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Molecular detection of emerging pathogens in low-resource settings

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Sandia team

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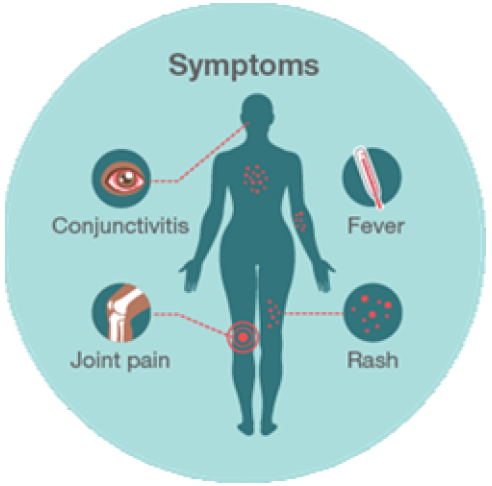
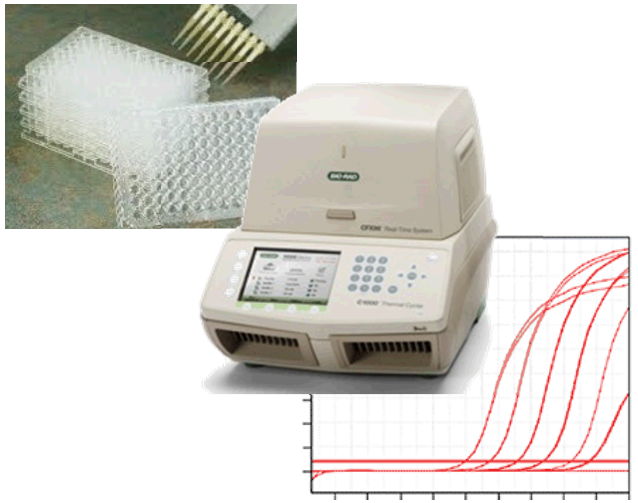
Conventional Laboratory Diagnostics for viral infections

- Diagnostic tests for viral infections include:
  - **Culture** –"gold standard" in many cases, but this is slow, technically challenging, has low sensitivity for many viruses (*e.g.* dengue, Zika) and exceedingly risky for others (*e.g.* Ebola).
  - **Serology** – detecting immune response to viruses; most sensitive late in infection (after seroconversion), but may suffer low specificity (*e.g.* cross-reactive response for flaviviruses)
  - **Nucleic acid detection**: detection of viral RNA; most sensitive early in infection (viremic phase)

- qRT-PCR has great sensitivity and precision but requires a well-equipped laboratory
  - Need to extract RNA (cleanup/concentrate)
  - Reagents require refrigeration
  - The instrumentation is (usually) power hungry and not portable, nor is the rest of the workflow.



www.cdc.gov



Challenges in Deployed Diagnostics

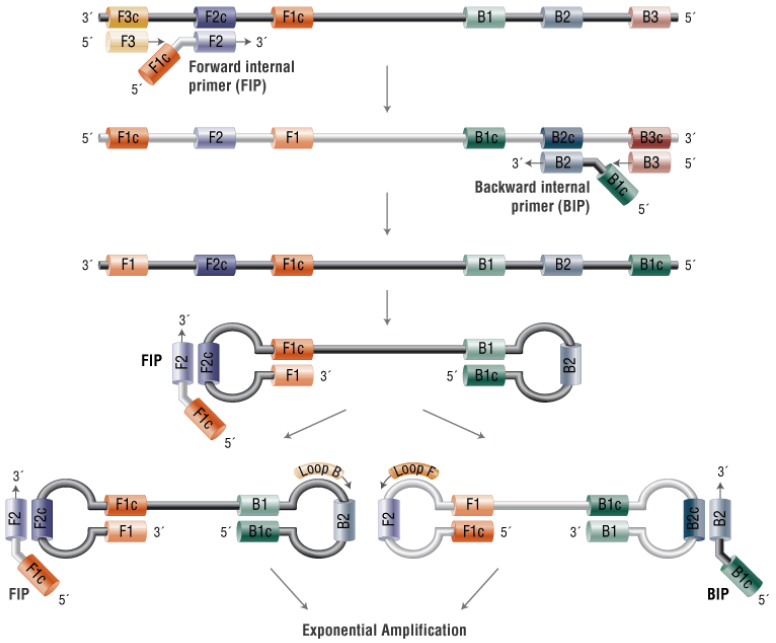
Sample transport: safety, stability, security, and speed?

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

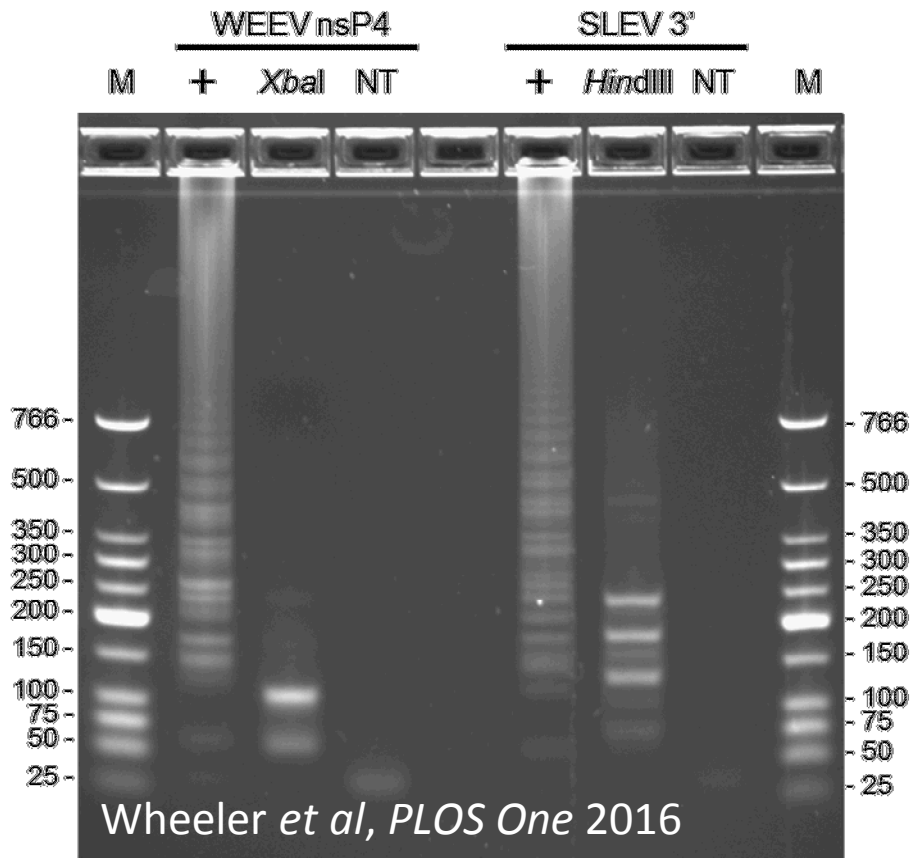
Complex reaction scheme involves strand displacement instead of thermal denaturation

For a deployed LAMP diagnostic:

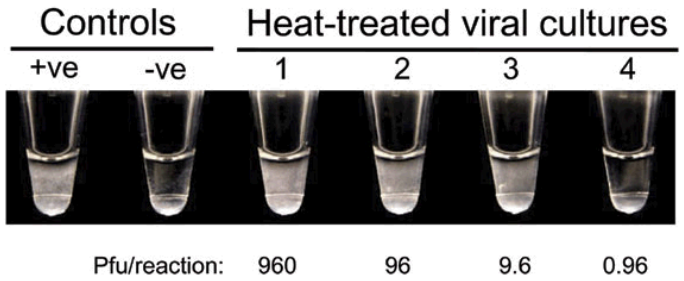
- *Closed tube* detection – don't want to open the tube after the reaction
- *Large discrimination* between positive and negative samples
- *Bright signals* – naked eye or simple detector
- *Target-specific*, vs detecting total DNA
- *Endpoint* is good enough for yes/no answer (LAMP is semi-quantitative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

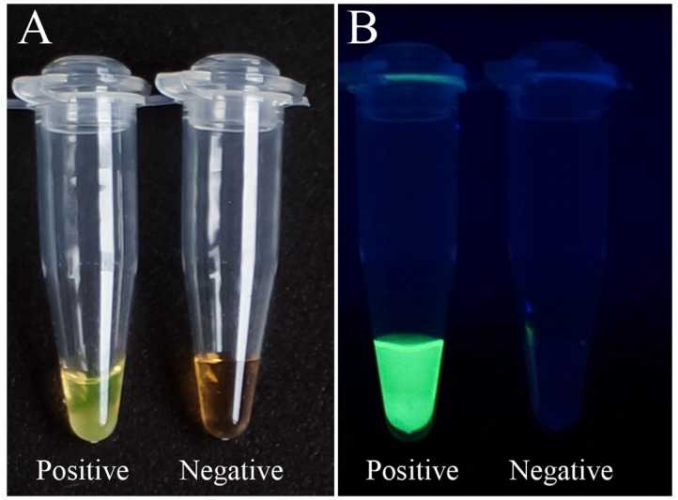


B. Turbidity (precipitation of Mg pyro-phosphate, from making a ton of DNA)



Jayawardena, *Emerg. Inf. Dis.* 2007

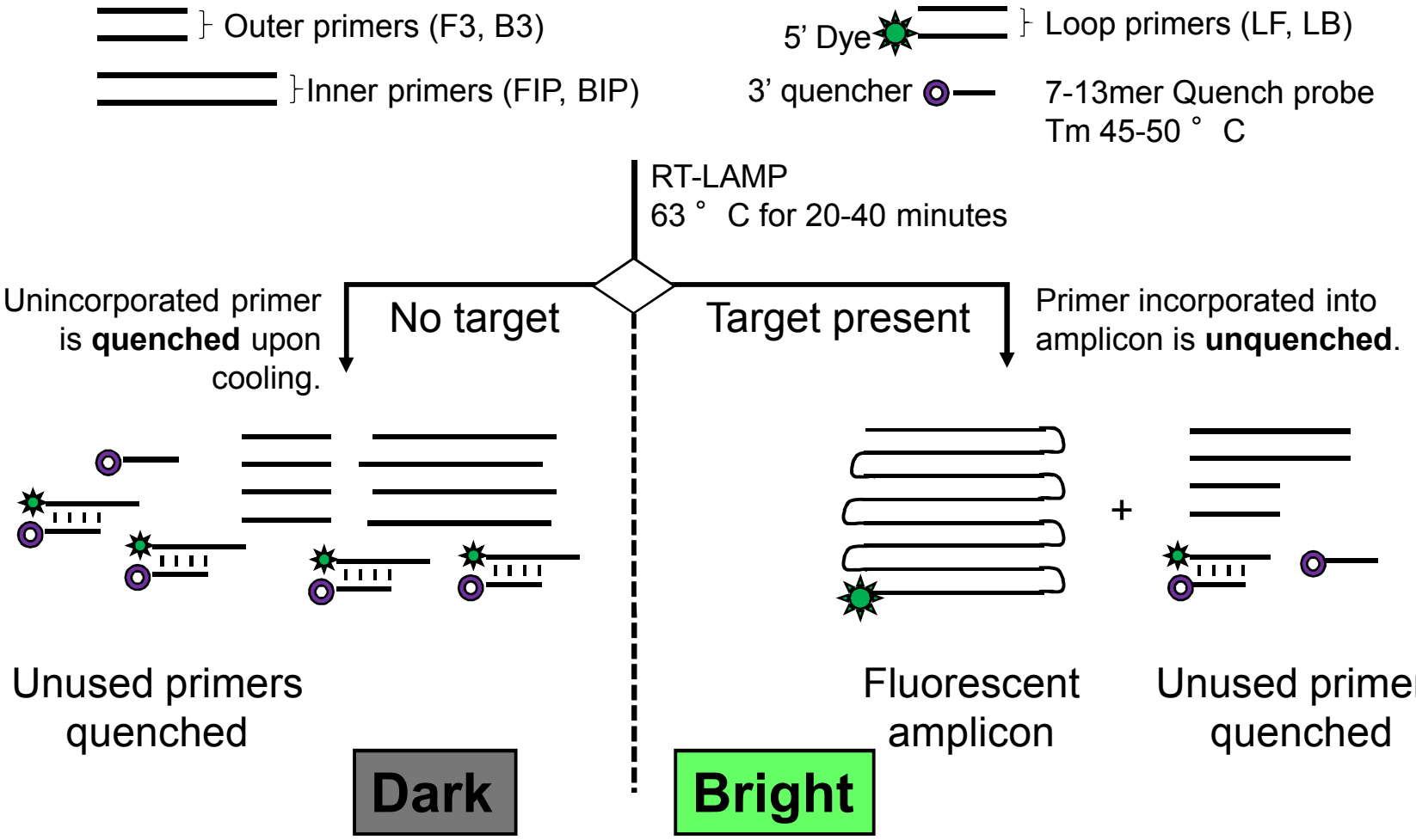
C. Post-reaction, open the tube and add a ton of SYBR Green



Nie *PLoS One* 2012

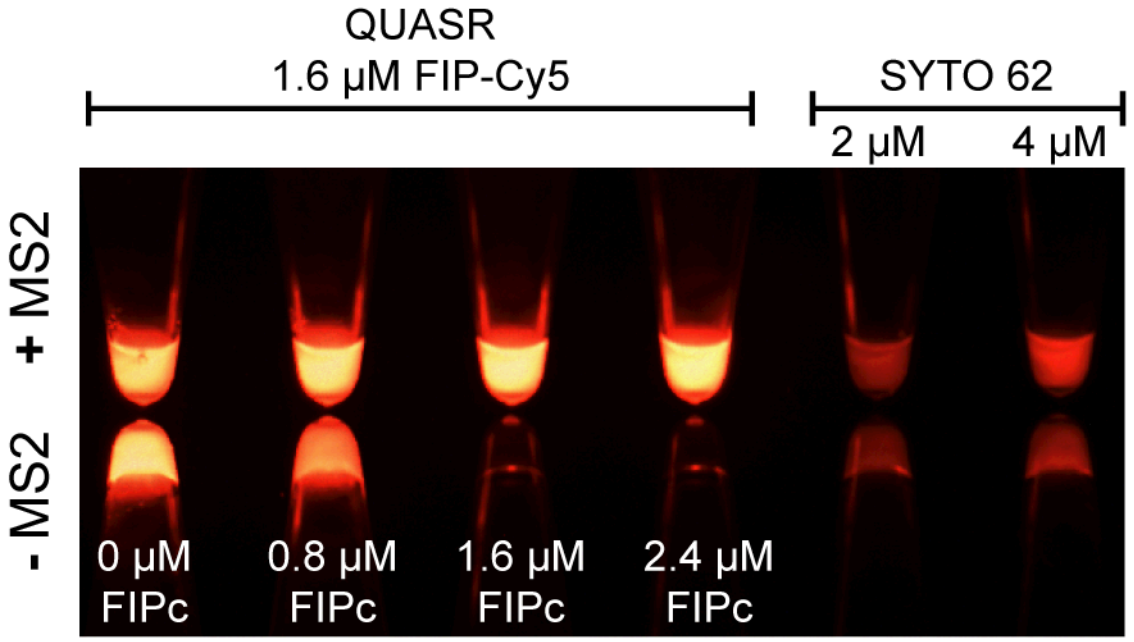
D, E, F, G... Color change and other nonspecific indicators of total DNA synthesis…

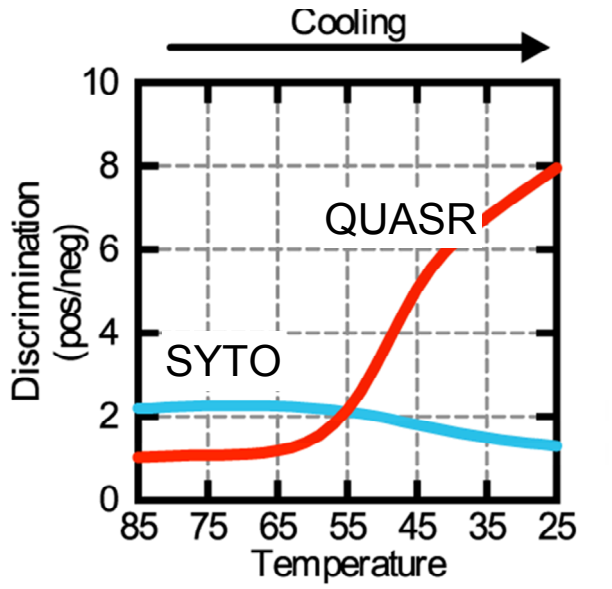
QUASR: Quenching of Unincorporated Amplification Signal Reporters



QUASR Proof of Concept (MS2)

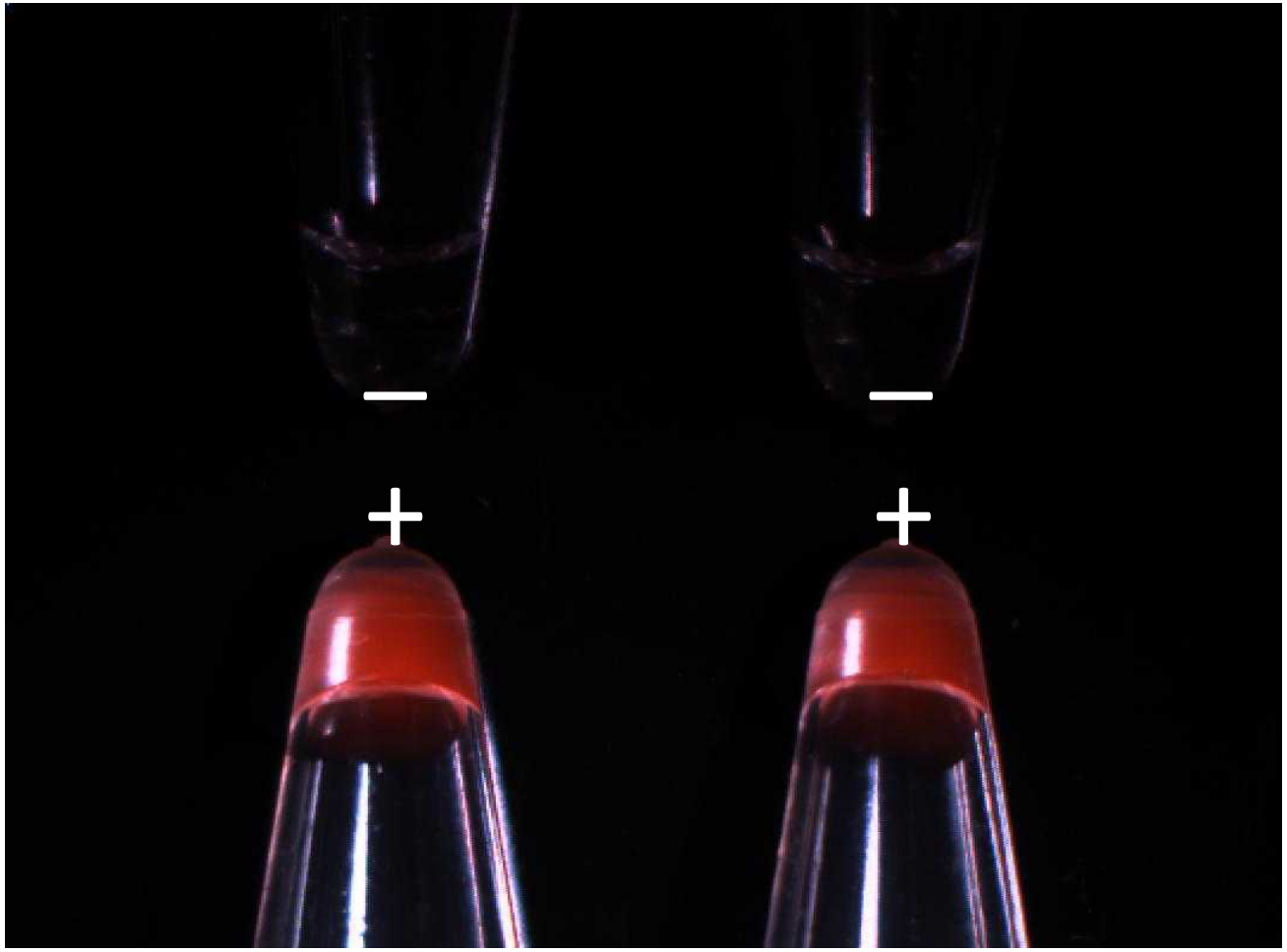
- QUASR signal develops as reaction cools below T_m of quench probe
- Contrasts to intercalating dye (*e.g.* SYTO) where discrimination is highest while hot
- Endpoint only, but closed-tube, very bright signals, and target-specific.





Ball *et al*, *Anal. Chem.* 2016

WNV QUASR assay (ROX label)



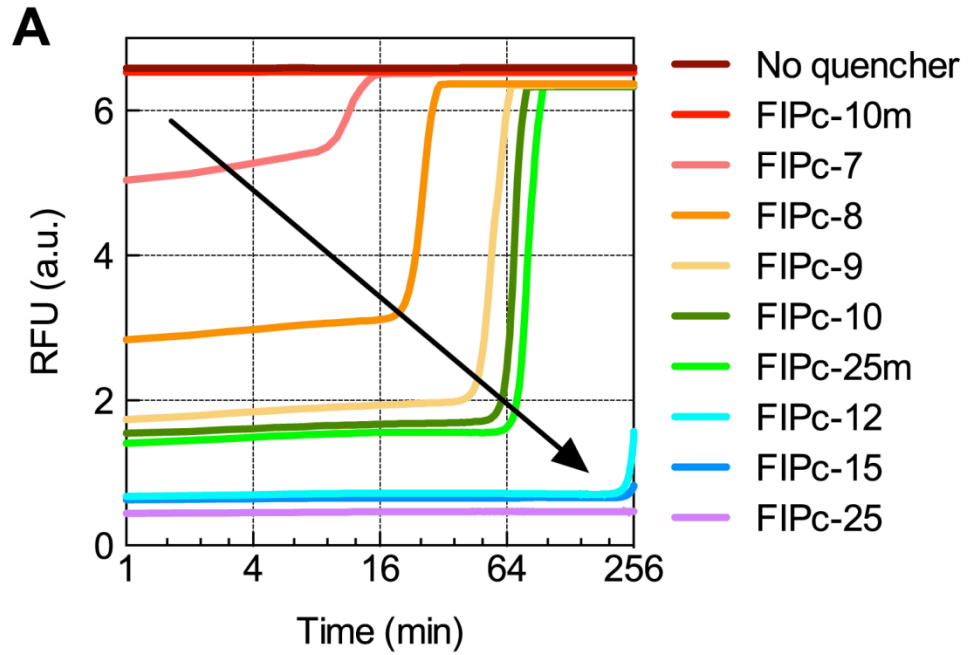
Published WNV RT-LAMP primer set from Parida *et al* adapted to QUASR detection

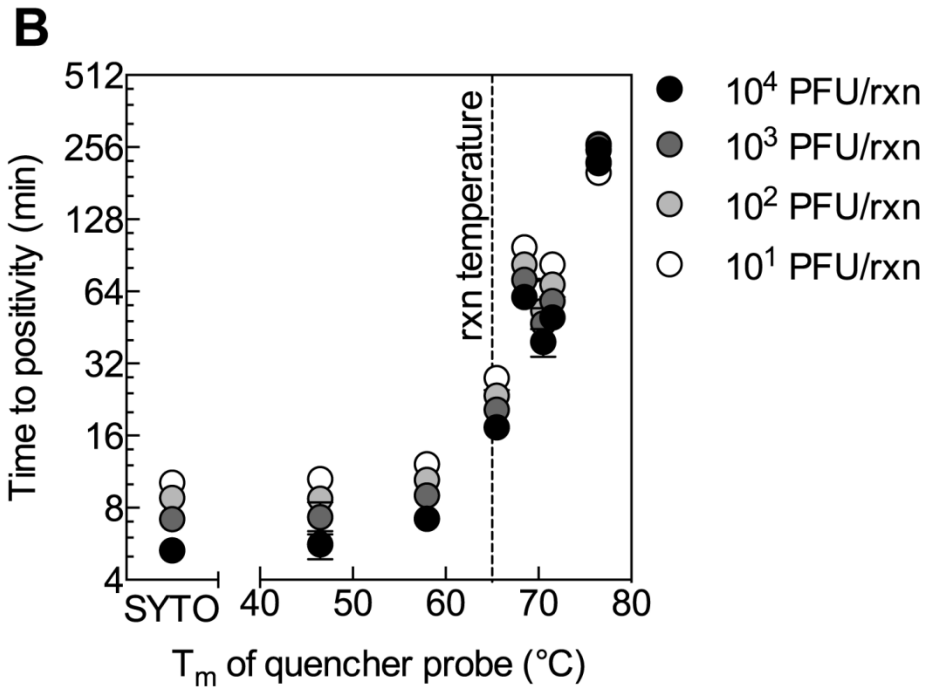
Color photo taken with green LED flashlight and magenta theatre lighting gel as a filter

- ☑ **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

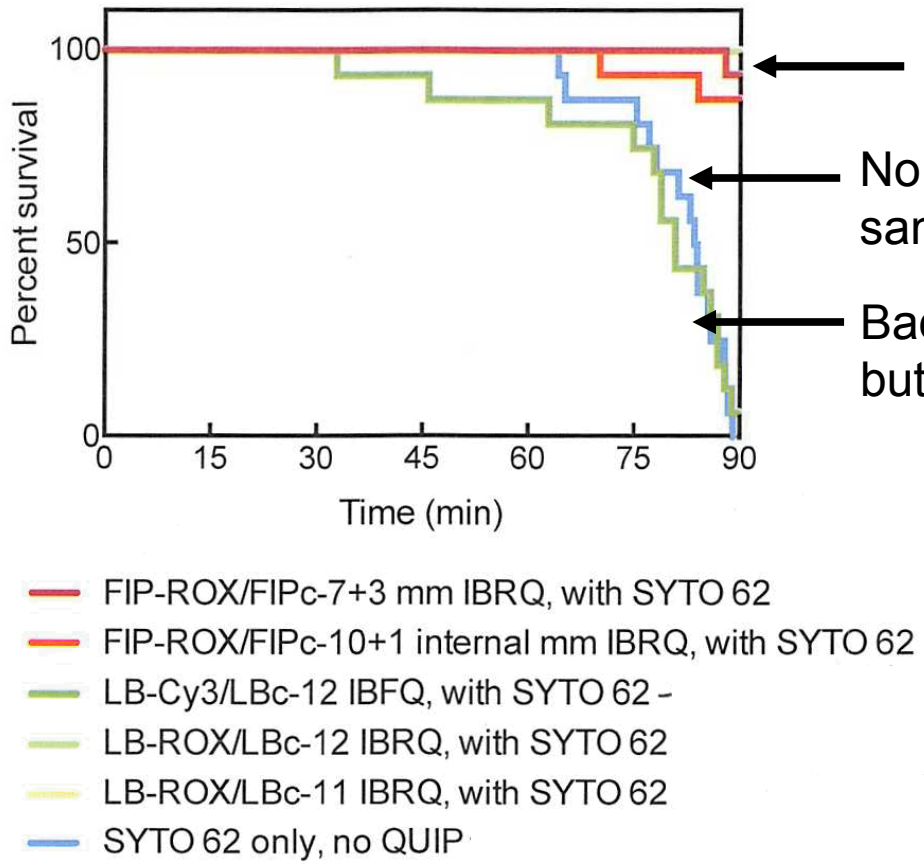




Ball *et al*, *Analytical Chemistry* 2016

QUASR can suppress detection of LAMP "false positive" amplification

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



C. Ball, *Analytical Chemistry* 2016

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

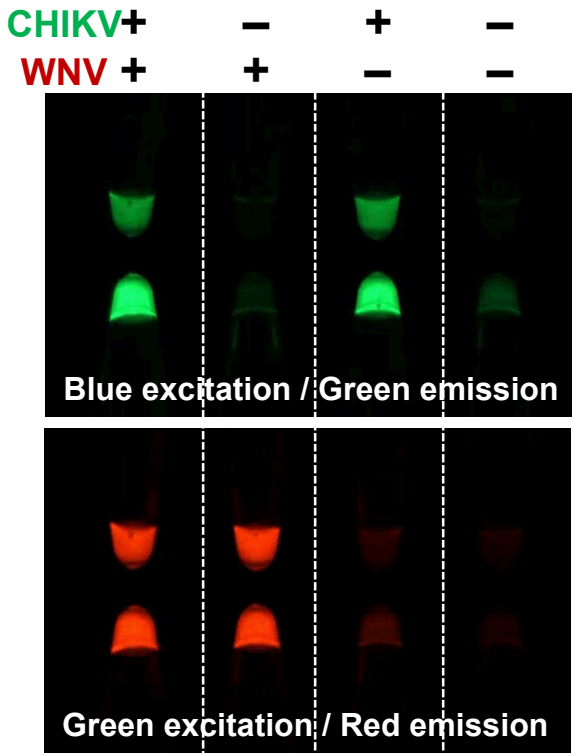
No QUASR: Spontaneous amplification in all samples, and visible with non-specific DNA dye

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

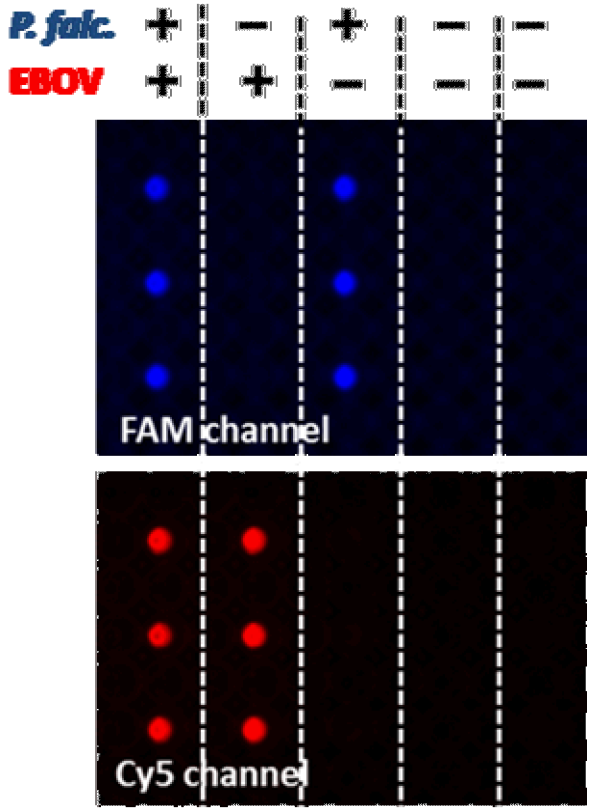
- "False positive" amplification usually occurs >40 minutes, so we define a cutoff of 30-40 minutes
- Even if a sample shows false positive with the SYTO dye, the QUASR signal is usually still negative
- *e.g.* 1/197 FP for QUASR, *vs* 67/145 FP for SYTO, in 90-minute extended rxns)

Multiplexing RT-LAMP with QUASR

(A) Chikungunya virus + West Nile virus



(B) *Plasmodium falciparum* + Ebola virus

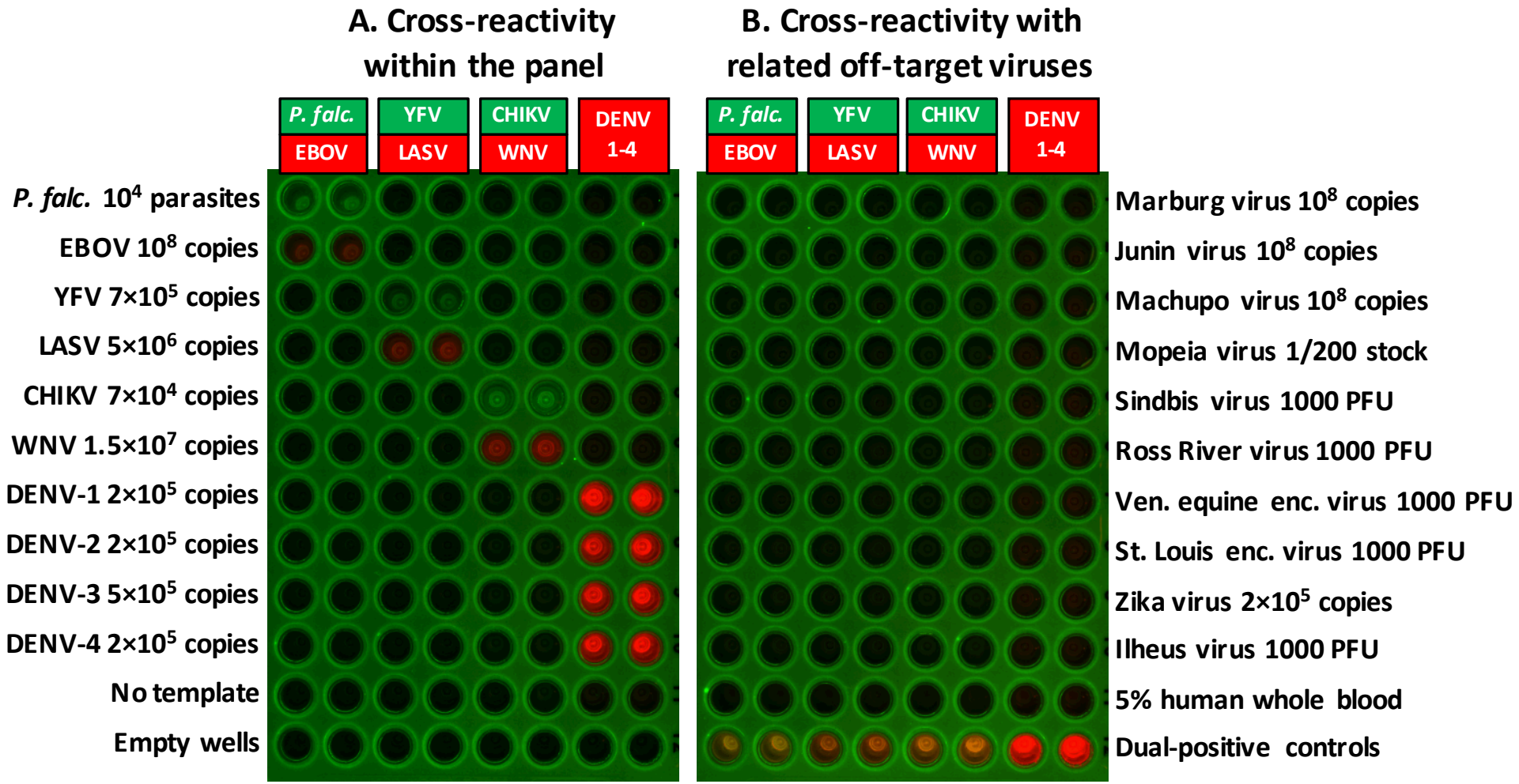


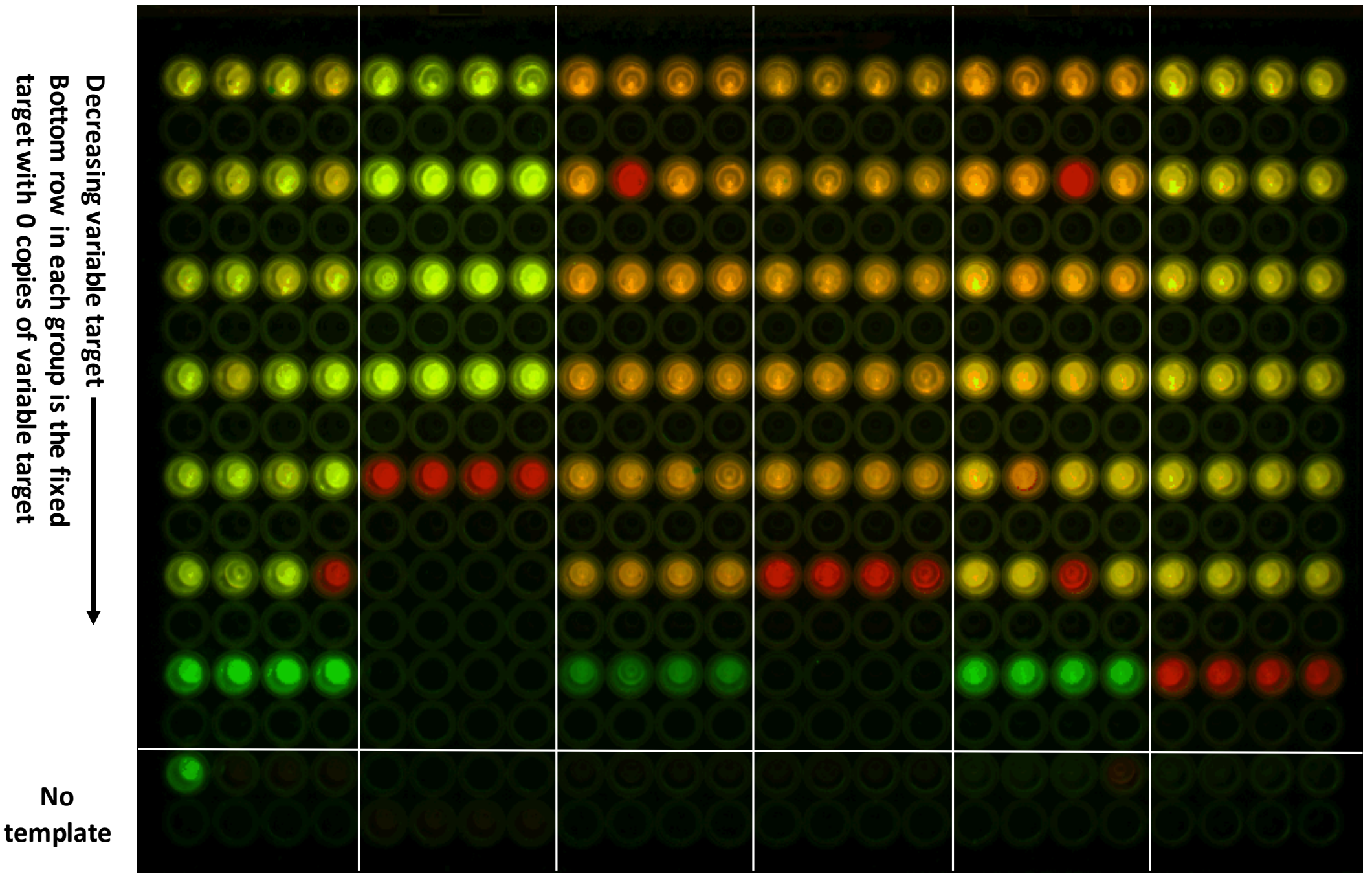
Yooli Light

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

LAMP historically was hard to multiplex

Multiplexed QUASR panel for febrile pathogens



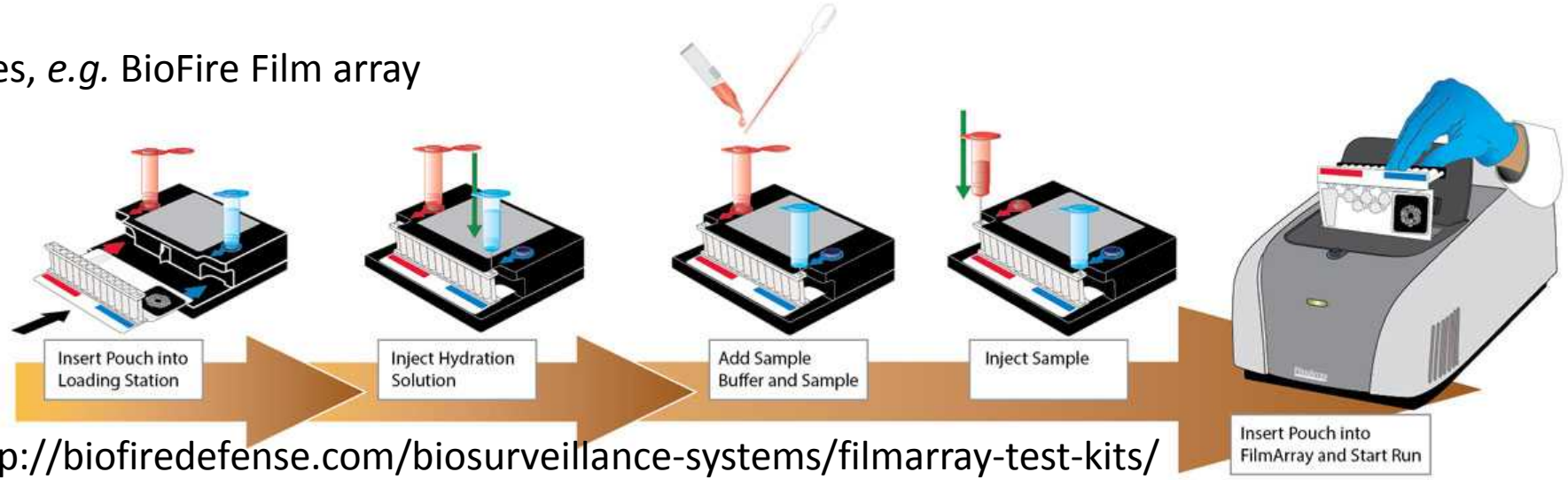

Pf 20 parasites
EBOV 10^8 – 2000 copies
Pf 10^4 – 20 parasites
EBOV 2000 copies
YFV 1400 copies
LASV 10^{11} – 10^6 copies
YFV $7{\cdot}10^6$ – 1400 copies
LASV 10^6 copies
CHIKV 220 copies
WNV 10^8 – 3000 copies
CHIKV $7{\cdot}10^6$ - 220 copies
WNV 3000 copies
Decreasing variable target
Bottom row in each group is the fixed target with 0 copies of variable target
No template

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

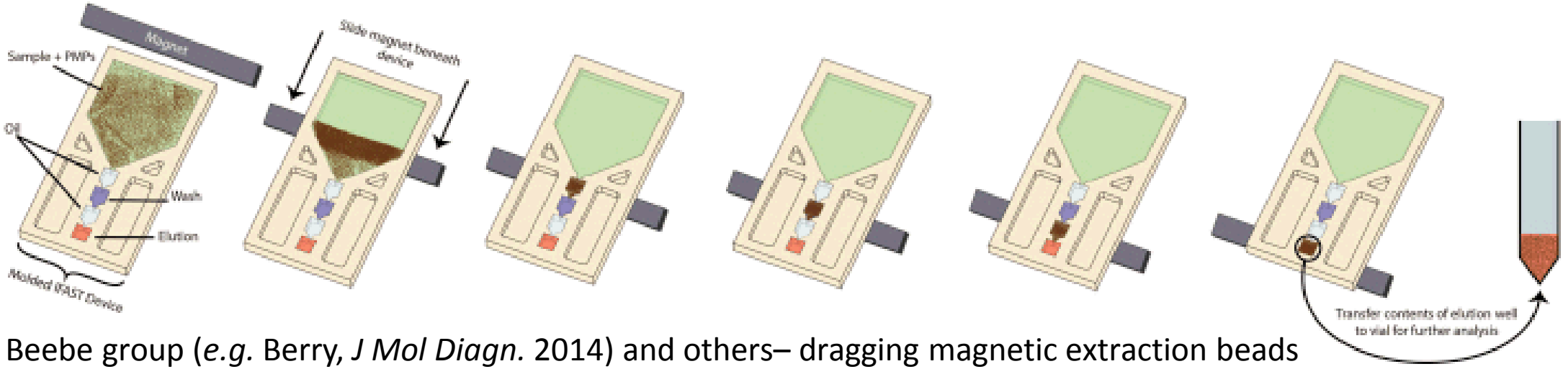
- For PCR, usually sample prep = DNA/RNA extraction
  - Serves to release DNA/RNA from the confines of cell or virus particles, remove inhibitory substances, and (in some cases) concentrate DNA/RNA
  - Usually some variation upon lyse / bind / wash / elute
  - Losses of DNA/RNA can occur at each of these 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 formats
- Others seek to change the paradigm

Adapting extraction for POC or field use

Fully integrated POC devices, *e.g.* BioFire Film array
Assay consumables & instrument perform a self-contained DNA/RNA extraction.

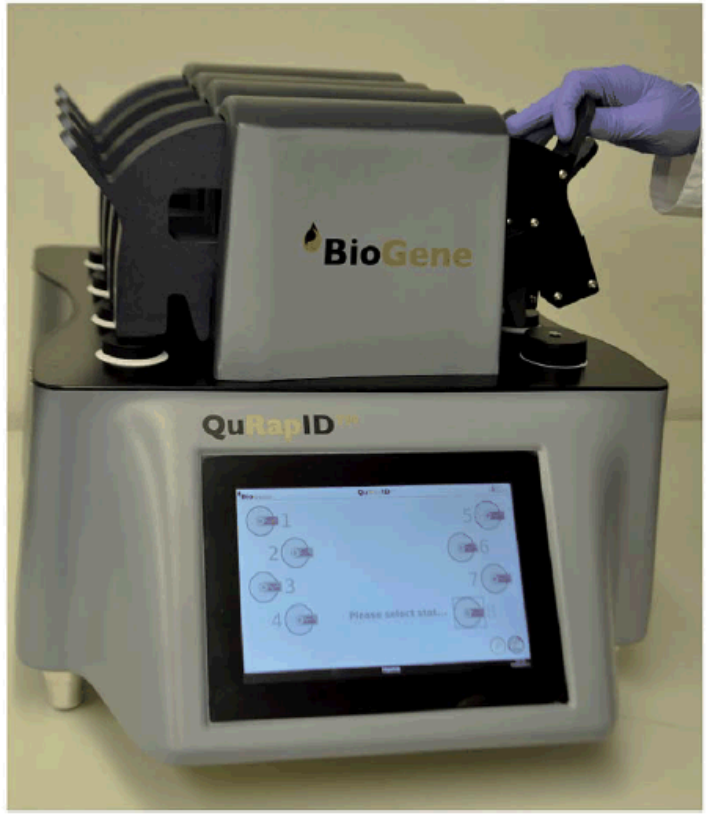


http://biofiredefense.com/biosurveillance-systems/filmarray-test-kits/

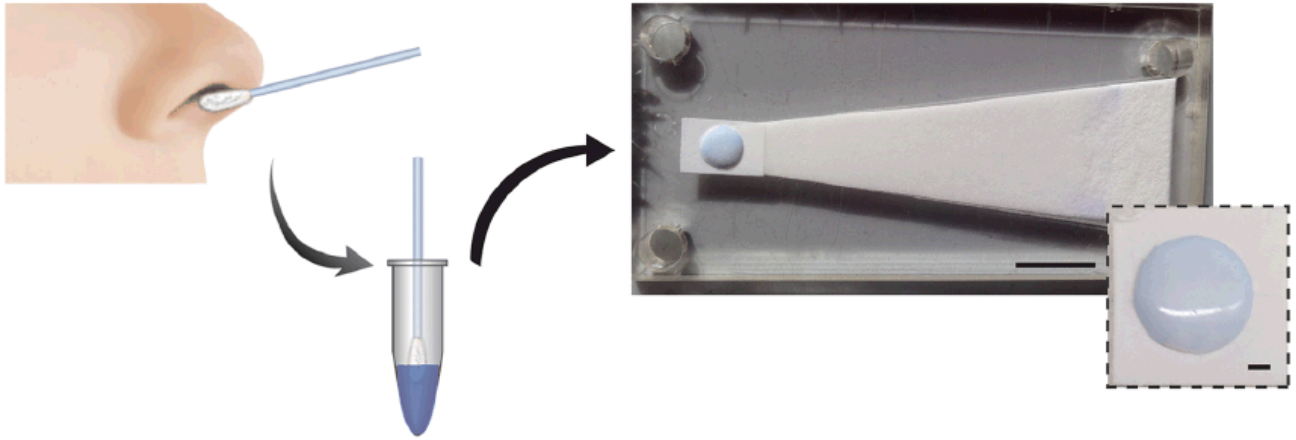


Beebe group (*e.g.* Berry, *J Mol Diagn.* 2014) and others– dragging magnetic extraction beads across interfaces (water/oil or water/air) to perform bind-wash-elute without pipetting

Changing the paradigm – novel approaches

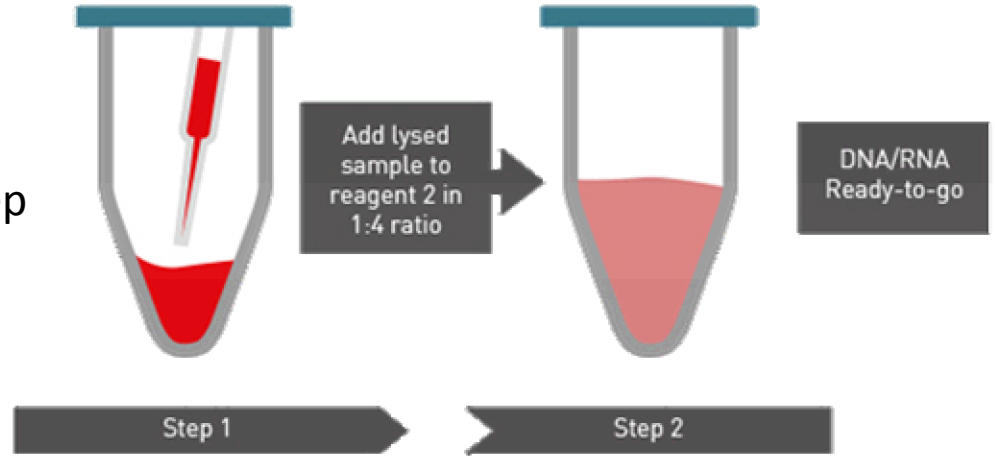


Shah *et al*, *Chem Sci* 2017 - Ebola qRT-PCR directly from whole blood using freeze-thaw lysis in a sub-zero thermal cycler, and inhibitor-resistant PCR reagents



Rodriguez *et al*, *Anal. Chem.* 2015 – Lyse, precipitate RNA, and capture on filter prior to isothermal amplification

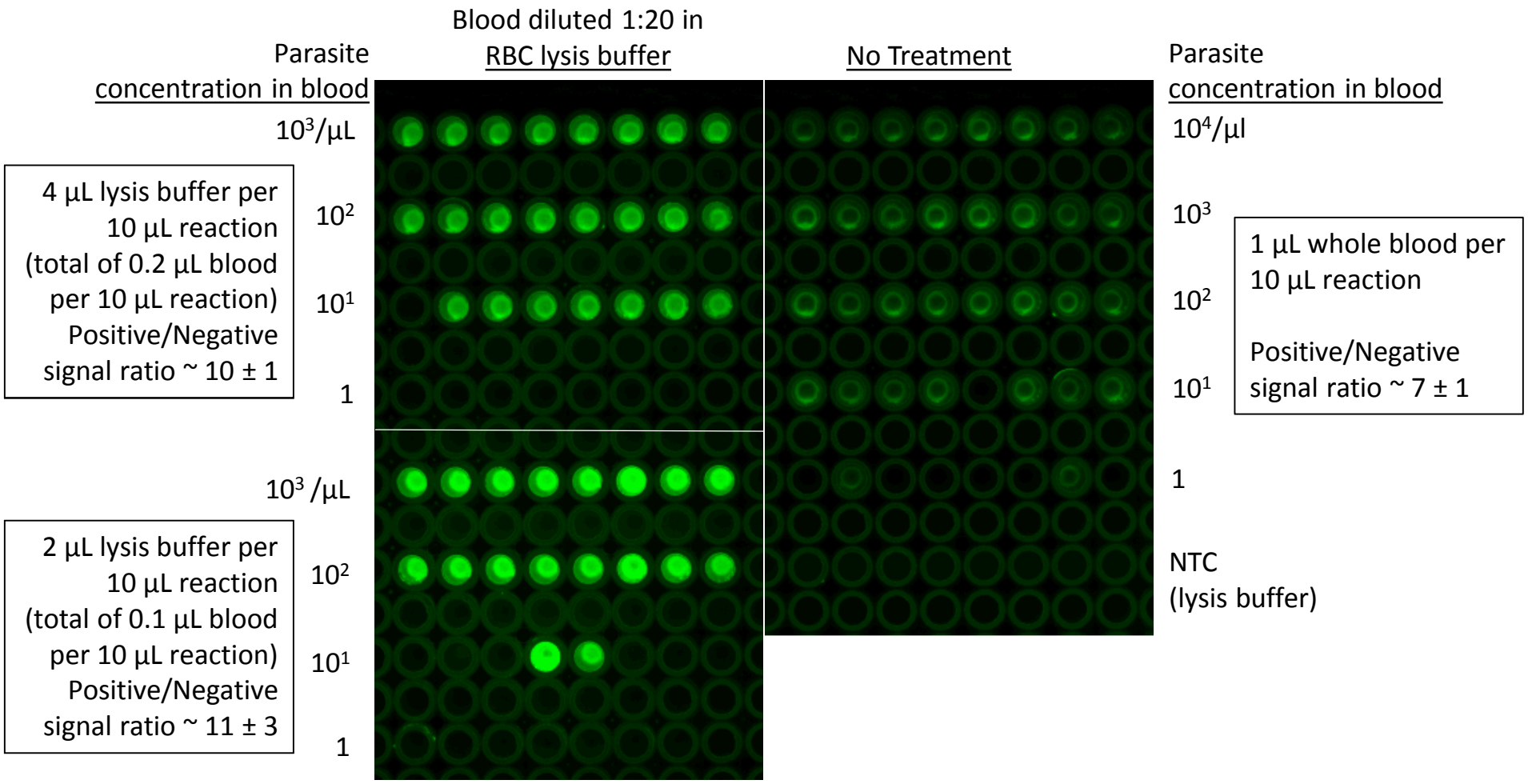
Arcis Biotechnology – 2-step dilution in proprietary reagents to lyse & extract PCR-ready DNA or RNA
http://www.arcisbio.com/



Further simplifying…

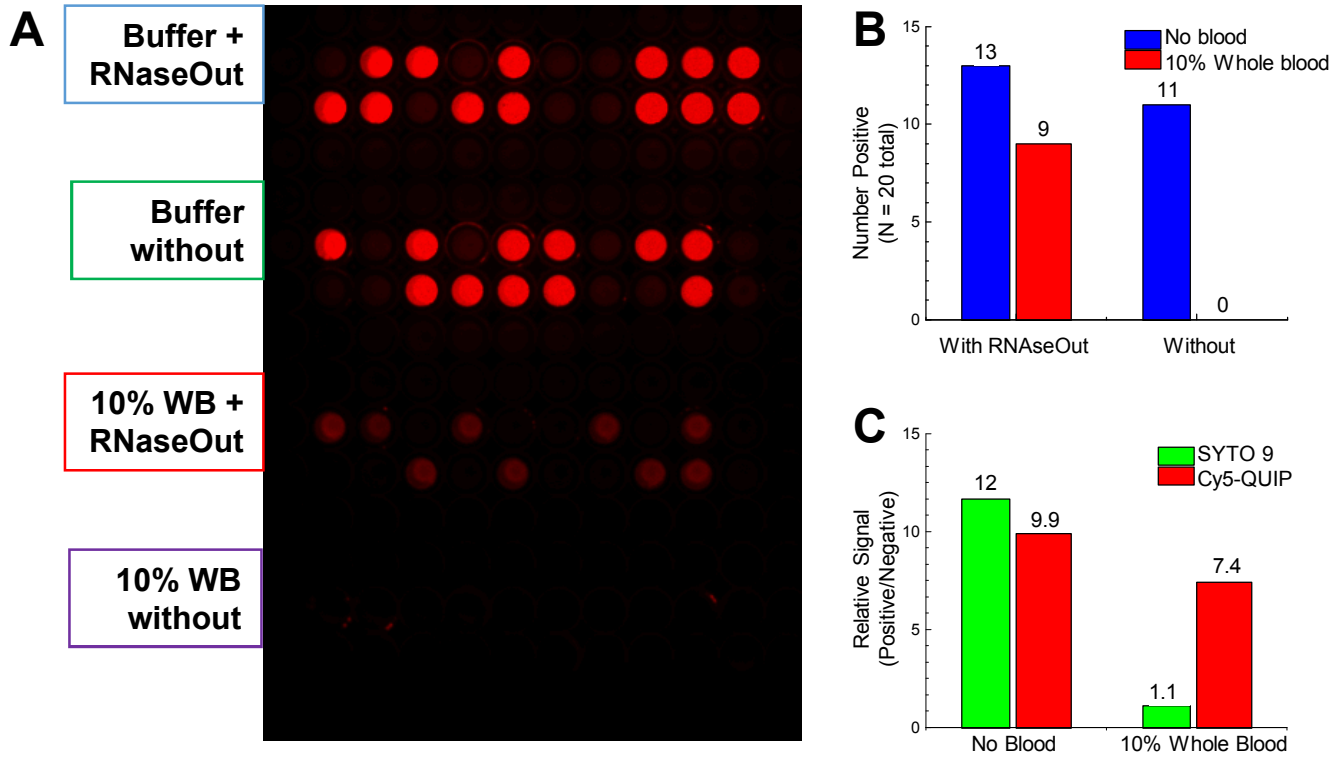
- Boiling or detergent lysis
- Or no sample prep at all (dilution)
- Many isothermal amplification techniques, and even PCR with novel variants of enzymes, can tolerate inhibitory substances such as blood
  - *Bst* DNA polymerase used in LAMP is known for its tolerance of crude samples
- We have tried 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

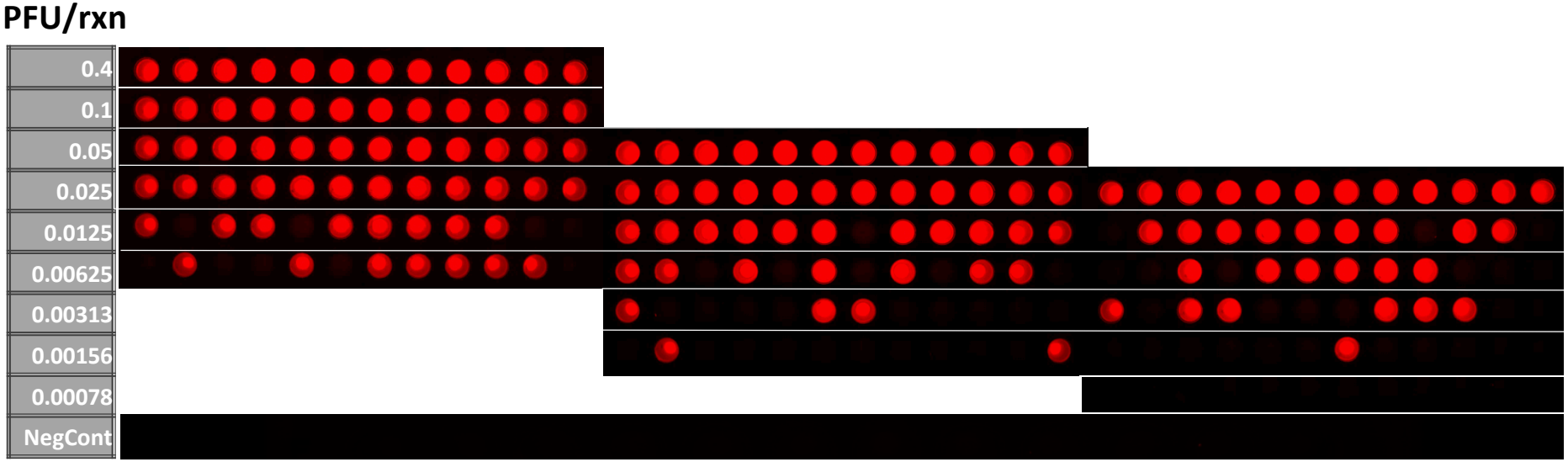


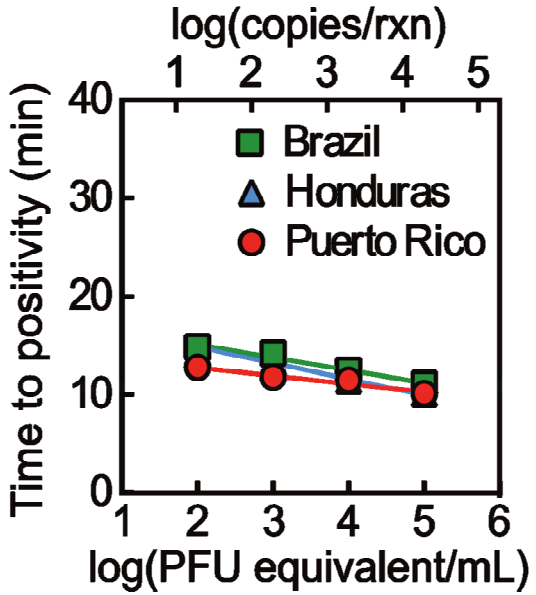
No Treatment: 1ul of whole blood sample
RBC lysis buffer : 1:20 diluted, 2 or 4ul added to rxn (so sampling 0.1 or 0.2 uL per reaction)
BIP-Cy3, 500ms exposure

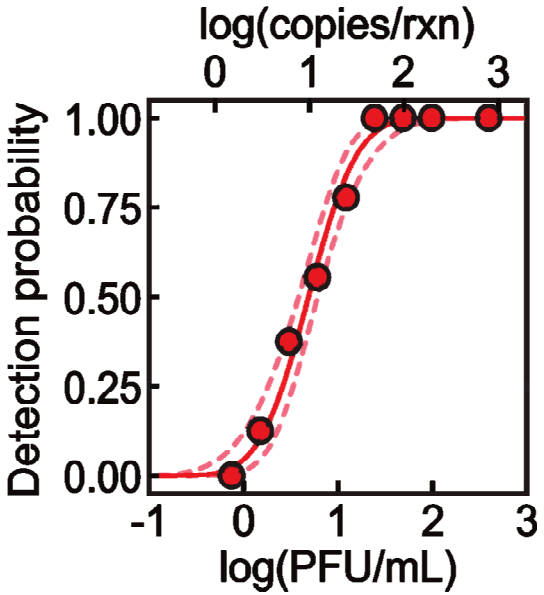
Addition of RNAse Inhibitor enables detection of Ebola RNA from whole blood



Zika RT-LAMP for Intact Virus (no extraction)







Sensitivity testing (10-30 replicates) with *intact virus* spiked into reaction buffer (no lysis/extraction)

<u>Detection probabilities</u>

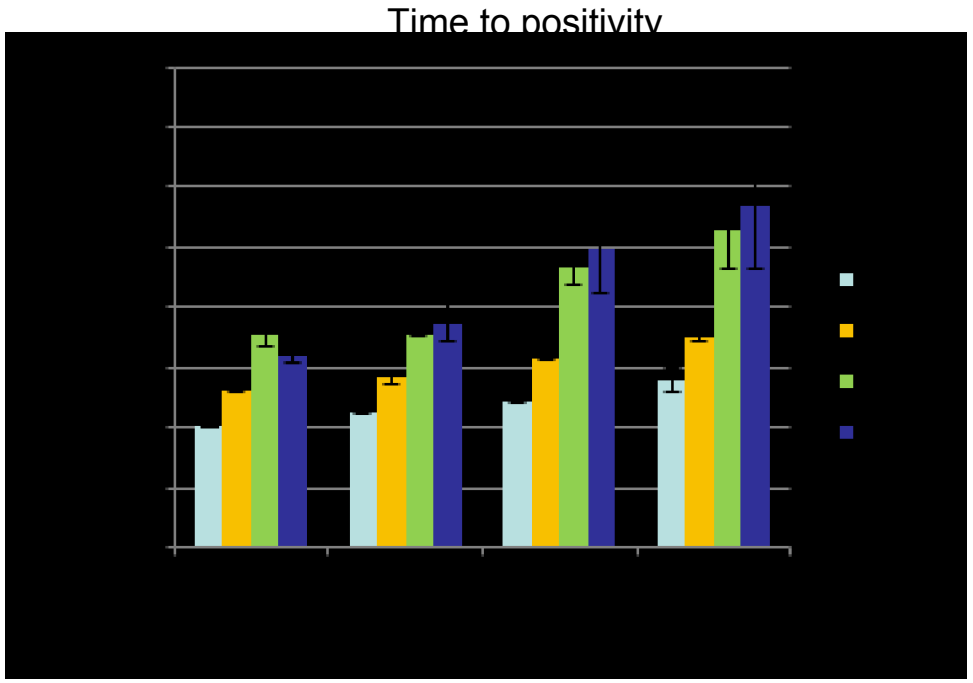
95% - 22 PFU/mL (5×10^4 copies/mL)

50% - 4.9 PFU/mL (10^4 copies/mL)

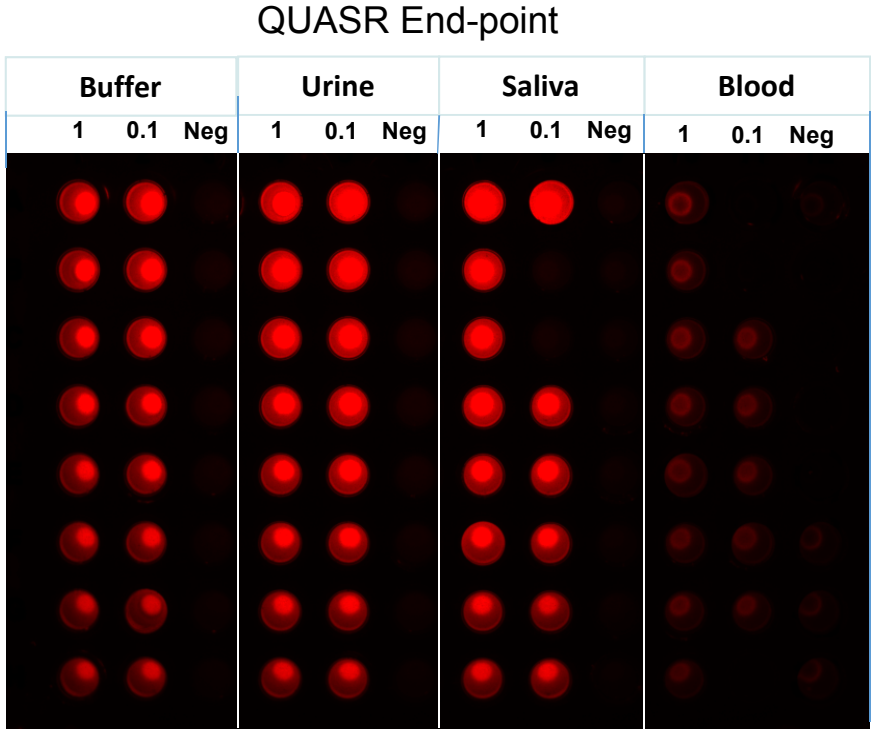
Priye *et al*, *Scientific Reports* 2017

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

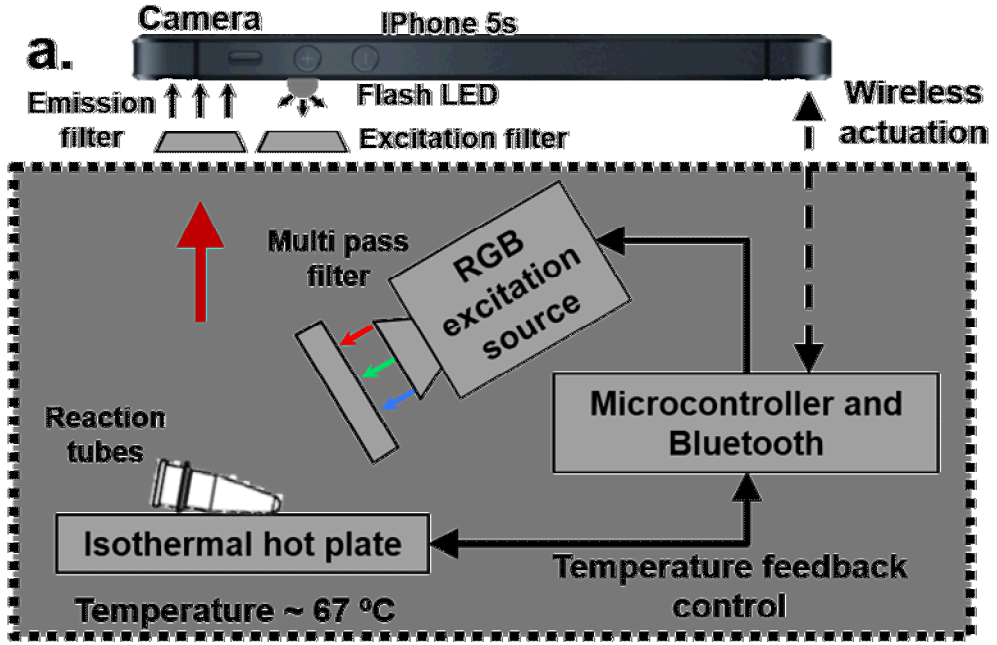


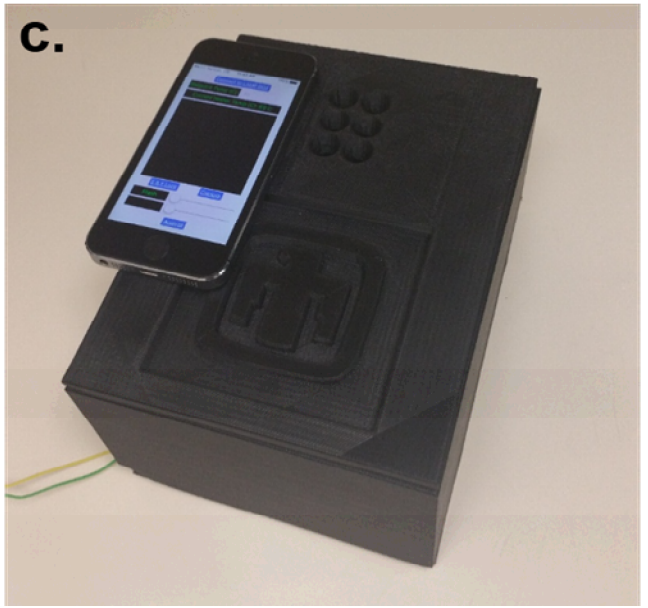
Priye *et al*, *Scientific Reports* 2017



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.

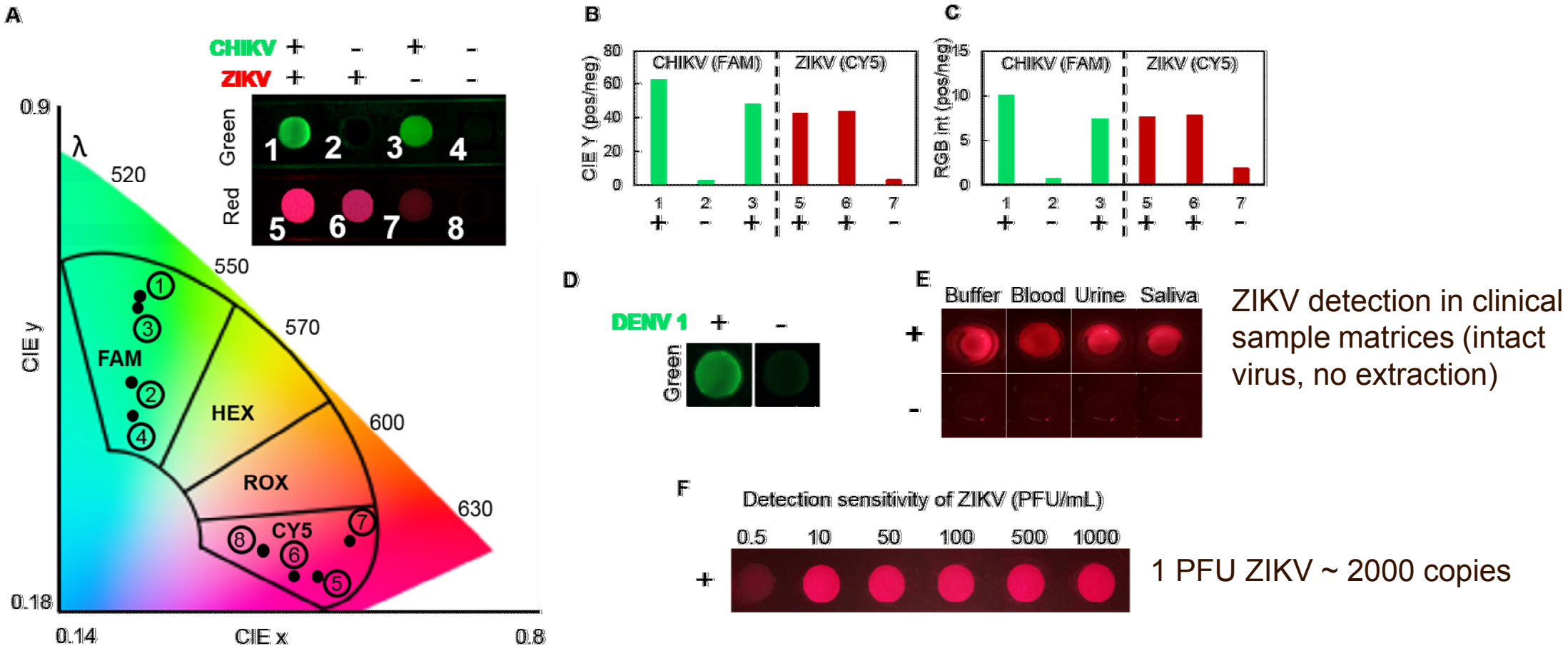




Priye *et al*, *Scientific Reports* 2017

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

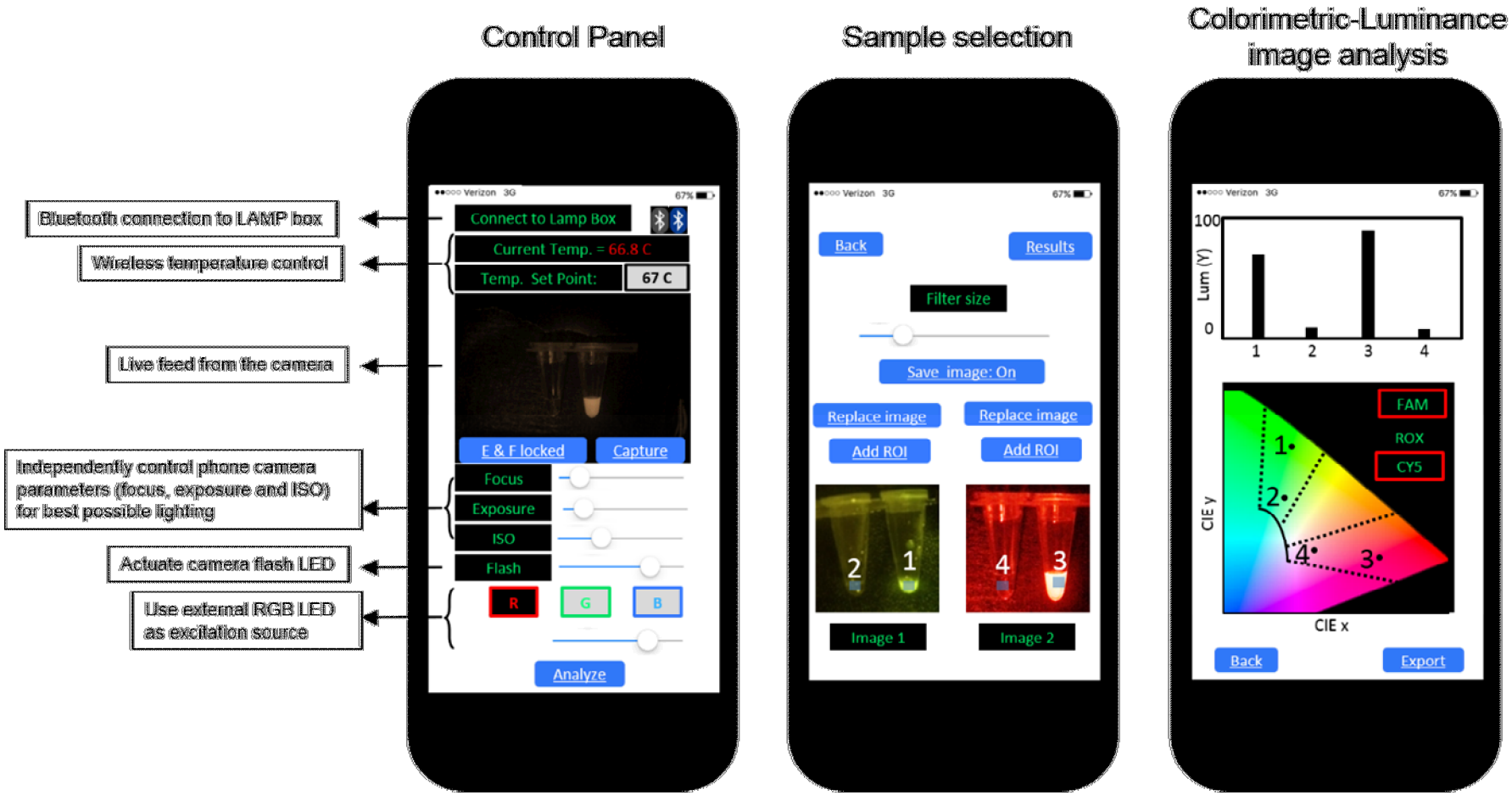


ZIKV detection in clinical sample matrices (intact virus, no extraction)

1 PFU ZIKV ~ 2000 copies

Priye *et al*, *Scientific Reports* 2017

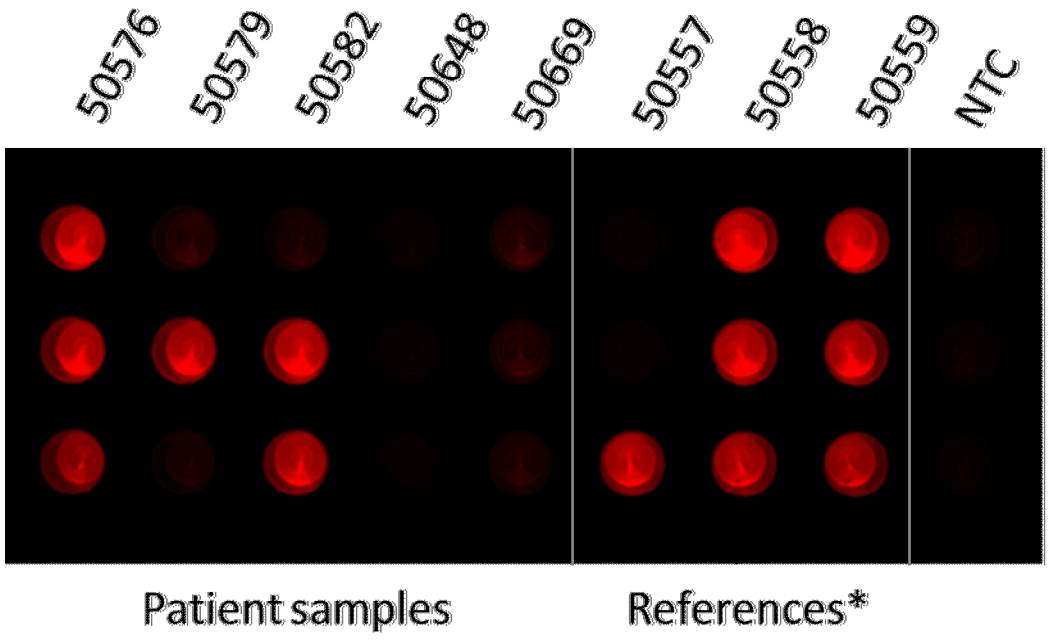
LAMP2Go App



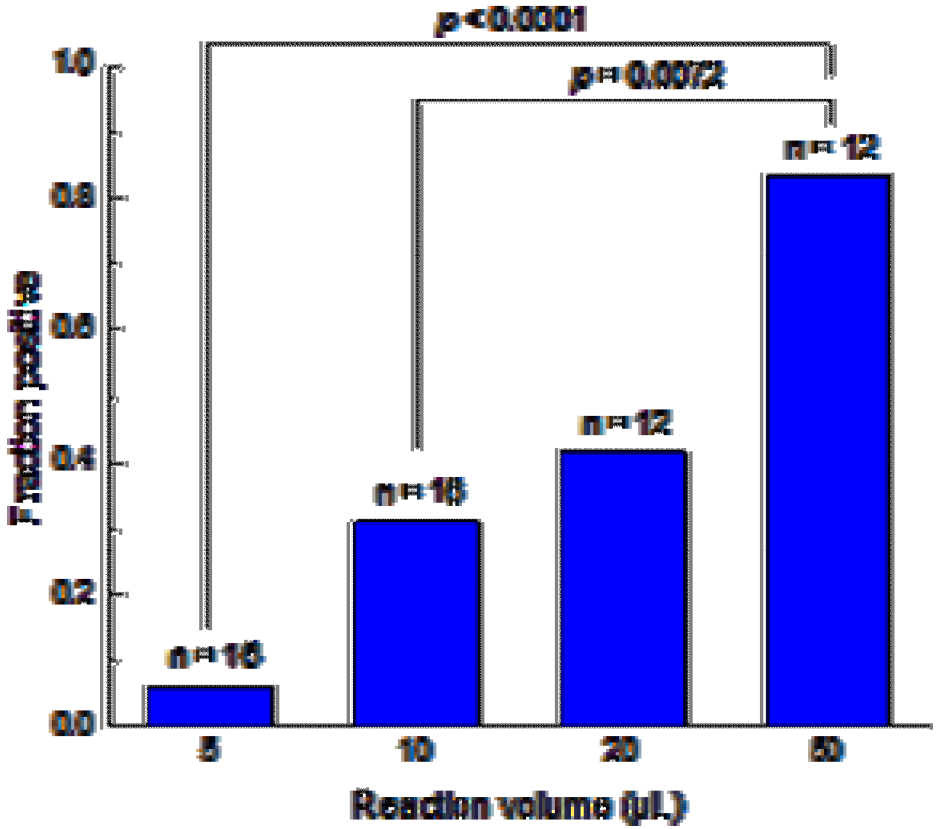
Versions for iPhone and Android
Older model phones ok (*e.g.* <$100 HTC Desire)

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)



Perspective on LAMP

- Based on number of publications, LAMP is one of the top contenders in the isorthermal/low resource/POC diagnostic space for “PCR alternatives”
  - Other top contender is Recombinase Polymerase Amplification (RPA)
- LAMP remains under patent by Eiken (through 2019) but it is relatively “open source” and amenable to DIY and hacking
- 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
- We are working on improving primer design *via* predictive modeling (thermodynamics and kinetics)

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, only 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).

Relevant Publications

S. Wheeler *et al*, Surveillance for Western Equine Encephalitis, St. Louis Encephalitis, and West Nile Viruses Using Reverse Transcription Loop-Mediated Isothermal Amplification, *PLoS One* 2016 (RT-LAMP with real-time monitoring, melt curve multiplexing, no QUASR)

C. Ball *et al*, Quenching of Unincorporated Amplification Signal Reporters in Reverse-Transcription Loop-Mediated Isothermal Amplification Enabling Bright, Single-Step, Closed-Tube, and Multiplexed Detection of RNA Viruses, *Analytical Chemistry* 2016 (description of QUASR method)

C. Ball *et al,* A simple check valve for microfluidic point of care diagnostics, *Lab on a Chip* 2016 (QUASR employed in microfluidic device)

A. Priye et al, A smartphone-based diagnostic platform for rapid detection of Zika, chikungunya, and dengue viruses, *Scientific Reports* 2017

N. Tanner *et al*, Simultaneous multiple target detection in real-time loop-mediated isothermal amplification, *Biotechniques* 2012 (DARQ technique, with real-time displacement of ‘probes’, partial inspiration for QUASR endpoint technique)

D. Rudolph *et al*, Detection of Acute HIV-1 Infection by RT-LAMP, *PLoS One* 2015 (uses a technique that resembles either QUASR or DARQ)