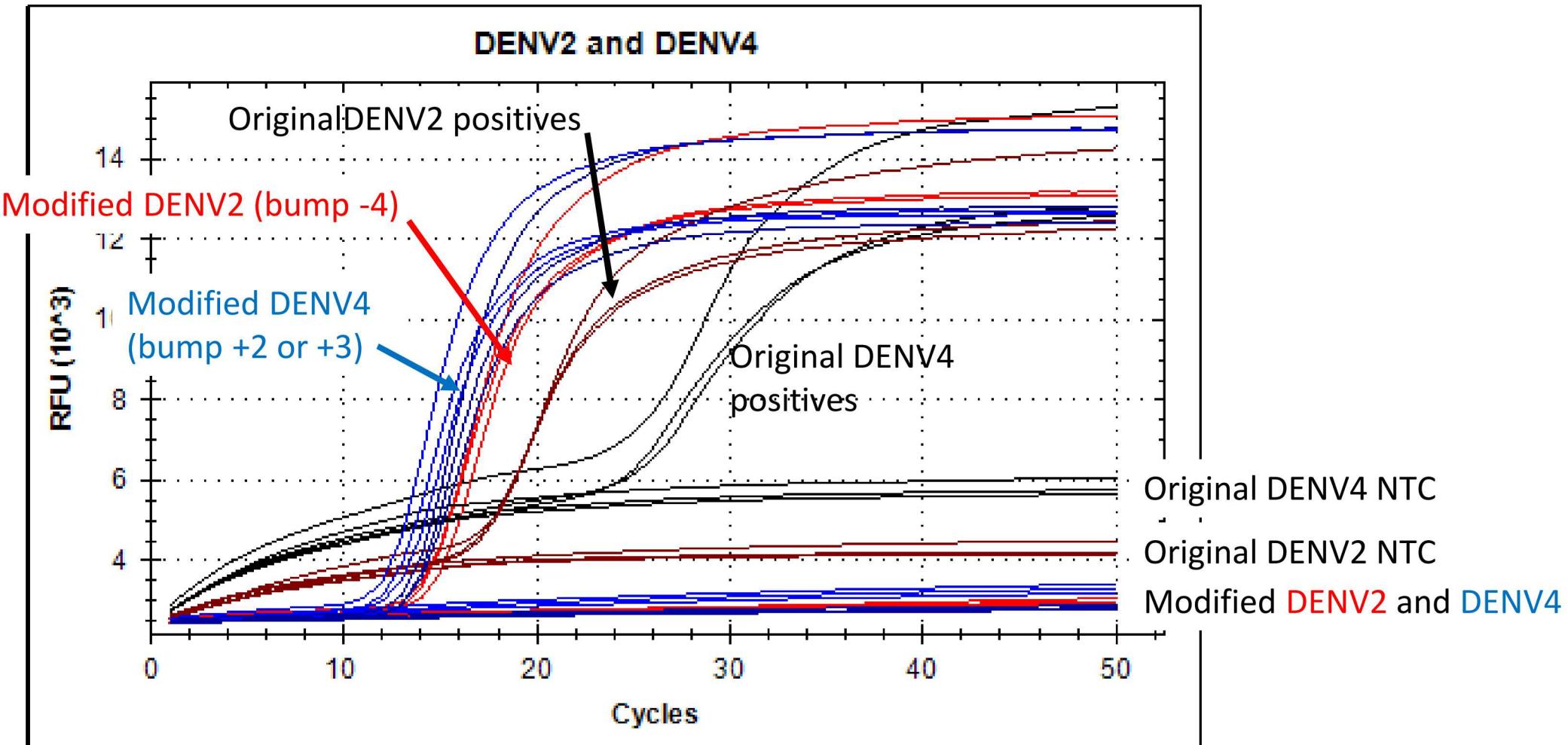
5'-tcatctcaccttggggccccccggtttagaggagacccctc->

III. **RESULTS**

<-ctcccccaqaggaga



Bumping F2 reduces background and speeds amplification in both cases



Thermodynamic basis for rising baseline?

- nearest-neighbor model for primer hybridization

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

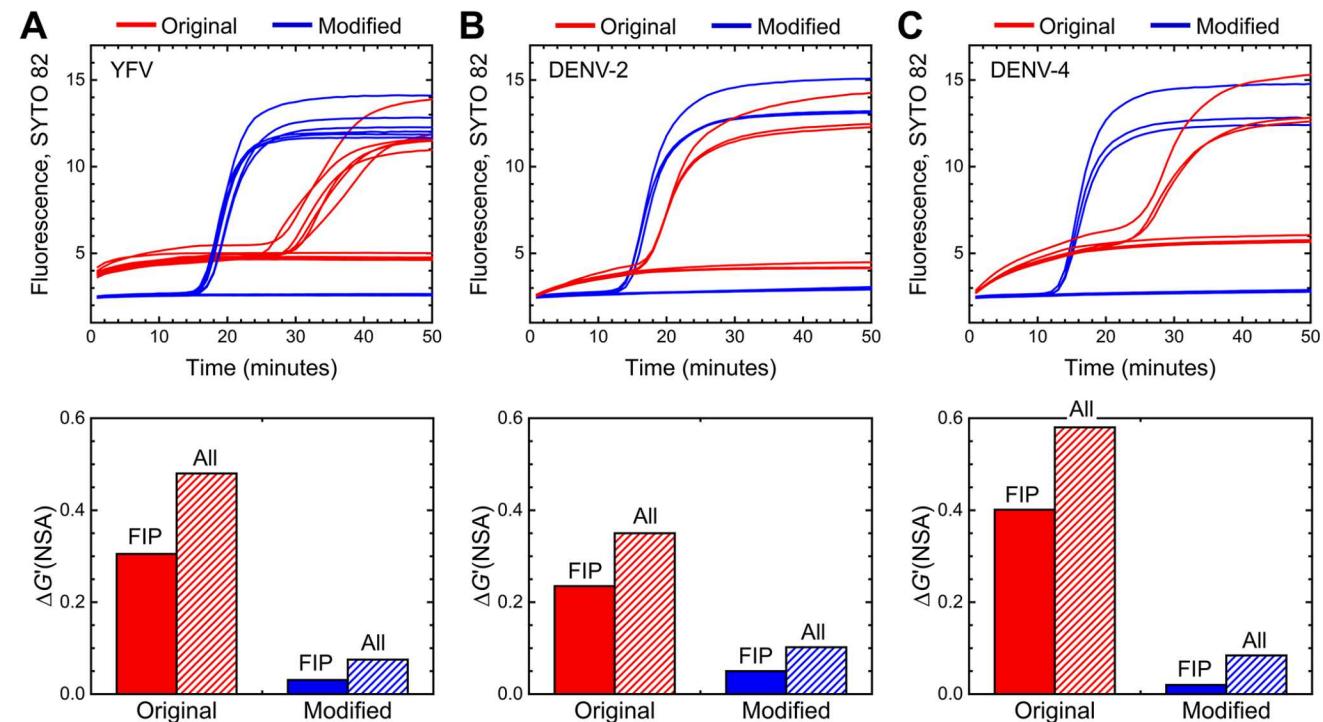
- Normalized $\Delta G'$ for all possible primer-primer interactions

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

- 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



Conclusions & Future Work

- QUASR modification to LAMP helps satisfy requirements for a point-of-care diagnostic
 - Many published LAMP primer sets can be improved with simple changes
- Smart phone can be used both to control simple instrumentation, score assays, and transmit/archive data
- “Direct” amplification from crude samples w/ no sample prep is possible in some cases
 - Limitation for pathogens present at low concentration
 - A conventional DNA/RNA extraction is not always necessary, and other techniques to concentrate pathogens without the typical silica-based extraction may be feasible
- So far, mostly tested in the lab with simulated samples
- Current estimate of smartphone device and assay is TRL4
 - Needs further work for user-friendliness and reliability
- Seeking partnerships for field testing & advancing TRL
 - Adaptable to other pathogens (bacterial/fungal/viral)
 - Adaptable to other sample types & scenarios (environmental samples, arthropods, plant pathogens, etc).