SAND2022-1219C

Sandia National Laboratories

Exceptional service in the national interest

LAMP & Isothermal Nucleic Acid Amplification: Ready for Prime Time?

Robert Meagher, Ph.D.

rmeaghe@sandia.gov

Moleular & Precision Med Tri-Con

February 21-23, 2021

SAND 2022xxxxxx

Sandia National Laboratories is a multimission laboratory managed and operated by National Technology & 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-NA0003525.

Acknowledgements

Sandia (and ex-Sandia) team

Yooli Light – Assay development and characterization

Taylor Moehling – Assay development and characterization

Aashish Priye – Smart phone platform & modeling

Cameron Ball – Portable devices, Assay characterization

Gihoon Choi – Portable devices

Sara Bird – Virology

Oscar Negrete - Virology

Collaborators

Lark Coffey (UC Davis)

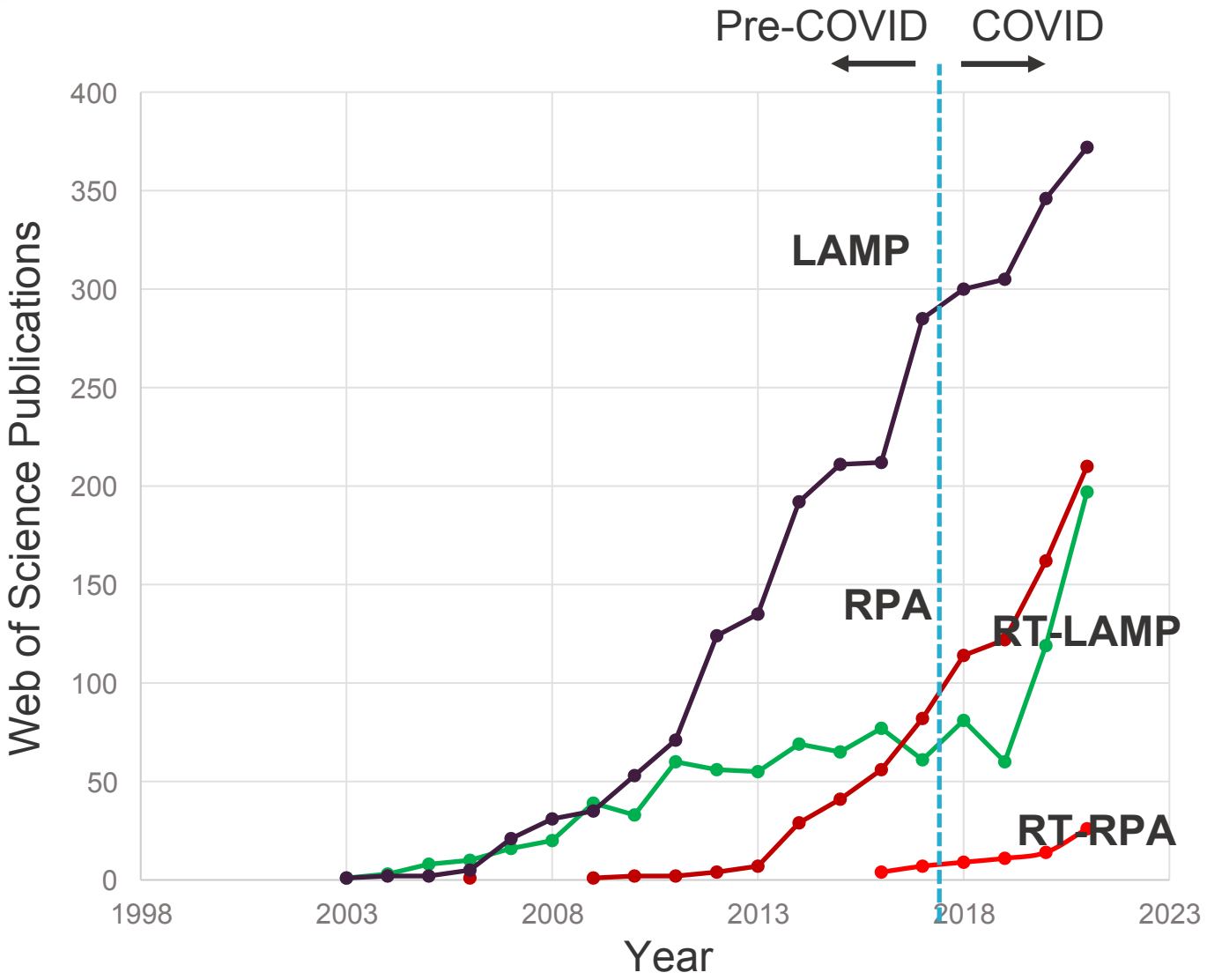
Scott Weaver (UTMB)

Slobodon Paessler (UTMB)

Funding

- Sandia Laboratory-Directed Research and Development (LDRD)
- NIH NIAID (R21-R33)
- DOE National Virtual Biotechnology Laboratory

Recent Publication Trends for Two Isothermal Amplification Methods



Percentage of publications associated with SARS-CoV-2

Year	2020	2021
RT-RPA	20%	55%
RT-LAMP	35%	69%

Web of Science Search terms:

- "Loop-Mediated Isothermal Amplification" (AND SARS-CoV-2)
- "RT-LAMP" OR ("Loop-Mediated…" AND Reverse)
- "Recombinase Polymerase Amplification" (AND SARS-CoV-2)
- "Recombinase Polymerase Amplification" AND "Reverse"

Changing Landscape for Isothermal Amplification Methods

Pre-COVID era

- "Low resource settings" often synonymous with the developing world
- Neglected tropical diseases (malaria, dengue, *etc*)
- Emerging diseases (Ebola, Zika)
- Plant & animal pathogens

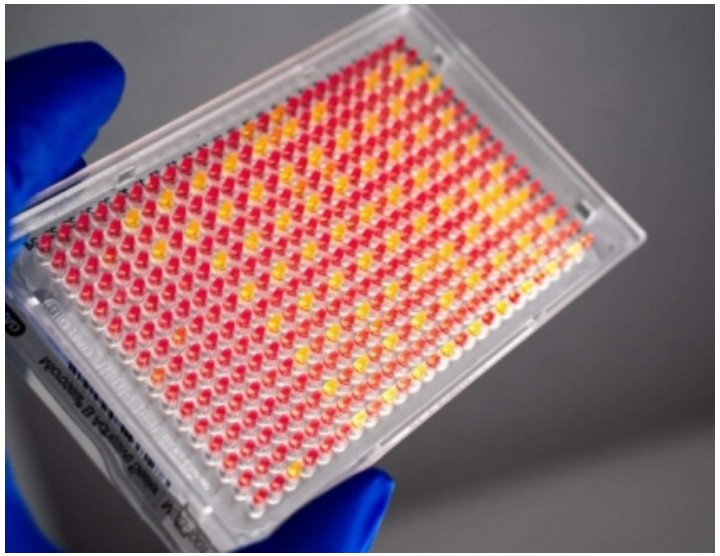
COVID era

- Diagnostic resources strained in all countries
- Contagious pathogens (COVID, Flu)
- Frequent testing associated with economic activity (work, school, travel)
- Non-traditional laboratory settings including home tests

Limited access to PCR is a common theme

The Setting Matters

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.



High-throughput laboratory (www.color.com)

Point-of-Care Rapid Tests (Image from ETHealthworld.com)

Home tests (Image from fortune.com)

LAMP – What is it good for? And why think twice?

PROs of LAMP

- Isothermal = lower power than thermal cycling
- Sensitive
- Fast: 15-45 minutes sample-answer
- **Able to handle crude or lightly processed samples without extraction**
- Crowd-sourced innovation

CONsiderations

- **Reputation for false-positives**
- **Usually LESS sensitive than qPCR**
- Speed depends on other factors
- Extraction-free applications need empirical testing on case-by-case basis
- Still requires care for temperature control.
- Complex reaction mechanism
- Complex primer design

Sample prep without bind/wash/elute

- Many isothermal amplification techniques, can tolerate inhibitory substances from clinical sample matrices.
- A separate lysis step is not always required for LAMP.
- Extraction-free approaches have become very popular for COVID-19 assays
- Three general types of sample matrix effects to consider:
  1. A substance in the sample matrix **inhibits the enzyme**
  2. The sample matrix contains buffering compounds or salts that **indirectly** affect the amplification
  3. The sample matrix contains **nucleases** that degrade the template.

Example: SARS-CoV-2 RT-LAMP in saliva or swabs

- Decrease in sensitivity is consistent with RNase degradation of template.
- Detergents can improve viral lysis at low temperature, but this exposes RNA to RNase.
- RNA degradation can happen in the time between viral lysis and reverse transcription.
- Heat lysis of virus does not irreversibly denature RNases.

Strategies for RNase mitigation

- High dilution
- Add carrier RNA to ‘occupy’ RNases
- Add RNase inhibitor
- HUDSON protocol: Heat to denature RNase

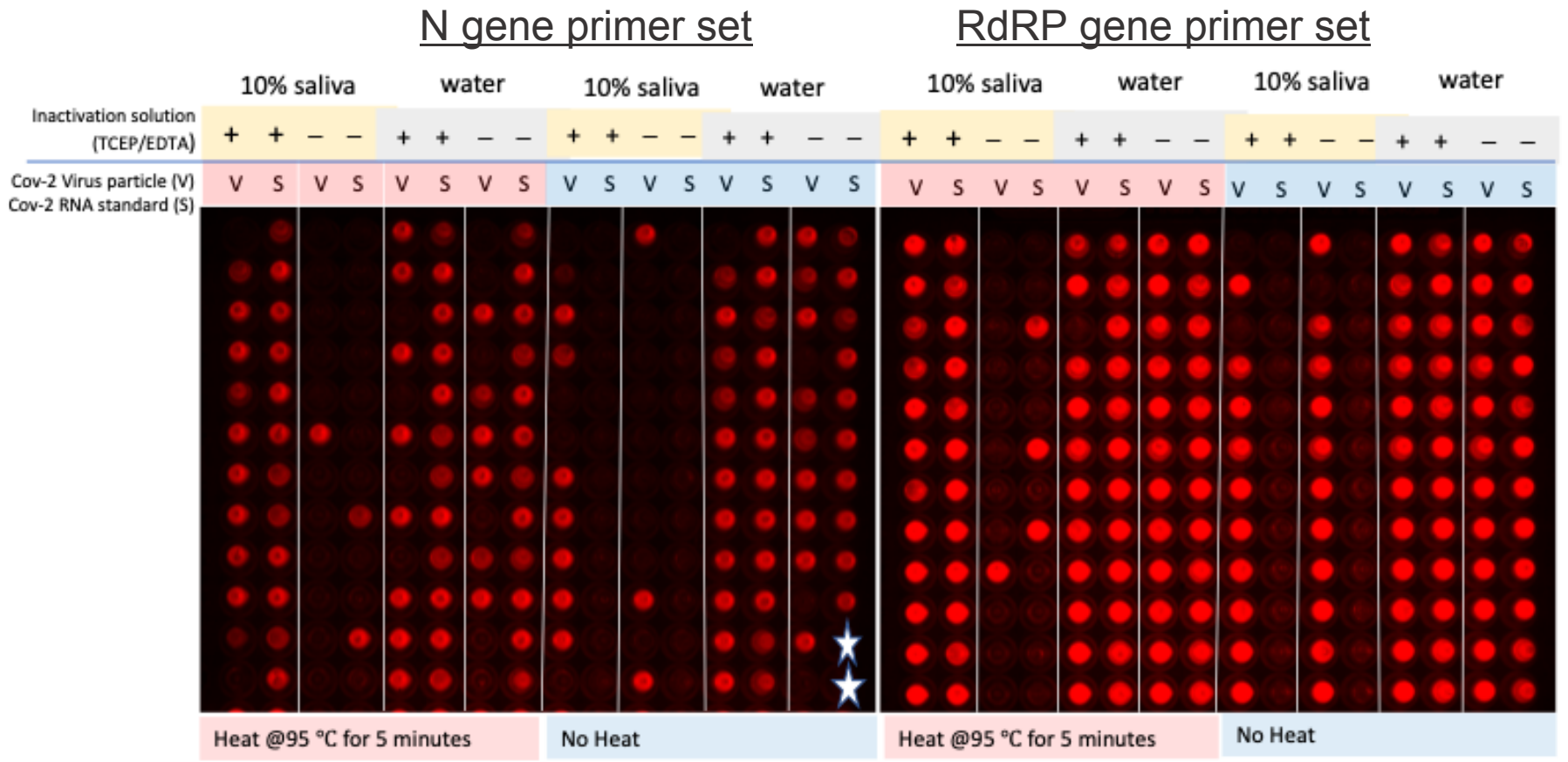
  + TCEP (reduces disulfides to prevent RNase refolding)

  + EDTA (chelates divalent cations; may inhibit RNases prior to denaturation)

  This protocol works for us, but requires dilution post-treatment to dilute EDTA

Pretreatment improves SARS-CoV-2 detection from saliva

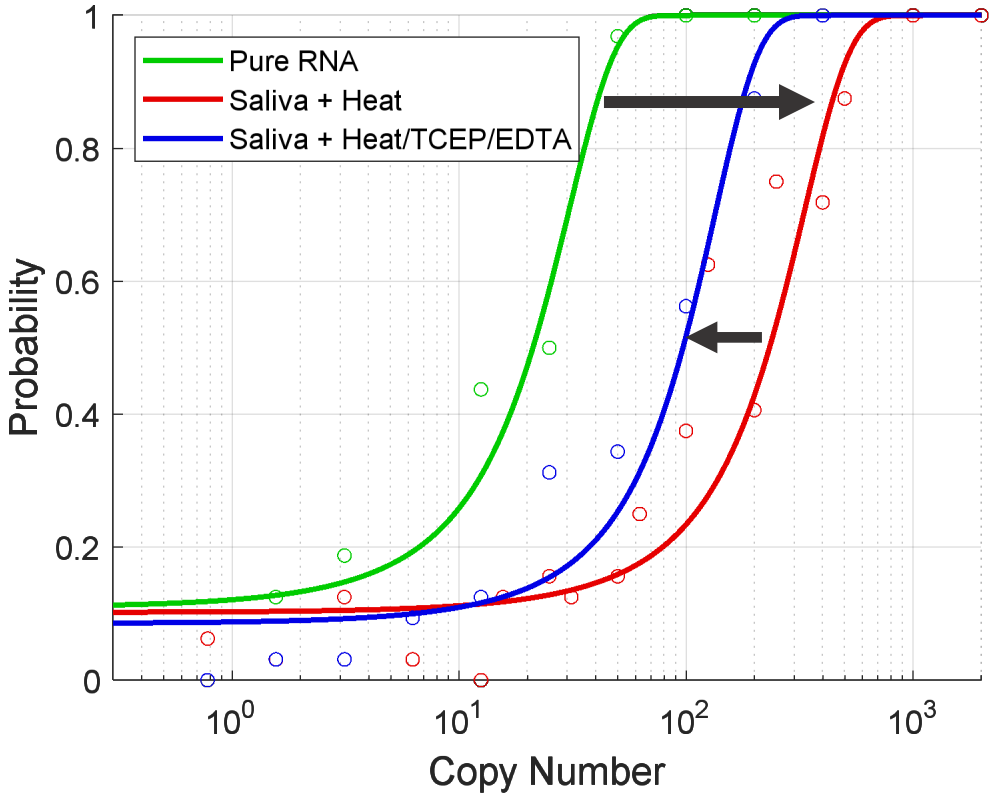
- 200 copies/reaction, V = whole virus (inactivated), S = RNA standard
- Heat + TCEP/EDTA improves detection of both whole virus and standard
- TCEP+EDTA without 95C heat improves whole virus but not standard (unprotected RNA is degraded)
- 95C heat without TCEP+EDTA is not sufficient to protect RNA.



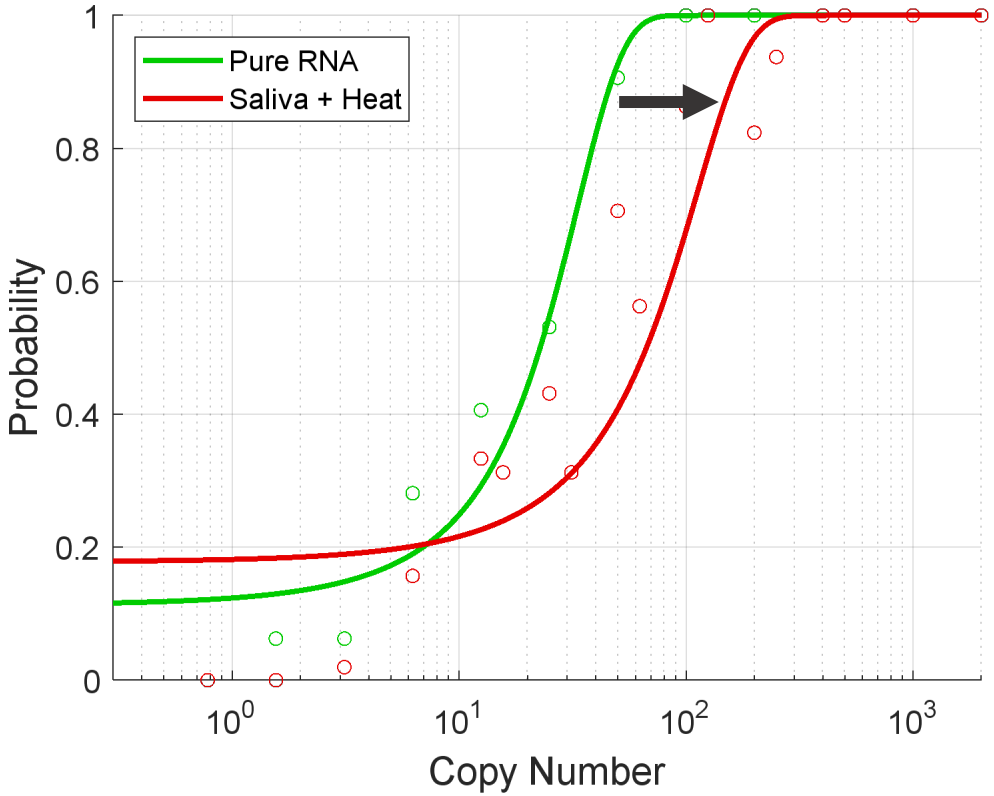
Quantitating effect of saliva & pretreatment on SARS-CoV-2 assays

- 10% saliva resulted in a larger performance hit to one primer set than the other
- Heat/TCEP/EDTA ‘recovered’ some performance but not back to level or pure RNA

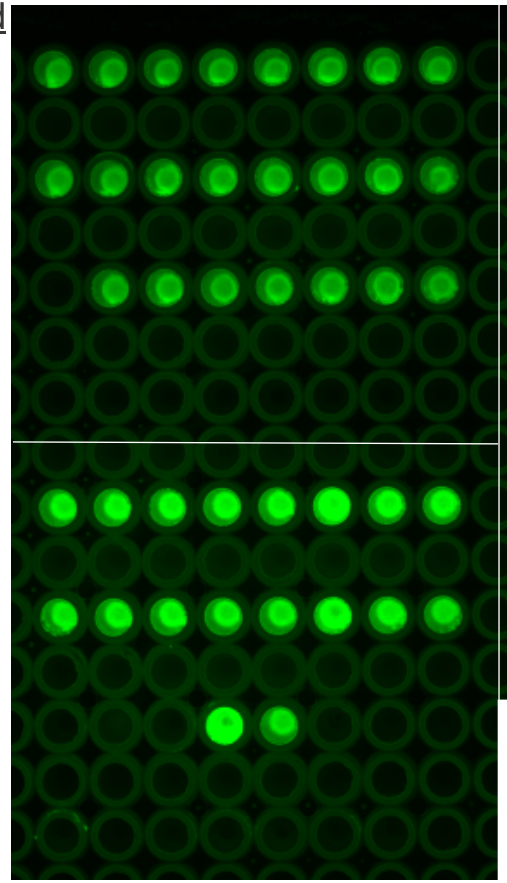
N gene primer set



RdRP gene primer set



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



Parasite concentration in blood

10^3/µL

10^2

10^1

1

Blood diluted 1:20 in RBC lysis buffer

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

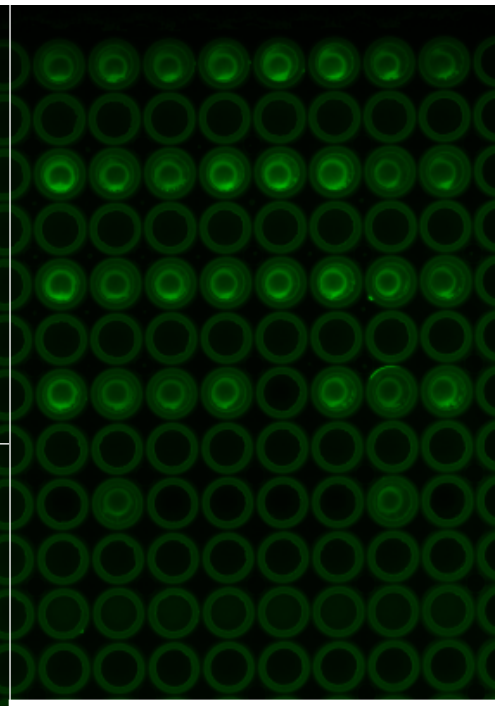
10^3 /µL

10^2

10^1

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 µL whole blood per 10 µL reaction

Positive/Negative signal ratio ~ 7 ± 1

1

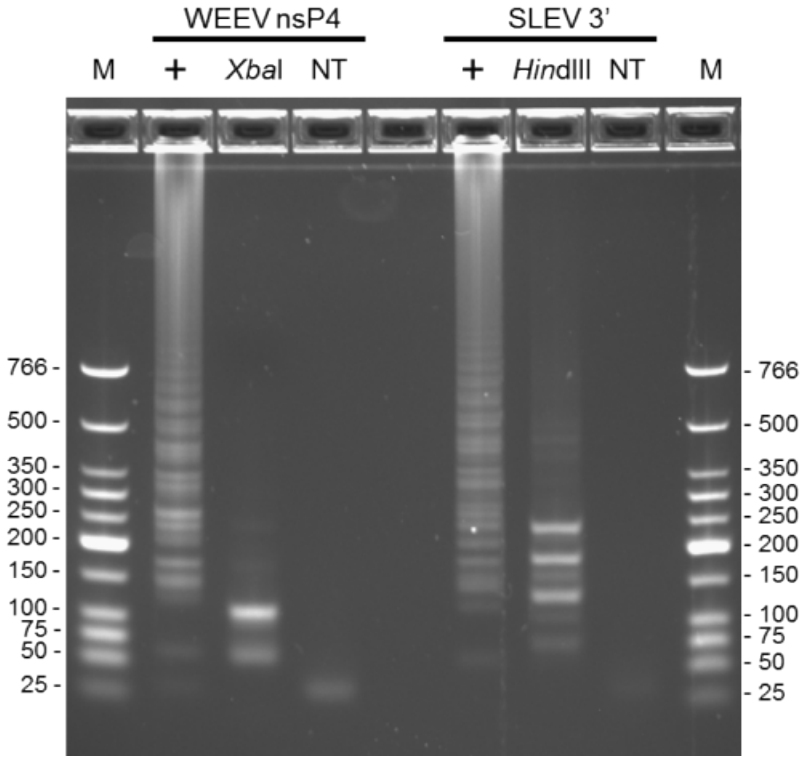
NTC (lysis buffer)

“No treatment” gives best LOD due to highest sample loading.
High blood loading does quench the fluorescent signal.

LAMP, specificity, and false positives

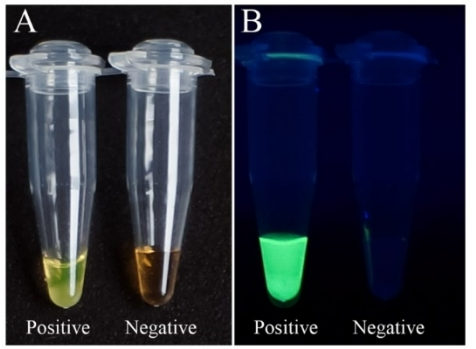
Several older (and some newer) methods to assess LAMP were **"open-tube"**

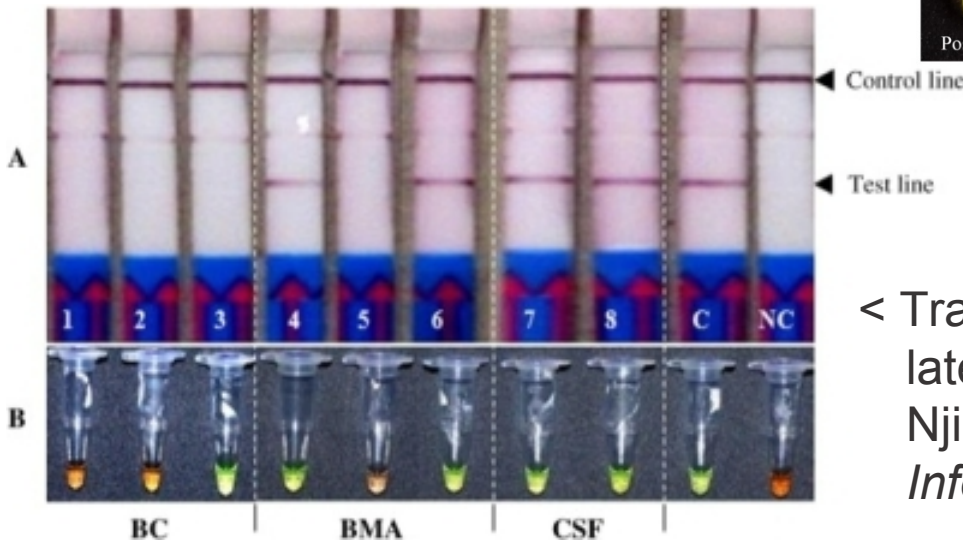
- Amplicon contamination almost inevitable when opening the tube!
- Absolutely requires **strict separation** of sample prep and post-reaction analysis



^ Gel with restriction digest, *e.g.* Wheeler *PLOS One* 2016

Post-reaction addition of SYBR Green, *e.g.* Nie *PLOS One* 2012 >



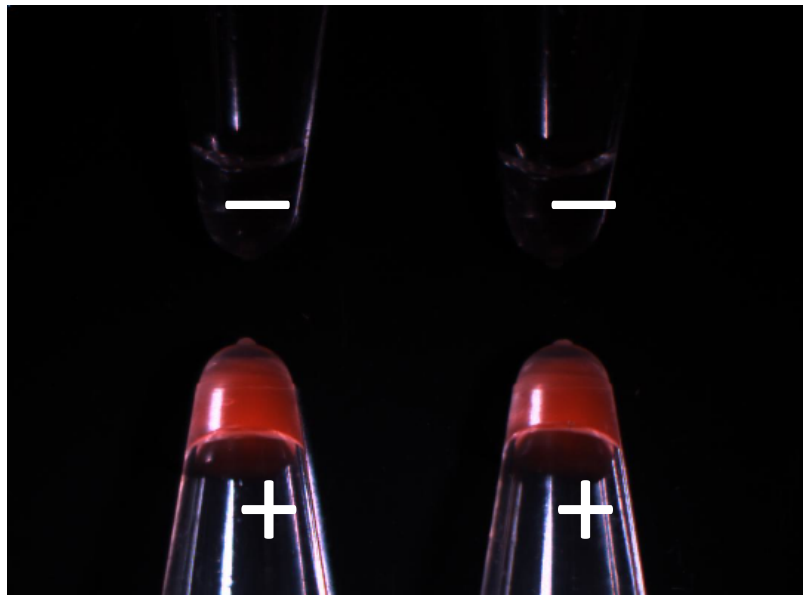


< Transfer to exogenous lateral flow assay, *e.g.* Njiru *Diagn. Microbiol. Infect. Disease* 2011

Closed-tube monitoring methods for LAMP mitigate false-positives due to amplicon contamination

Closed-tube methods are the norm now

- especially **colorimetric methods**
- intercalating dyes (*e.g.* SYTO dyes)
- Self-contained transfer to lateral flow
- pseudo-probe based methods (*e.g. QUASR)*



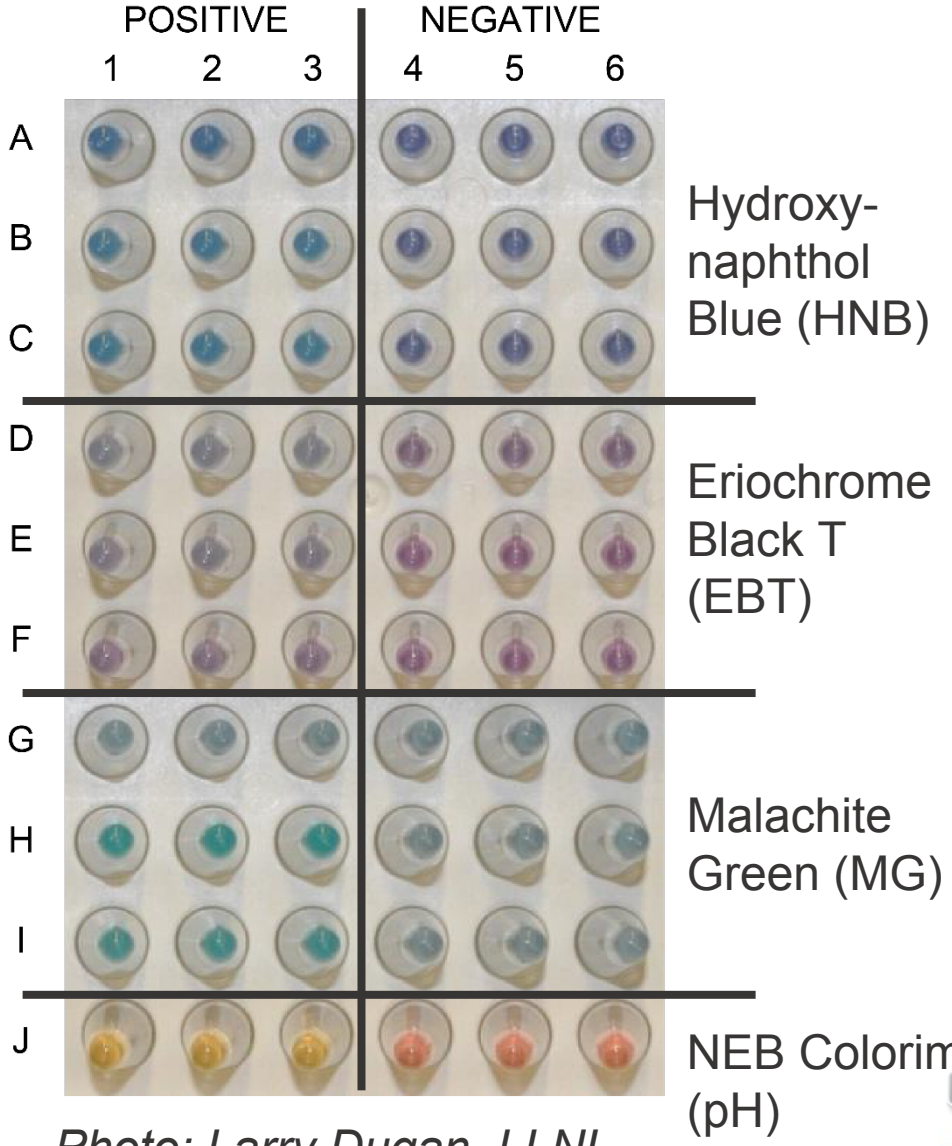
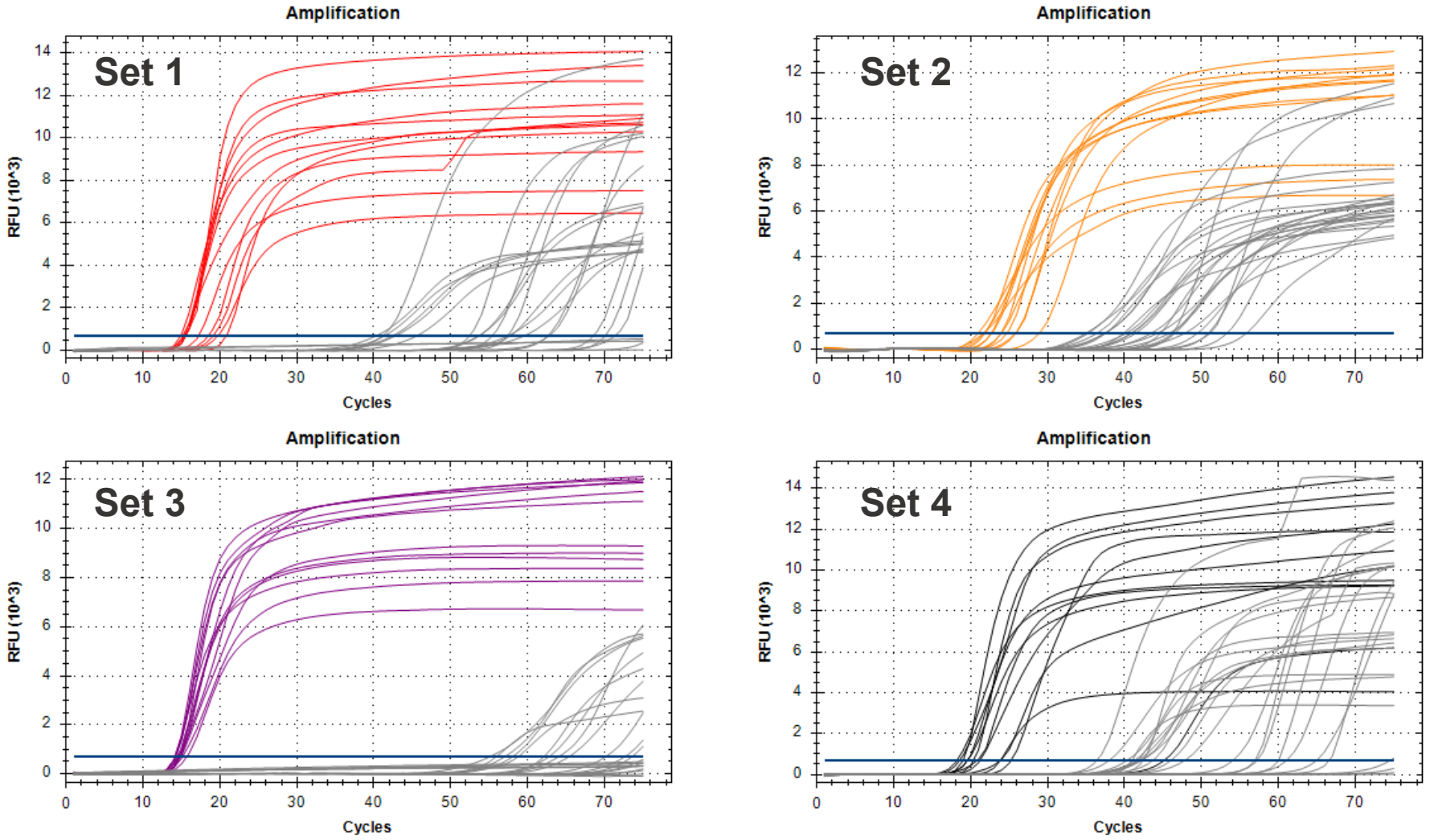


Photo: Larry Dugan, LLNL

Non-contamination-associated false-positives persist

***Example**: Screening candidate primer sets for COVID-19 (real-time monitoring with SYTO 9). **Colored traces**: 50 RNA copies. **Grey traces**: no-template controls*



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

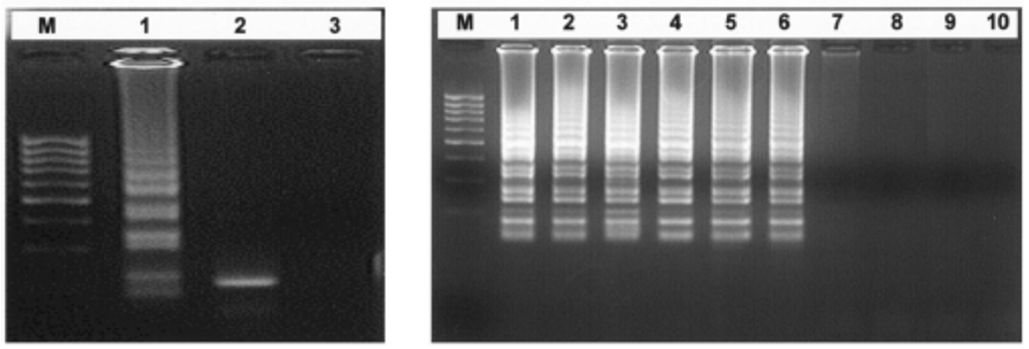
Real-Time Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of West Nile Virus

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

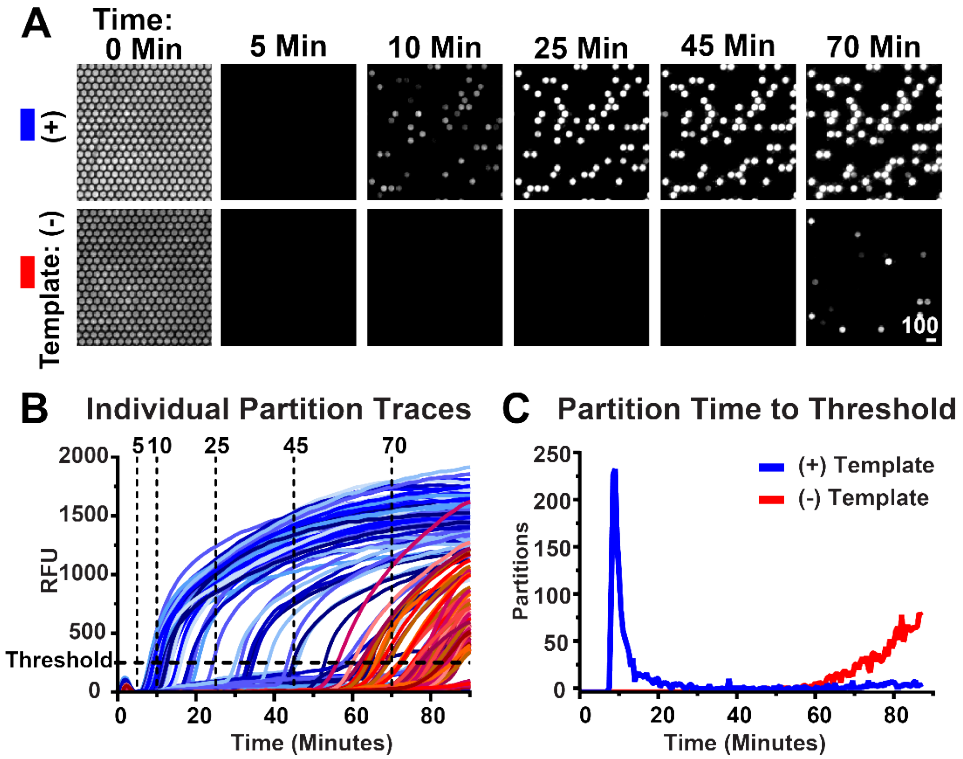
We excised bands and sequenced some of the products of true & false positives

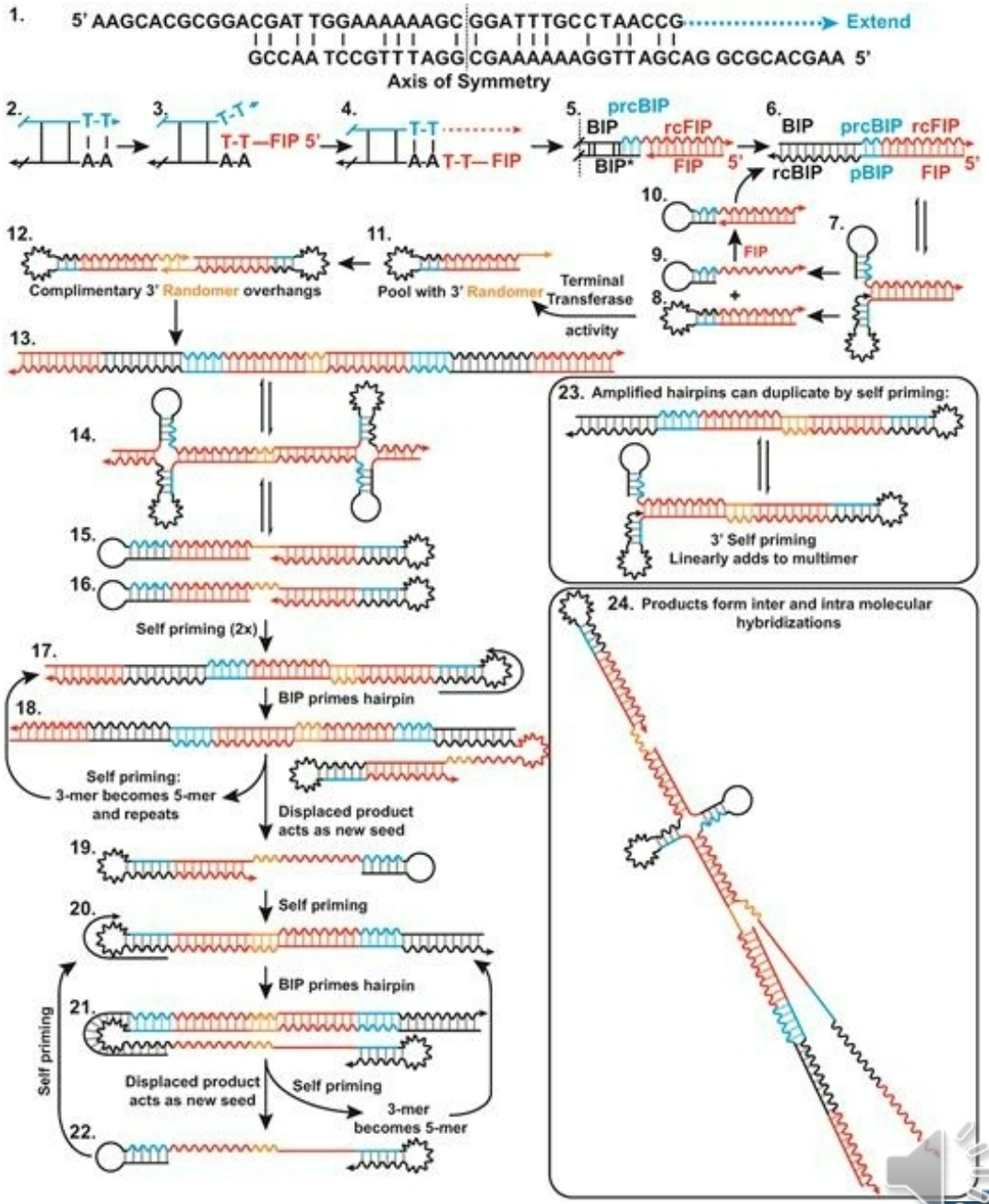
Sequencing LAMP false-positives

True positive amplicon structure (*n* = 9 samples)		False-positive amplicon structure (*n* = 6 samples)	
[+LB, -BIP, -inner region, +FIP, -LF]	3/9	[+FIP, -BIP, -LF]	1/6
[+LF, -FIP, +inner region, +BIP]	4/9	[+LF, -BIP, -FIP]	3/6
[+LF, -FIP, +inner region, +BIP(partial), +LB, -BIP]	1/9	[+LF, +BIP, -FIP(partial)]	1/6
[+LF, -BIP, -FIP]	1/9	[+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

Other recent studies





- Rolando *et al*, *Anal. Chem.* 2019 (as reprinted in Moehling *et al*, 2021): Digital LAMP in microwell partitions
- Rolando *et al*, *Nucl. Acids Res.* 2020: putative mechanism of non-template amplification involving **primer-dimer extension**, **template switching** and **terminal transferase**

Other primer-related artifact: primer-dimer self-amplification

- We observe that some primer sets demonstrate a rising baseline when monitoring in real time with a SYTO dye.
- This includes published primer sets that were originally described for use with *colorimetric endpoint detection.*

Original:

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

DENV-2 assay: demonstrates rising baseline in real-time monitoring

Versus

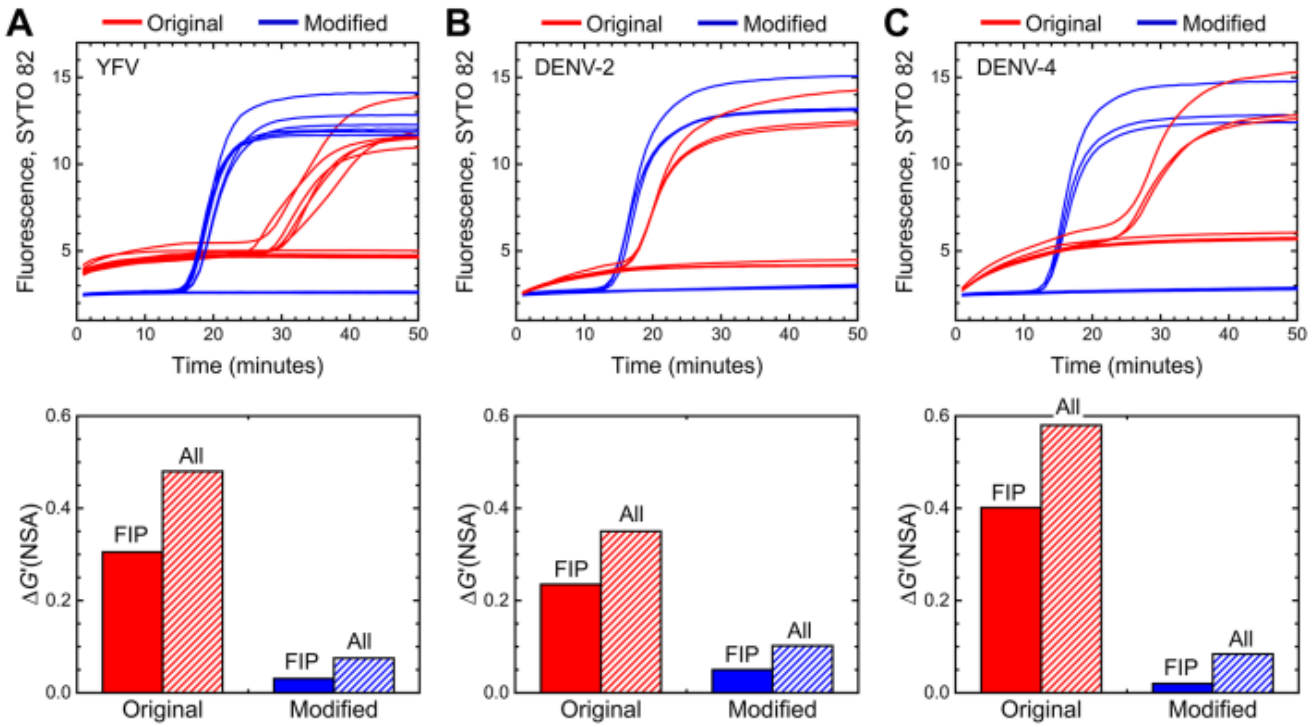
F2 region nudged 4 bases in 5' direction on template

```
5-tcatctcaccttgggccccccctagcggttagaggagacc->
                 || | || ||| | | ||| || | ||
               <-ccagaggagattggcgatccccccgggttccactctact-5
```

Primer revised to reduce 3'-end complementarity of primer-dimer, while still fitting sequence alignment

Thermodynamic prediction of nonspecific amplification?

Small adjustments to primers to minimize ΔG'(NSA) eliminate baseline rise and improve rate of reaction



Meagher *et al*, *Analyst* 2018

- 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 ΔG' of primer interactions

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

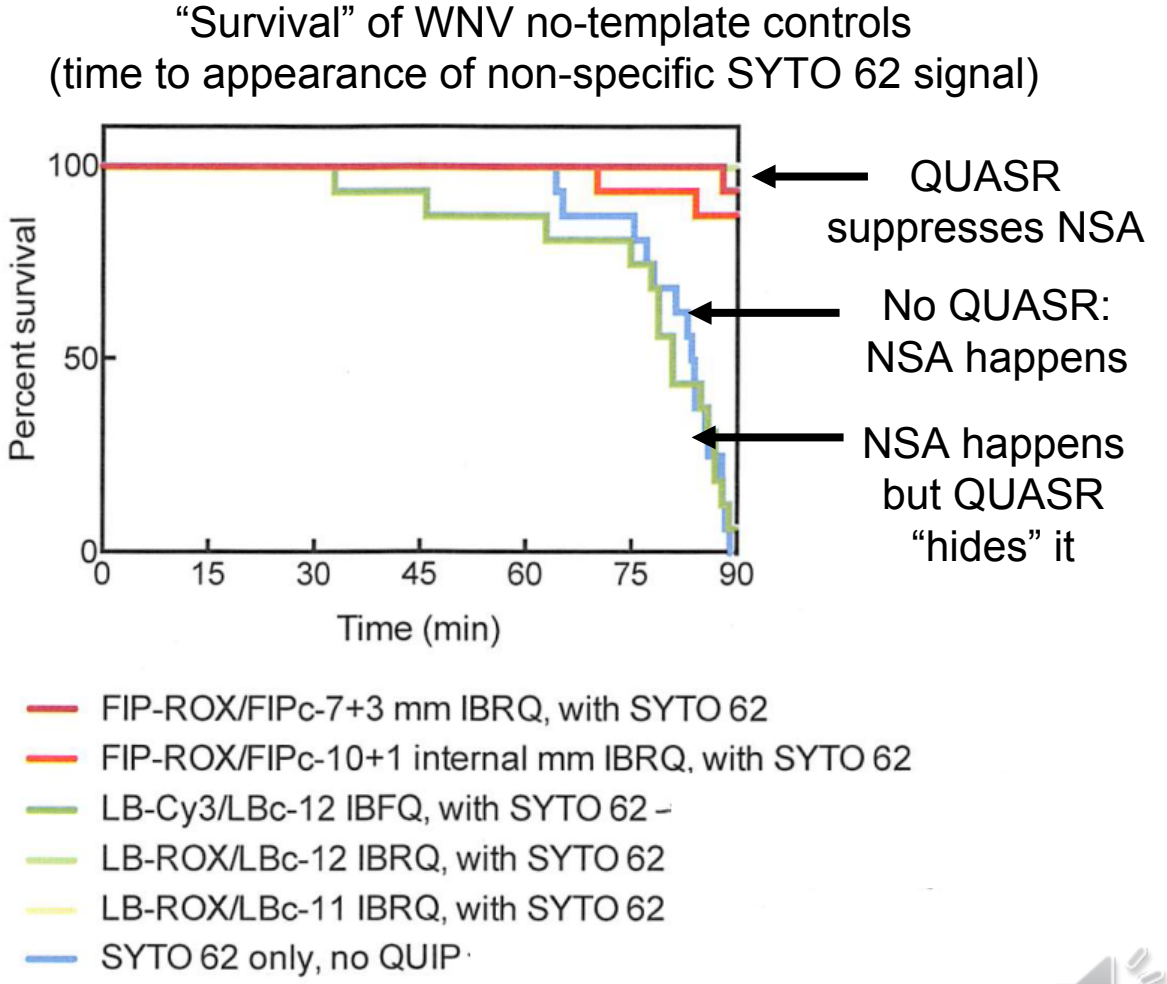
- Anecdotally, ΔG'(NSA) may also be predictive of primer sets prone to "false positives"

Why do isothermal methods suffer from false positives when PCR doesn't?

- qPCR usually uses a probe to look for a sequence internal to the amplicon.
- Hydrolysis probes (*e.g.* Taqman) are fundamentally incompatible with strand-displacing mechanisms like LAMP.
- qPCR isn't immune to false-positives, but they should arise late (*e.g.* Ct > 40).
- PCR is a discrete process with a predictable scale: the cycle
  - 1 doubling of amplicon per cycle, independent of the kinetics of the individual primer set
  - Amplifiable primer structures require ≥ 1 cycle to form
- Isothermal methods are continuous processes without a uniform time scale
  - Each primer set and amplification, including primer-initiated false-positives, follows its own kinetics, which is presently not predictable.

Suppressing LAMP false-positives

- Primer design to avoid amplifiable dimers
- Time-gated approach
  - Non-specific amplification (NSA) happens late
- Move away from non-specific detection techniques
  - NSA may still occur, but if you don't detect them, it's not as much of a problem
  - E.g. QUASR example at right

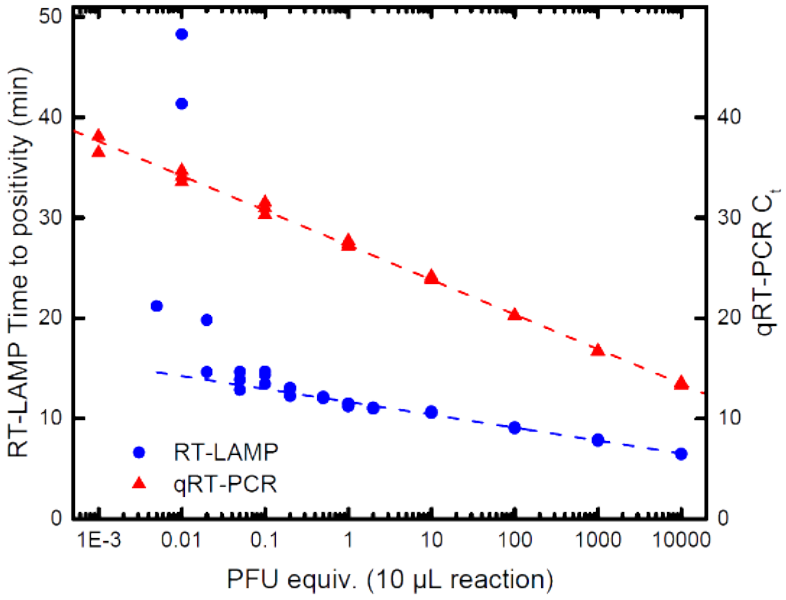


C. Ball, *Analytical Chemistry* 2016

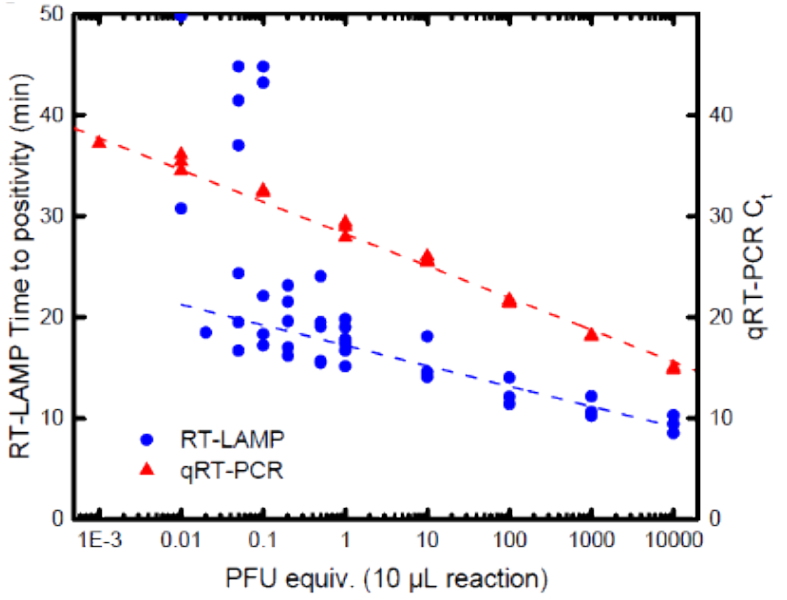
On LAMP sensitivity

- qPCR: some variability about what threshold to use for calling something “positive”: Ct < 40? Ct < 38? Ct <35?
- Many studies that try to directly compare RT-LAMP to RT-qPCR find LAMP becomes less reliable at sample concentrations corresponding to Ct 30-35.

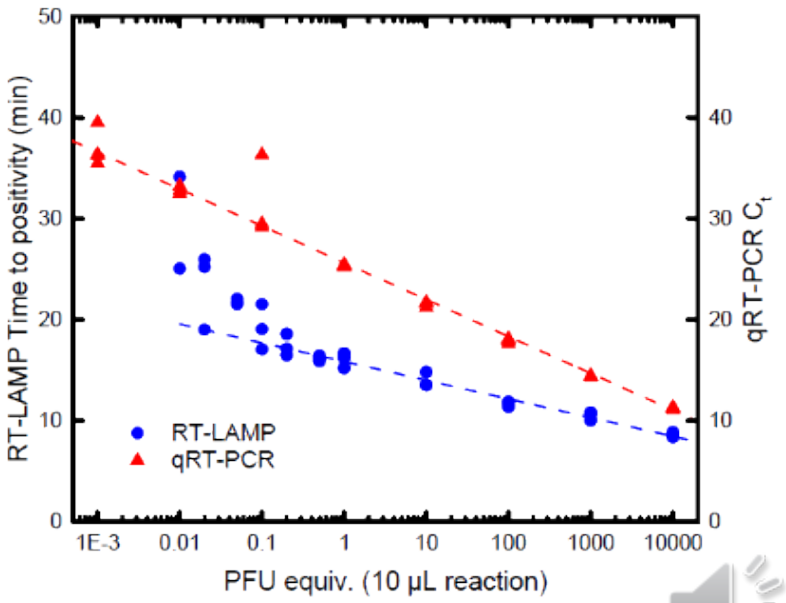
WNV



SLEV



WEEV

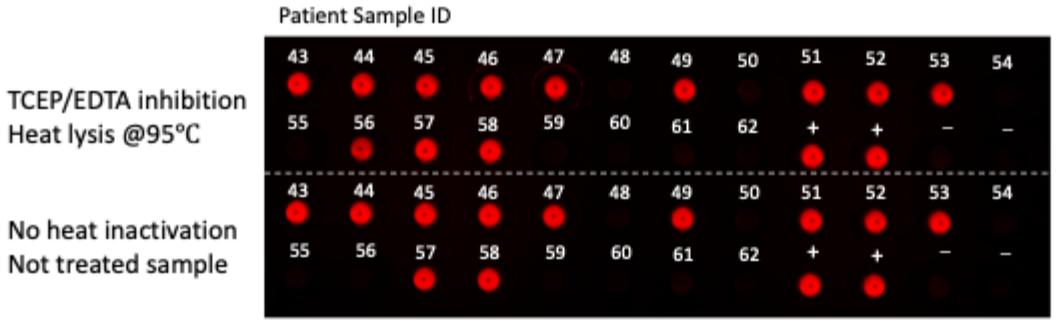


Wheeler et al, PLOS One 2016

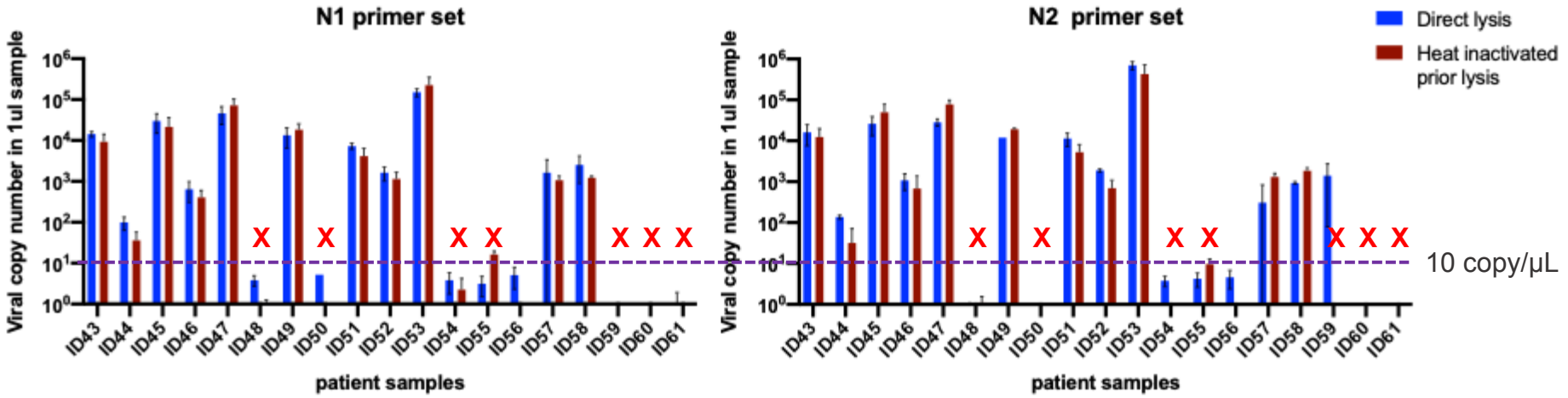
RT-LAMP *vs.* RT-qPCR for COVID NP Swabs

- Small in-house study with a panel of banked positive NP swabs indicates RT-LAMP method is failing primarily on low viral load samples (including borderline undetectable by RT-qPCR)

RT-QUSAR LAMP on Nasal Specimens without Sample Preparation (20% sample volume in 25 ul reaction)

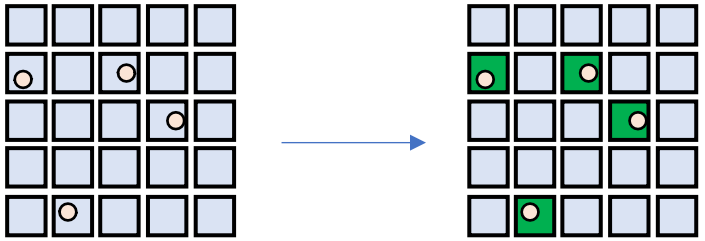


RT-qPCR on Cov-2 viral RNA Purified from Patient Nasal Specimens (20% sample volume in 10 ul reaction)

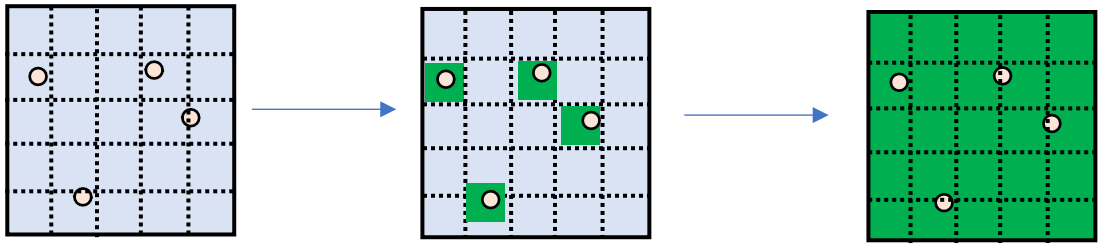


Probability of detection scales with sample volume

Option A: Many small reactions



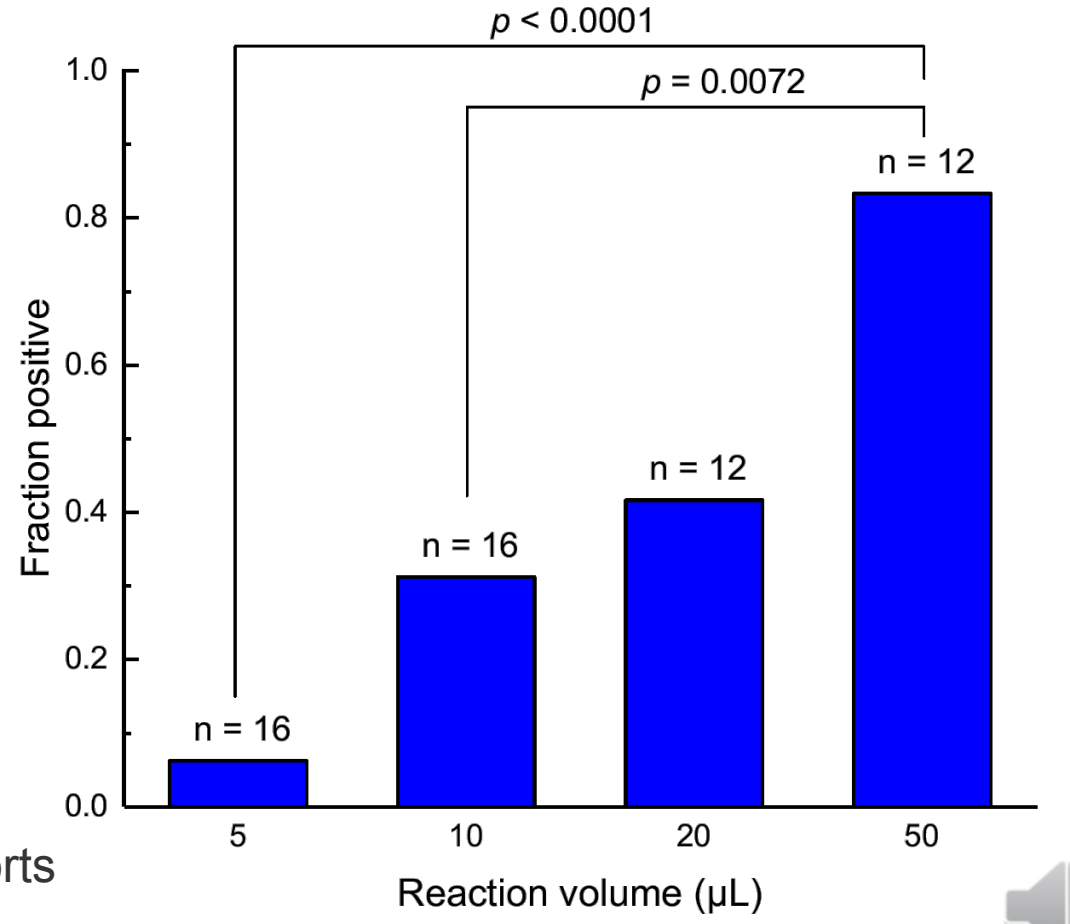
Option B: One large reaction



- For an *individual* reaction, P(A) < P(B)
- For an *ensemble* of small reactions of same volume as a single large reaction, P(any A) ≈ P(B) (?)
- Example: Wei *et al*, *Scientific Reports* (2021) 11:5448 reports **500 µL** "HP-LAMP" assays with 5 µL heat-treated saliva

Experimental demonstration with WNV RNA

- Constant template concentration
- Constant ratio of template volume/reaction volume



LAMP vs PCR sensitivity: Final thoughts

- RT-LAMP seems less likely than RT-qPCR to achieve few-copy detection limit.
  - Is there something fundamental about RT-LAMP that makes this difficult?
- Comparisons across methods (PCR, LAMP, other isothermals) are inevitably tied up in questions of specifics of assay design.
  - Well-controlled, head-to-head comparisons between methods are rare.
  - Literature is full of meaningless “My assay is better than your assay” claims.
- Noteworthy paper: Zhang *et al*, *Biotechniques* 2020, 69(3):178-185, “Enhancing colorimetric loop-mediated isothermal amplification speed and sensitivity with guanidine chloride “

Further Reading

EXPERT REVIEW OF MOLECULAR DIAGNOSTICS
2021, VOL. 21, NO. 1, 43–61
https://doi.org/10.1080/14737159.2021.1873769

Taylor & Francis
Taylor & Francis Group

REVIEW

LAMP Diagnostics at the Point-of-Care: Emerging Trends and Perspectives for the Developer Community

Taylor J. Moehling [a,*], Gihoon Choi [a,*], Lawrence C. Dugan [b], Marc Salit [c] and Robert J. Meagher [a]

[a]Sandia National Laboratories, Biotechnology & Bioengineering Dept., Livermore, CA, USA; [b]Lawrence Livermore National Laboratory, Biosciences & Biotechnology Div., Livermore, CA, USA; [c]Joint Initiative for Metrology in Biology, SLAC National Accelerator Lab and Departments of Bioengineering and Pathology, Stanford University, Stanford, CA, USA

ABSTRACT

Introduction: Over the past decade, loop-mediated isothermal amplification (LAMP) technology has played an important role in molecular diagnostics. Amongst numerous nucleic acid amplification assays, LAMP stands out in terms of sample-to-answer time, sensitivity, specificity, cost, robustness, and accessibility, making it ideal for field-deployable diagnostics in resource-limited regions.

Areas covered: In this review, we outline the front-end LAMP design practices for point-of-care (POC) applications, including sample handling and various signal readout methodologies. Next, we explore existing LAMP technologies that have been validated with clinical samples in the field. We summarize recent work that utilizes reverse transcription (RT) LAMP to rapidly detect SARS-CoV-2 as an alternative to standard PCR protocols. Finally, we describe challenges in translating LAMP from the benchtop to the field and opportunities for future LAMP assay development and performance reporting.

Expert opinion: Despite the popularity of LAMP in the academic research community and a recent surge in interest in LAMP due to the COVID-19 pandemic, there are numerous areas for improvement in the fundamental understanding of LAMP, which are needed to elevate the field of LAMP assay development and characterization.

ARTICLE HISTORY
Received 12 November 2020
Accepted 6 January 2021

KEYWORDS
Loop-mediated isothermal amplification; point-of-care; diagnostics; SARS-CoV-2

Contact:
rmeaghe@sandia.gov