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Thermodynamic Predictions of Primer Interactions and Impact on LAMP Assay Performance

Robert Meagher – Sandia National Laboratories, Livermore, CA - rmeaghe@sandia.gov

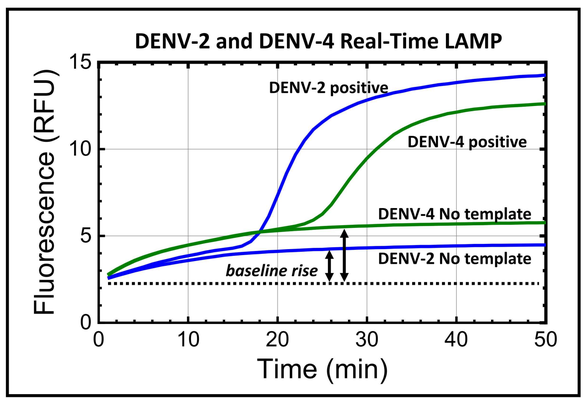
Sandia National Laboratories

U.S. DEPARTMENT OF ENERGY

Introduction: Loop-mediated isothermal amplification (LAMP) has emerged as one of the most popular isothermal "PCR alternatives" for detection of pathogen DNA & RNA in low-resource settings. Unlike qPCR assays, which comprise a pair of primers bounding a discrete amplicon, and optionally a probe binding internal to the amplicon, LAMP features a complex arrangement of 4-6 primers, with 6-8 binding regions. And compared to qPCR, the rules for primer design are not well-understood by many practitioners of LAMP: a few software packages are available for LAMP primer design, but empirical testing of multiple sets is usually necessary. In our experience, published primer sets vary widely in quality, and there are no agreed-upon metrics for how to evaluate or compare LAMP primers or other isothermal assay primers (*i.e.* there is nothing analogous to the MIQE guidelines for qPCR assays).

This poster describes our efforts to understand and correct a common observation with real-time monitoring of LAMP assays: rising baselines. For more detail see our recent publication: Meagher *et al*, *Analyst* (2018), **143**: 1924-1933.

Case study: "Pan-dengue" primers from literature (Lau, *PLOS One* 2015)
This set of 24 primers comprises four sets of semi-specific primer sets for the four serotypes of DENV. The primers nest within a highly conserved part of the 3'-UTR. Based on sequence alignments they are collectively a good match for many genotypes of each serotype. But when tested individually, we found the DENV-1 and DENV-3 sets performed nicely whereas the DENV-2 and DENV-4 featured a **rising baseline** that we attributed to formation of unstable but apparently amplifiable primer dimers:



Primer-dimers identified for DENV-2:

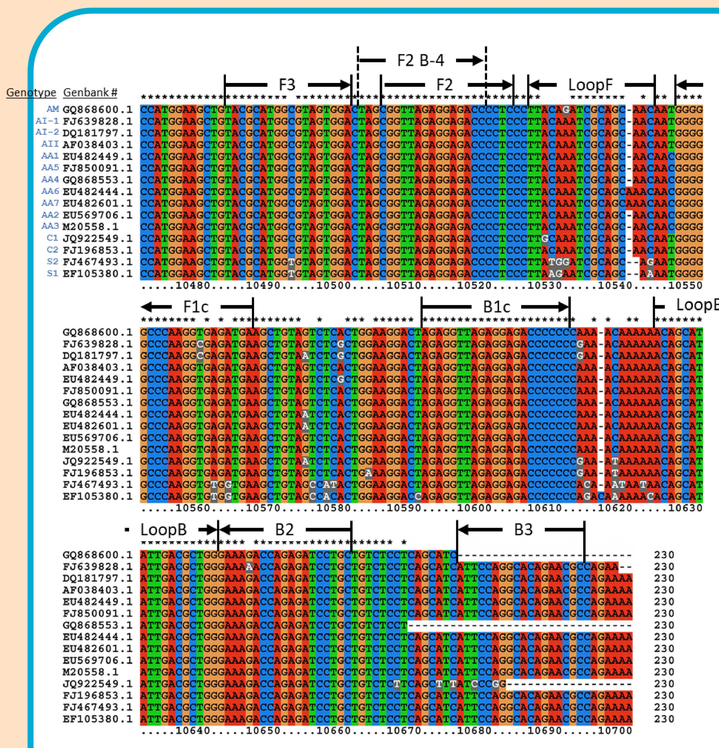
- Would you expect these structures to be stable enough to extend at 63 °C?

```
Self-Dimers:

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


          Cross Primer Dimers:

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



Corrective action: We "bumped" the F2 region of the FIP primer slightly to move the region of homology away from the 3' end of the FIP primer. But the allowable "bumps" are constrained by two features: there isn't much spacing between the F3, F2, and LoopF primer regions, and for a highly variable target like DENV we need to make sure our changes don't result in a loss of specificity. In this case, we were lucky that a useful "bump" to F2 didn't greatly affect the specificity of the priming site!

What happens when we "bump" primers? We reduce the $\Delta G_{hybridization}$. Effectively this means that at the reaction temperature, a smaller fraction of the primer exists in this dimer state. Having an extendable 3' end also matters, although the rules for *Bst* DNA polymerase and its variants aren't well understood, and Meagher, *Analyst* 2018 also describes a case we caught where *Bst* 2.0 can apparently extend a hairpin from a 3' mismatch!

```
"Bumped" F2 primer self-dimer
5-tcatctcaccttgggccccccctagcggttagaggagacc->
               || | || ||| | | ||| || | ||
             <-ccagaggagattggcgatccccccgggttccactctact-5

versus Original F2 primer self-dimer
5-tcatctcaccttgggccccccggttagaggagacccctc->
                          |||| | | ||||
                      <-ctccccagaggagattggcccccccgggttccactctact-5
```

Fewer consecutive matches near 3' end of primer means less stable, less amplifiable dimers

But wait… there's more! With 6 primers per assay, there are many possible primer-primer interactions. As the simplest possible approach, we tried to capture the thermodynamics of all possible primer-primer interactions (although we didn't pay special attention to the 3' end):

Nearest-neighbor model for primer hybridization:

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

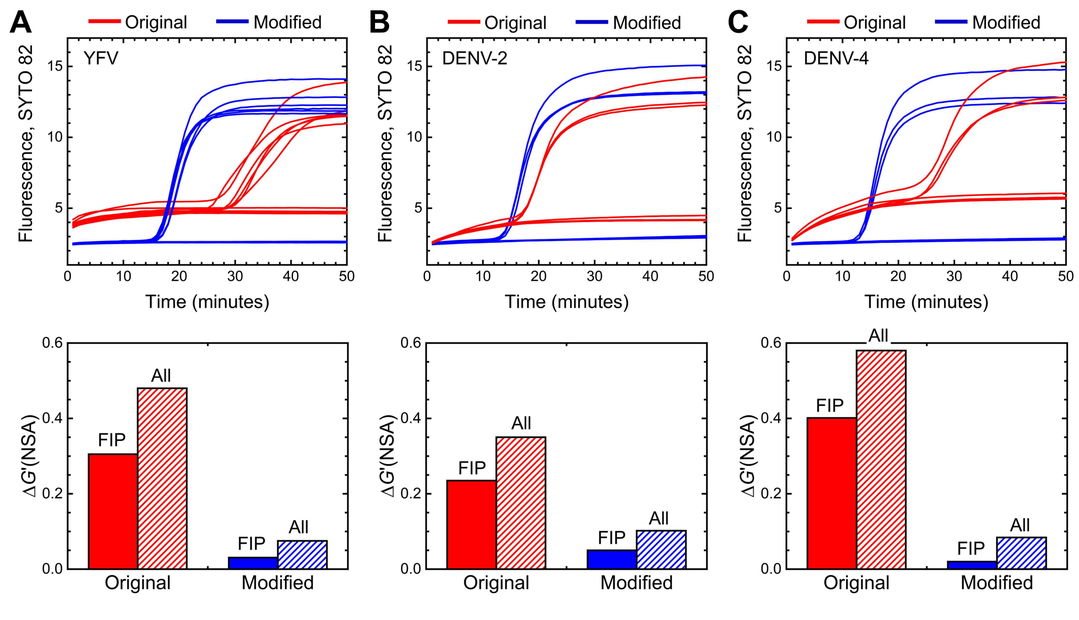
Normalized $\Delta G'$ for all possible primer-primer interactions

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

This parameter should be predictive of the probability of non-specific amplifications, and, we hypothesized, should predict rising baseline!

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

We tested primer bumping with three "pathological" pathogen primer sets: DENV-2, DENV-4, and YFV (from Kwallah, 2013). The identification of the worst primer dimer and the analysis of how to bump was all performed manually, but in each case we found that we greatly reduced the baseline rise, and improved the time to positive detection. In the case of YFV, the modification also improved probability of detection at low copy number. And in each case, bumping a single primer *greatly reduced $\Delta G'(NSA)$,* computed both for the bumped primer, and for the entire set.



Conclusion: although based on a small number of examples, our work suggests *$\Delta G'(NSA)$* could be a simple parameter to consider for optimization in LAMP primer design software to improve assay speed and reduce rising baselines. We also suspect this parameter might also help predict the highly undesirable phenomenon of "false positives" (exponential amplifications without a template), which occur sporadically with certain LAMP primer sets.

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