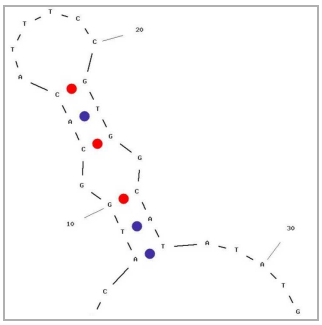

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# LAMP-QUASR Primer Design

**This protocol is published without a DOI.**

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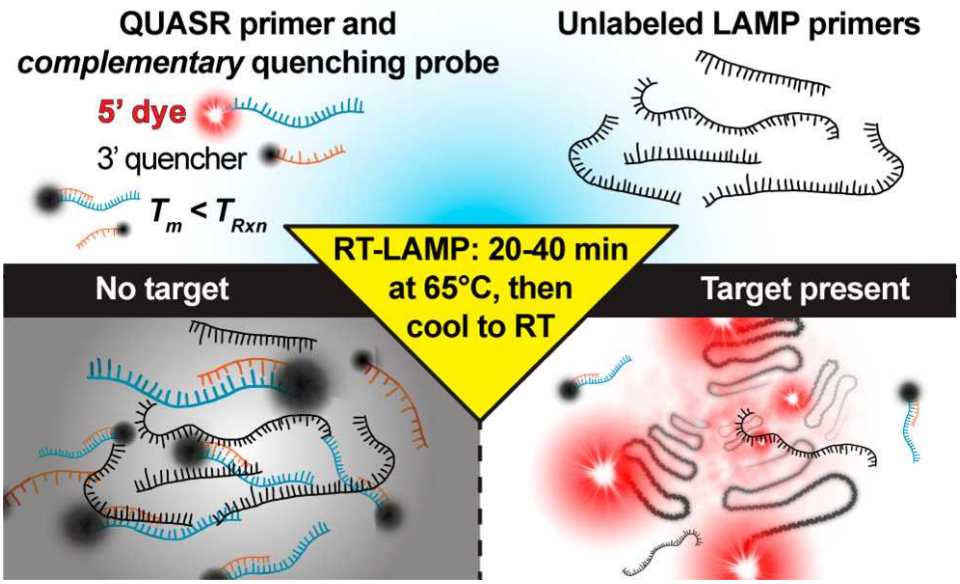
COMMENTS 0

ABSTRACT

This protocol is designed for the design and adaptation of a LAMP primer set in order for it to be used with the QUASR technique. In order to do this, it requires an already designed (and hopefully experimentally validated) LAMP primer set. The steps indicated in this protocol are the ones we follow to adapt a previously validated primer set for their use in LAMP-QUASR reactions.

QUASR is "a simple technique that allows closed-tube, target-specific detection, based on the inclusion of a dye-labeled primer that is incorporated into a target-specific amplicon if the target is present. A short, complementary quencher hybridizes to unincorporated primer upon cooling down at the end of the reaction, thereby quenching the fluorescence of any unincorporated primer" (Ball et al., 2016).




Principle of QUASR detection in LAMP or RT-LAMP (Ball et al., 2016)

Therefore, using QUASR-LAMP allows for specific sequence detection and reduces the false positives often associated with unspecific amplification, and it only requires a minor modification of one of the LAMP reaction primers.

This protocol mostly focuses on the software and criteria we use to decide which primer of the set should be picked, modified with a fluorophore, and used as a template for quencher probe design. Some steps are based on the criteria used by Priye et al. (2017), with several modifications and additions of our own.

PROTOCOL CITATION

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## Analyze existing primer set

1 In order to choose a primer for QUASR probe design, is important to understand the parameters present in a LAMP primer set.

One way to do this, is by **checking the complete set of primers (including full-length FIP and BIP) for self-dimerization and/or cross-dimerization** using the **Multiple Primer Analyzer** tool provided by Thermo Fisher and observing the results for primer dimer formation.

*The default sensitivity value should be 3, for which few to none dimers should be expected. We recommend changing the sensitivity value to **2** and observe the self-dimers and cross-dimers that form.*

Note

When we are designing LAMP primers from scratch, we often use this sensitivity setting and reject any set with long regions of complementarity (over 7 bp) or amplifiable primer dimers (with 4-5 bp of dimerization at the 3' end of one sequence), specifically the ones between FIP, BIP, LF and LB, as they are present in a much higher concentration that F3 or B3 in the reaction.

**In case the primer set has already been experimentally validated, it is still important to consider these interactions as a guide to see which sequence has less probability to form dimers and, therefore, is most suitable for QUASR modification.**

2 It is also important to check any **hairpin structures** formed by each primer and analyze them in terms of their stability and location among the sequence.

This can be achieved by analyzing each **individual primer** using the **OligoAnalyzer** tool provided by *Integrated DNA Technologies*, with default settings except adjusting Mg++ concentration to **8 mM**, the default for a LAMP reaction.

Note

**This is a critical step, as usually the sequence with the less number and most unstable secondary structures is the best candidate for QUASR primer modification.**

**Things to consider for this selection**

- In OligoAnalyzer, the **number of secondary structures is often irrelevant** in comparison to more important parameters like the position and Tm of each hairpin.
- The need to further analysis of the hairpin structure depends on its **stability and Tm**.

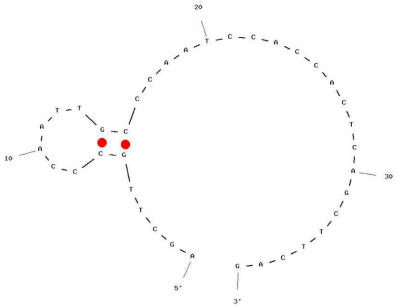
- Any structure with a Tm below 35ºC **and** a ΔG above -1 kcal/mol could be easily **ignored**, as they would not be present at the incubation temperature of a LAMP reaction.

- Any structure with a Tm above 55ºC **or** a ΔG below -3 kcal/mol should lead to the **rejection** of this primer for QUASR-modification, as these hairpins would remain at incubation temperature and therefore interfere with the primer-probe interaction.

- Structures with a Tm between 35ºC and 55ºC **or** a ΔG between -3 and -1 kcal/mol should be **taken into account** as criteria for QUASR-primer selection. In this case, the **position** of the hairpin is vital as a secondary structure occupying the 5' end of the primer would interfere with the binding of the quenching probe, and a structure on the 3' end could lead to extendable primer dimers. **A primer hairpin on the center position is often preferred over one with a hairpin in a 5' or 3' end,** even when it has a somewhat higher Tm or lower ΔG.

Expected result

| structure | Image | ΔG (kcal.mole$^{-1}$) | $T_m$ (ºC) | ΔH (kcal.mole$^{-1}$) | ΔS (cal.K$^{-1}$mole$^{-1}$) |
|---|---|---|---|---|---|
| 1 |  | -0.39 | 31.1 | -19.7 | -64.75 |
| 2 |  | -0.34 | 29.7 | -21.4 | -70.65 |
| 3 |  | -0.28 | 29.5 | -18.7 | -61.79 |
| 4 |  | -0.15 | 26 | -45.4 | -151.78 |
| 5 |  | -0.12 | 26.6 | -22.3 | -74.39 |
| 6 |  | 0.55 | 21.8 | -50.6 | -171.55 |
| 7 |  | 0.55 | 17.8 | -22.3 | -76.65 |



*Example of a primer very suitable for QUASR modification, indicating a table with the hairpins it forms (left) and a larger detail of structure 1 listed on the table (right).*

3 Choose an individual primer for further QUASR modification. If possible, prefer a **FIP or BIP labelled** primer, as they are present in a higher concentration in the reaction and therefore produce results with higher contrast.

## QUASR Probe: Design and Analysis

4 Design a quencher probe, by choosing the complementary sequence of the first 10-13 bp of the labelled primer chosen in **step 3.**

*We recommend starting with 13 bp and reduce the length of the probe according to the requirements indicated in the steps below.*

5 Determine the Tm of the quenching probe, which should have a value **below 55ºC.**

**If the designed probe does not fulfill this requirement,** go to step #4 **and reduce the length of the quenching probe by 1 bp until the correct Tm is achieved.**

Note

A recommended tool for this is Tm calculator, with the adequate primer and Mg (8mM) concentration.

Considering that the quenching probe is at 1,5x times the concentration of the fluorescent primer, the probe concentration should be 2400 **nM** if you are using a FIP/BIP primer for QUASR or 1200 **nM** if you are using LF/LB.

6 Analyze, using **Multiple Primer Analyzer**, if the addition of the quenching probe to the primer set leads to the the formation of new cross-dimers.

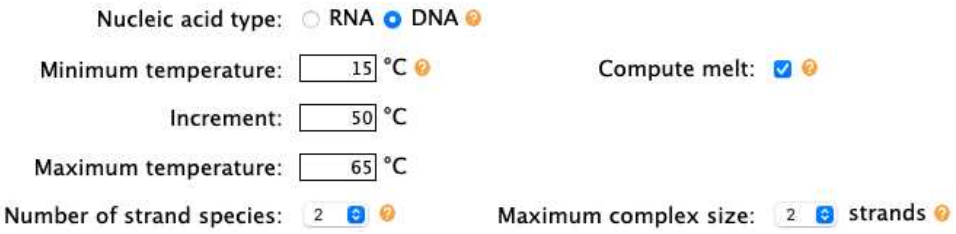
Keep in mind that a **very stable dimer between the quenching probe and the fluorescent primer is expected**, as this interaction is the basis of how QUASR works.

Note

However, there should be few (hopefully none) interactions between the quenching probe and other primers of the set, as this could lead to weak quenching and the occurrence of false positives.

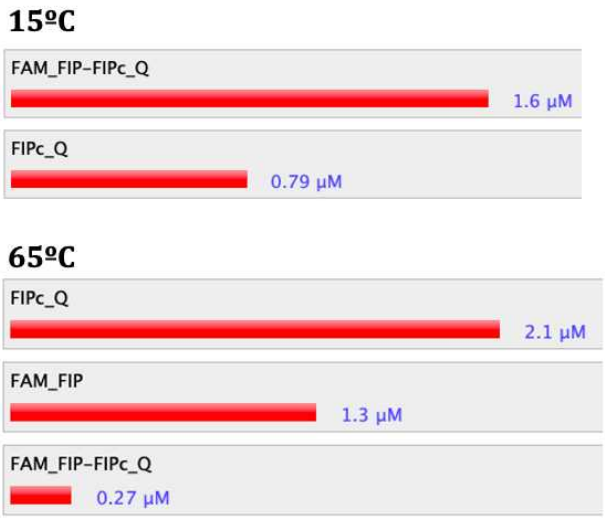
*Sometimes, a 5-6 bp complementarity region between the probe and another primer could be accepted, as long as is not on a non-fluorescent FIP/BIP primer or the 3' end of a LF/LB primer.*

7 Analyze the **interactions between fluorescent primer and quenching probe at different temperatures** using **NUPACK** with the following settings:




Remember the concentrations of primer (with fluoropohore) and quenching probe are **1.6 and 2.4 µM, respectively, if a FIP/BIP primer is used** or **0.8 and 1.2 µM, respectively, if a LF/LB primer is used**.

Expected result




*Here is an example result of an analysis we made. The relevant things to look at is to make sure that all of the fluorescent primer (FAM_FIP in this case) is hybridizing with the quenching probe (FIPc_Q) at room temperature (15ºC), but is mostly free to form amplicons at LAMP reaction temperature (65ºC).*

*In this specific case, even though FAM_FIP is not completely free at 65ºC, still a considerable amount of the primer would be able to participate in the reaction, indicating a good result.*

## Comparison and Selection

8 **If you have a quenching probe of 11-13 bp**, reduce its length by one base pair. Then, go to step #5 and repeat the analysis to step 7 to see if the change in length of the quenching probe leads to less dimer formation and/or better results in the NUPACK analysis.

*This simple step **should not be overlooked**, as we have seen this small change can reduce the stability of primer dimers and significantly change the proportions observed in the NUPACK analysis, leading to bigger discrimination between positive and negative reactions.*

8.1 If the shortest quenching probe designed still has more than 10 bp, repeat **step 8** and compare the results between different probes.

8.2 If **another primer** of the set also seems suitable for QUASR modification, it is recommended to repeat **steps 4 to 8** with this primer and compare the results between different possible quenching probes.

9 **Select a suitable QUASR primer based on the criteria indicated in step 2 and the results obtained from the previous analyses.**

- If possible, prefer a **FIP or BIP labeled primer**, as they are present in a higher concentration in the reaction and therefore produce results with higher contrast
- If you plan to test two QUASR primers, we recommend trying out with one FIP or BIP-labeled primer and another LF or LB-labeled primer

10 Order the fluorescent-labelled primer and its corresponding quenching probe, and test them experimentally in your LAMP-QUASR reactions.

Note

*As a recommendation, we add a FAM fluorophore on the 5' end of the labelled primer and a Iowa Black® Hole Quencher at the 3' end of the quenching probe, but feel free to use any fluorescent molecules of your choice.*