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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

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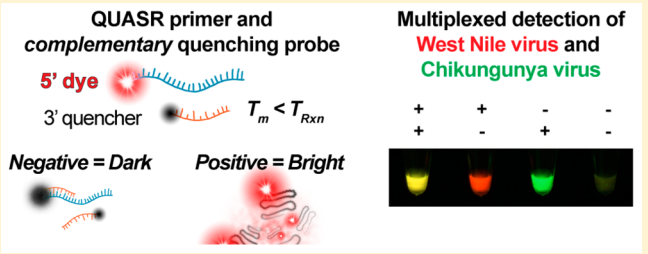
Supporting Information

ABSTRACT: Reverse-transcription-loop-mediated isothermal amplification (RT-LAMP) has frequently been proposed as an enabling technology for simplified diagnostic tests for RNA viruses. However, common detection techniques used for LAMP and RT-LAMP have drawbacks, including poor discrimination capability, inability to multiplex targets, high rates of false positives, and (in some cases) the requirement of opening reaction tubes postamplification. Here, we present a simple technique that allows closed-tube, target-specific detection, based on 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 fluorescence of any unincorporated primer. Our technique, which we term QUASR (for quenching of unincorporated amplification signal reporters, read "quasar"), does not significantly reduce the amplification efficiency or sensitivity of RT-LAMP. Equipped with a simple LED excitation source and a colored plastic gel filter, the naked eye or a camera can easily discriminate between positive and negative QUASR reactions, which produce a difference in signal of approximately 10:1 without background subtraction. We demonstrate that QUASR detection is compatible with complex sample matrices such as human blood, using a novel LAMP primer set for bacteriophage MS2 (a model RNA virus particle). Furthermore, we demonstrate single-tube duplex detection of West Nile virus (WNV) and chikungunya virus (CHIKV) RNA.



Loop mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification technique that is a useful alternative to polymerase chain reaction (PCR) for low-cost or point-of-care diagnostics for infectious disease. The technique can be coupled with reverse transcription (RT-LAMP) for detection of RNA targets, e.g., RNA viruses.[1,2] LAMP (and RT-LAMP) is generally regarded as highly specific and highly sensitive, but a major challenge for LAMP in point-of-care applications is the detection of amplification without requiring cumbersome manipulations or elaborate instrumentation. Furthermore, the available detection mechanisms used in LAMP are not easily amenable to multiplexing to distinguish multiple targets in a single reaction, e.g., for syndromic panels or variant strains of pathogens. In contrast, spectral multiplexing techniques exist for PCR that enable detection of 2−4 targets per reaction (e.g., TaqMan, Thermo Fisher Scientific). Our aim was to develop a single-step, closed-tube, and multiplexable detection method for use with LAMP and RT-LAMP.

Detection of amplification in LAMP (or RT-LAMP) occurs either at the reaction end point or in real-time (quantitative). We have observed that LAMP and RT-LAMP have a narrower quantitative range than corresponding qPCR or qRT-PCR assays. Furthermore, in a point-of-care setting, end point monitoring for a positive or negative result is preferable to quantitation for simplicity of interpretation for nonexperts. End point detection in LAMP is usually accomplished by one of the following techniques: observing turbidity;[3] running product on a gel to observe a banding pattern;[1] adding intercalating dye such as SYBR Green[4] or SYTO dyes[5] to observe a color change and/or fluorescence; adding manganese-quenched calcein to generate fluorescence upon amplification;[6] or adding a colorimetric indicator such as hydroxynaphthol blue[7] or pH-sensitive dyes[8] to generate a color change upon amplification.

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Each of these existing techniques has specific advantages and disadvantages. In our experience, the turbidity produced by LAMP is subtle and difficult to see by the naked eye. Alternatively, running the product on a gel or postreaction addition of SYBR Green requires opening the tube after amplification, which presents a risk for amplicon contamination. While several of the SYTO family of dyes (notably SYTO 9, SYTO 62, and SYTO 82) are noninhibitory for closed-tube end point or real-time detection, fluorescence observations must be performed at elevated temperature for maximum discrimination between positive and negative amplifications. Manganese-quenched calcein detection is reported to suffer from inhibition from manganese.[7] The color change resulting from hydroxynaphthol blue may be too subtle for some users (particularly those with color vision deficiency) to distinguish without instrumentation. Although the color change from pH-sensitive dyes can be quite striking, this technique relies upon weakly buffered reaction mixtures and may not perform well with crude or buffered samples (e.g., 10% blood or soils). Furthermore, none of these techniques is sequence specific but rather detect total amplification. These detection methods are thus prone to detection of nonspecific amplification, which can occur with LAMP (and other nucleic acid amplification techniques including PCR) even in the absence of the specific target.

The lack of target specificity further means that the above detection techniques cannot be multiplexed to allow detection of more than one target in a single reaction. Several reports describe multiplexing by means of performing a postreaction restriction digest and running the product on a gel;[9] this requires opening the tube plus several additional processing steps. Other reports describe multiplexing techniques for LAMP or other isothermal strand displacement techniques based on displacement of a bound quencher;[10] fluorescence resonance energy transfer (FRET);[11] a combination of labeled primers and intercalating dyes;[12] or strand displacement of a quencher bound to a probe targeting the loop region of the amplicon (DARQ).[13] However, techniques relying upon strand displacement of a probe can inhibit the LAMP reaction.

We have developed a novel approach for end point determination of LAMP and RT-LAMP reactions, based upon quenching of unincorporated amplification signal reporters (QUASR). Our technique is named after the extremely luminous celestial objects known as quasars. Like its namesake, QUASR is capable of producing an extremely bright signal. In this report, we first outline the operating principles of QUASR. Then, we highlight QUASR's superior end point discrimination ability compared to SYTO dye using bacteriophage MS2 as a model RNA virus. Furthermore, we demonstrate the feasibility of QUASR detection of MS2 in a reaction containing 10% whole blood. Next, we apply QUASR to perform single-tube duplex detection of RNA from two mosquito-borne viruses: West Nile virus (WNV) and chikungunya virus (CHIKV). Briefly, we spotlight QUASR's excellent resistance to false positives. Finally, we close with a discussion of how QUASR LAMP relates to the previously reported DARQ LAMP technique.[13]

MATERIALS AND METHODS

LAMP Primer Design. LAMP Designer v1.13 software (Premier Biosoft) was used with default parameters to scan for suitable LAMP primer sets for bacteriophage MS2 (GenBank NC_001417.2). Primer sets were analyzed by BLAST, comparing to all viral sequences in GenBank, to determine likelihood of cross-reactivity with other viruses. Primer sets were also evaluated to minimize hairpin formation and self-dimerization using OligoAnalyzer and mFold programs (IDT, Coralville; retrieved October 6, 2015; http://www.idtdna.com/Scitools). The MS2 primer set reported here targets the MS2 replicase (RNA-dependent RNA polymerase) gene, and sequences are shown in Table S1. RT-LAMP primer sets for WNV and CHIKV were obtained from published literature[2,14] and are also listed in Table S1.

Viral Templates. MS2 phage was obtained from ATCC (15597-B1) (Manassas, VA). MS2 phage was diluted in water and used directly in assays, without propagation or extraction of RNA.[15] An MS2 RNA standard (United States Biological) was also used in some assays for quantitation. WNV (isolate L-CA-04 SAC-04-7168, GenBank accession no. DQ080059) and CHIKV (strain Ross, GenBank accession no. AF490259) were cultured and quantitated by plaque assay, and RNA was extracted as described in supplementary methods. **Safety**: WNV and CHIKV culture requires biosafety level 3 (BSL-3) containment and protocols. Genomic RNA from positive-sense RNA viruses such as WNV and CHIKV should be treated as potentially infectious material.

QUASR Primer Design. QUASR primers and their complementary quenching probes were designed using IDT's online OligoAnalyzer tool (v3.1) with parameters adjusted for LAMP reaction conditions. Fluorescently labeled primers for QUASR detection of MS2, WNV, and CHIKV were selected by avoiding primers that were likely to form stable hairpins. The melting temperature of the fluorescent primer-quenching probe complex was designed to be significantly lower than 65 °C (at least 5 °C lower). Primers, dye-labeled primers, and quenching probes were ordered from Integrated DNA Technologies (Coralville, IA). Primers and their quenching probe sequences are reported in Tables S1 and S2.

RT-LAMP Assays. RT-LAMP was performed in 10 μL reaction volumes in thin-walled PCR strip tubes, 96-well plates, or 384-well plates. The reaction mixture had a final composition (after adding water or template) of 1× Isothermal Amplification Buffer (New England Biolabs, NEB no. B0537S) supplemented with an additional 6 mM $MgSO_4$ (NEB no. B1003S, final 8 mM $MgSO_4$), 1.4 mM each dNTP (NEB no. N0447L), 0.32 units/μL Bst 2.0 WarmStart DNA polymerase (NEB no. M0538M), 0.2 units/μL AMV reverse transcriptase (NEB no. M0277T, or Life Science Advanced Technologies no. AMVRTT-5), and 2 μM (or in some instances without) SYTO 9, 62, or 82 detection dyes (Life Technologies no. S-34854, no. S-11344, and no. S-11363). In some instances, 0.8 M Betaine (Sigma no. B-0300) was added to the reactions.

Primers were used in the amounts typically recommended for LAMP: 0.2 μM each for outer primers F3 and B3; 1.6 μM each for inner primers FIP and BIP; and 0.8 μM each for loop primers LF and LB. Quenching probes were typically added at 1.5× the concentration of the corresponding fluorescently labeled primer. Other concentrations were used in experiments as reported in the figures.

For RT-LAMP with 10% human blood, 20U of RNaseOUT (Thermo Fisher Scientific, MA) and 1 μL of human whole blood (Innovative Research, MI) were added to a final volume of 10 μL reaction containing the RT-LAMP mixture listed above. Experiments in buffer or blood were conducted with 10 replicates per group and replicated independently 3 times by 2 different operators. Differences between groups were tested by

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2-way ANOVA and post hoc analysis by Tukey's test with an alpha of 0.05.

RT-LAMP with real-time fluorescence monitoring was carried out in a BioRad CFX96 or CFX384, using detection channels 1 (FAM), 2 (HEX), and 5 (Cy5) for monitoring SYTO 9, 82, and 62 dyes, respectively. Reactions were incubated at a constant temperature of 63−65 °C for 50−70 min, with plate read steps at intervals of 1 min (in the BioRad CFX96 (CFX384), this is accomplished with a 48 s (38 s) single-temperature cycle followed by a plate read which takes approximately 12 s (22 s) in all-channel mode). Incubation was typically followed by inactivation of the enzyme at 95 °C for 2 min, followed by cooling to 25 °C in 0.1 to 1.0 °C increments. Time-to-positivity values were determined using the BioRad CFX Manager software, using baseline-subtracted curves, and a single threshold value autocalculated by the CFX manager for SYTO signal.

Duplexed WNV and CHIKV RNA detection was accomplished by adding both primer sets in at one-half their normal concentration. A 2 to 100 PFU equivalent of each viral RNA was added to the appropriate reactions.

End point images were taken with a color camera (Point Grey Research, no. CMLN-13S2C-CS, Richmond, BC) or an iPhone 6 (Apple, Cupertino, CA). Fluorescence was excited with a 10 W LED (LEDEngin, Inc. no. LZW4, Blue-465 nm, Green-523 nm, or Red-623 nm). Filters were used for excitation (480/30 BP, 520/40 BP, or 622/36 BP) and emission (535/40 BP, 550 LP, or 620/60 BP) (Edmund Optics, Barrington, NJ; Chroma Technologies, Bellows Falls, VT; or Thorlabs, Newton, NJ) with the high power LED and color camera. For detection by eye or iPhone 6, an LED flashlight served as the excitation source, and a single layer of plastic lighting gel (LEE Filters, Andover, Hampshire, U.K.; filter no. 113 (red) or no. 158 (green and duplexed)) was used as an emission filter. The iPhone image was acquired using the app Manual Camera−Custom Exposure and Controls (v. 1.7, KendiTech). Image exposure was adjusted using a standard photography white balance card set. No color adjustments were made.

RESULTS AND DISCUSSION

We schematically illustrate the principle of the QUASR technique in Figure 1. The QUASR technique relies upon simply using a primer (for LAMP, either the inner primers FIP or BIP, or the loop primers, LoopF and LoopB are suitable) labeled with a fluorophore at the 5′ end. As amplification proceeds, the fluorophore-labeled primers are incorporated into the amplicon. Also included is a short quencher probe, typically with 7−13 bases complementary to the 5′ end of the labeled primer. The quencher probe is modified at the 3′ end with a dark quencher (e.g., Iowa Black or Black Hole quencher). Critically, the melting temperature of the quenching probe annealed to the labeled primer (typically <55 °C) must be well below the temperature of the LAMP amplification (typically 60−65 °C), such that during the amplification the quenching probe is dissociated and does not participate in or inhibit the reaction. We have found that an internal mismatch in the quenching probe can reduce the complex melting temperature when necessary while preserving specificity. At a defined end point (typically 30−45 min of incubation), the reaction is stopped and cooled down by removing the reaction tubes to ambient temperature. Upon cooling, any free primer that has not been incorporated into an amplicon hybridizes with the

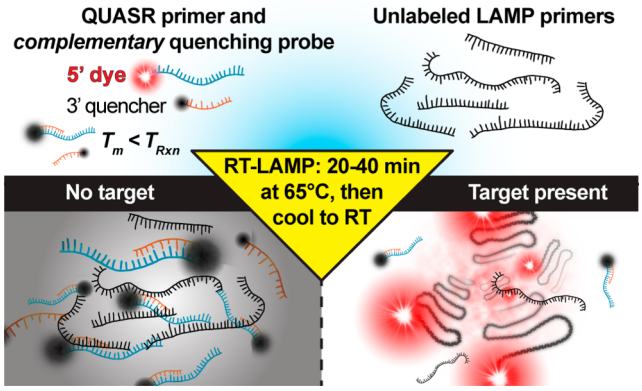

Figure 1. Principle of QUASR detection in LAMP or RT-LAMP. One of the loop primers (LF or LB) or inner primers (FIP or BIP) is labeled with a dye. The reaction mixture also contains a short probe, labeled with a dark quencher at the 3′ end, and complementary to 7−13 bases at the 5′ end of the dye labeled primer. The quench probe is present at slight excess relative to the labeled primer and has Tm > 10 °C below the temperature of the LAMP reaction, such that it remains dissociated during the amplification. After incubation, the reaction is cooled to ambient temperature, resulting in dark quenching of fluorescent primers (negative reactions) or highly fluorescent amplicons (positive reactions).

quenching probe, resulting in close proximity between the fluorophore and the quencher. However, any labeled primer that has been incorporated into an amplicon is unavailable to hybridize with the quenching probe and thus remains bright. Excess quenching probe ensures that fluorescence is fully quenched in negative reactions (Figure 2). Labeled FIP or BIP primer generally provides brighter signal than labeled LoopF or LoopB primer, since the former are used at twice the concentration of the latter in the LAMP reaction and thus incorporated to a higher degree into amplicons. Besides adding a fluorophore to the chosen primer and including an excess of complementary quenching probe, QUASR does not require altering any LAMP or RT-LAMP reaction conditions (e.g., time of amplification, primer concentration, or temperature).

QUASR at room temperature outperforms SYTO dyes at end point discrimination. We have previously found the SYTO family of intercalating dyes (particularly SYTO 9, SYTO 82, and SYTO 62) to be useful for routine closed-tube detection in LAMP, because these dyes are noninhibitory to LAMP at relatively high concentrations of 2−10 μM, thus offering bright signals at a variety of wavelengths. However, thanks to the high degree of DNA synthesis and excess of nucleotides in LAMP, a successful QUASR amplification results in a high degree of incorporation of labeled primers into an amplicon and thus a high residual fluorescence that allows even clearer discrimination between positive and negative reactions. We demonstrate this in Figure 2, using bacteriophage MS2 as a model RNA virus[16] for detection by QUASR RT-LAMP. As expected, it is apparent from Figure 2A that fluorescence in no template control QUASR reactions is strongly quenched with the addition of complementary FIPc probe. QUASR yields a darker negative signal and a brighter positive signal with 1.6 μM FIP compared to either 2 μM or 4 μM SYTO 62, thus affording greater signal discrimination. As seen in Figure 2, the QUASR technique detects amplification optimally at room temperature. Negative QUASR reactions look nearly identical to positive QUASR reactions at elevated temperatures, where the fluorophore-labeled primer and quench probe are dissociated

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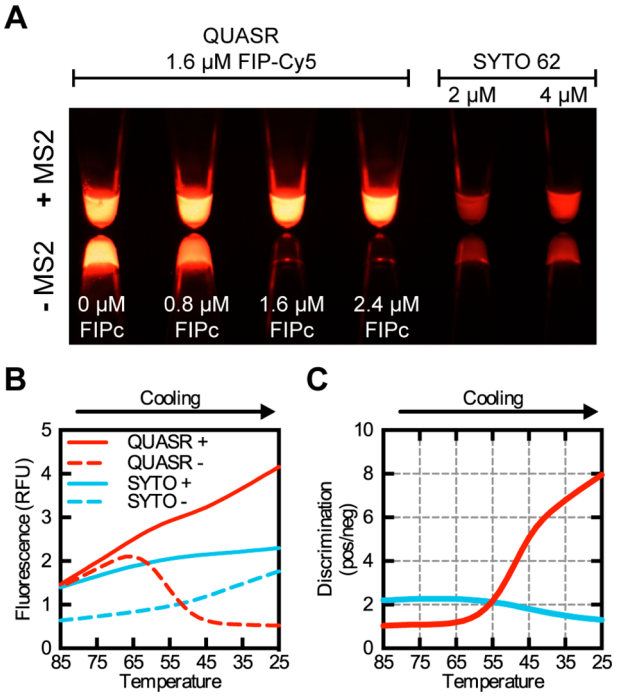

Figure 2. QUASR improves end point discrimination between positive and negative reactions compared to an intercalating dye: (A) comparison of room temperature end point detection with QUASR versus the intercalating dye SYTO 62 for RT-LAMP amplification of MS2 phage in PCR tubes. The top row of tubes shows positive reactions and the bottom row of tubes shows negative reactions. The four pairs of reactions on the left utilize QUASR via FIP-Cy5 with varying amounts of complementary quenching probe, FIPc, for detection. The two pairs of reactions on the right utilize SYTO 62 for detection. (B) Annealing curves for QUASR (1.6 μM FIP-Cy5 with 2.4 μM FIPc) and SYTO 62 (4 μM) reactions postamplification, by monitoring fluorescence in the Cy5 channel, while cooling from 85 to 25 °C in a real-time PCR machine. (C) Signal discrimination (positive/negative fluorescent signal) as reactions cool from 85 to 25 °C.

in solution but become very dark as the temperature drops below the annealing temperature of the quench probe (Figure 2B). In contrast, positive QUASR reactions typically become brighter as they cool due to the temperature dependence of fluorescence quantum yield (Figure 2B). The combined effect is greatly increased signal discrimination as the reaction cools (Figure 2C). By comparison, discrimination between positive and negative reactions with intercalating SYTO dyes is optimal at higher temperatures (below the melting temperature of the amplicon). We plot signal discrimination as the ratio between the fluorescence from positive and negative reactions in Figure 2C. At room temperature and without background subtraction, the discrimination between positive and negative reactions is 8:1 for QUASR but only minimal for SYTO 62. With background subtraction (based on water only controls), QUASR discrimination approaches 700:1 at ambient temperature. The fluorescence from QUASR is sufficiently strong that it can be observed by eye, even indoors with the lights on, using a colored LED flashlight for excitation and a colored plastic film (theater lighting gel) acting as an emission filter. This makes it convenient to use in the absence of specialized equipment.

QUASR provides robust detection in the presence of crude sample matrixes. In Figure 3, we show that QUASR detection of MS2 with a Cy5-labeled primer provides better discrimination than SYTO 62 in a reaction mixture containing 10% whole human blood. Amplification in the presence of whole blood can be observed by monitoring the SYTO 62 signal in a real-time PCR machine, with a similar time to positivity as a reaction without blood, meaning that the RT-LAMP itself is not strongly inhibited. However, the absolute rise in signal with SYTO 62 is weak, perhaps due to complexation of the intercalating dye with components in blood. The Cy5 fluorophore used in QUASR, by contrast, is less sensitive to the presence of blood. Consequently, less sample processing may be required for LAMP or RT-LAMP point-of-care diagnostics or surveillance measurements from materials like soils. Unlike pH sensitive dyes,[8] QUASR remains compatible with these types of crude, buffered samples.

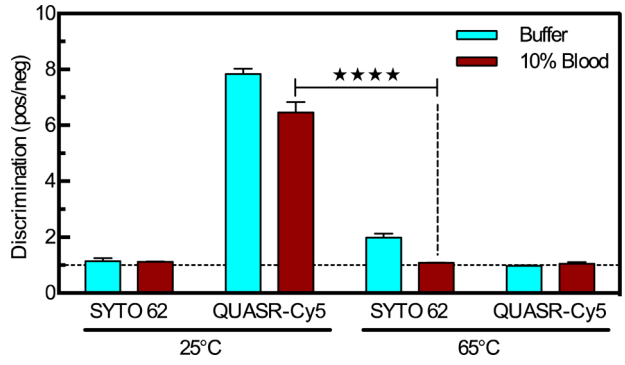

Figure 3. QUASR enables room temperature discrimination between positive and negative RT-LAMP reactions in 10% whole blood. In contrast, discrimination with SYTO 62 is completely lost in the presence of whole blood. Comparison by Tukey's test within 10% blood group following ANOVA, $P < 0.0001$. Other differences were statistically significant but not shown.

By combining multiple QUASR primer sets specific for different targets, spectrally multiplexed detection can be achieved, as demonstrated in Figure 4 for WNV and CHIKV. WNV is now endemic to the United States and regularly affects birds, livestock, and humans, causing severe symptoms and sometimes death.[17] CHIKV is an emerging virus globally,[18] with autochthonous transmission first reported in the United States in 2014.[19] Both viruses are transmitted by mosquito bites and present similar initial symptoms. A multiplexed assay for WNV and CHIKV would be useful for point-of-care diagnostics and vector-borne disease surveillance.[20] In Figure 4, the bright red (WNV) and green (CHIKV) fluorescent signals generated by the target-specific QUASR are easily distinguishable from negative reactions despite duplexing, which halves the concentration of each labeled primer set in the reaction. Simultaneous color detection is possible by examining the fluorescence overlay (Figure 4C), exciting with a green LED and observing through a 550 nm long pass filter Figure S3) or even exciting fluorescence with a blue LED and observing through an amber-colored gel filter, as captured by a smartphone in Figure 4D. The color difference in Figure 4D is more apparent when viewed in isolation, as shown in Figure S4. Because of its robustness, simplicity, and ability to multiplex, QUASR RT-LAMP could lower testing costs and expand access to diagnostics and biosurveillance tools in low resource settings where real-time monitoring is impractical. For such applications, discriminating positive from negative at a defined end point (e.g., 30 min of amplification) to provide a yes-or-no answer is instrumentally simpler than real-time

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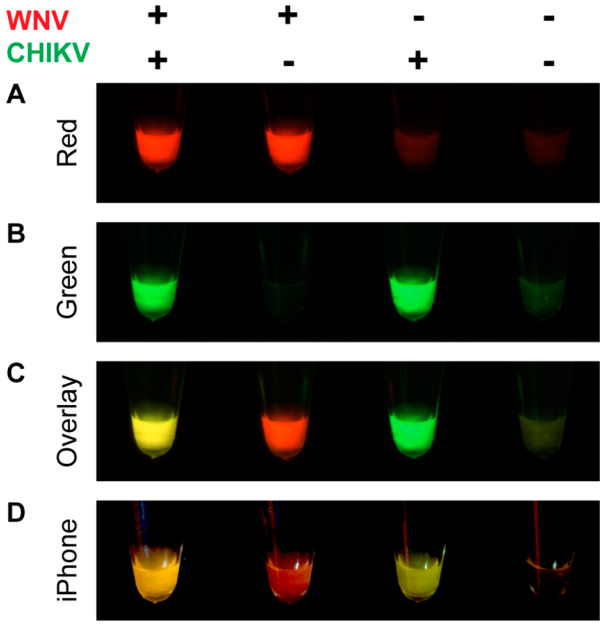

Figure 4. Multiplexed visual detection of WNV/CHIKV by QUASR RT-LAMP. 100 PFU equivalent of each viral RNA was used in each reaction where indicated by a plus sign. No template controls are indicated with a negative sign. WNV positives appear bright red when excited with green light (A), and CHIKV positives appear bright green when excited with blue light (B). A composite overlay of the images shows that the combination appears yellow (C). The image from an iPhone 6 using an unfiltered blue LED excitation source and a plastic theater gel as an emission filter confirms multiplexed detection (D).

quantitative detection and is simpler for a nonspecialist to interpret. In cases where quantitative detection of nucleic acids is desirable, qRT-PCR still outperforms qRT-LAMP, even when using refined methods.[21] Nevertheless, we note that one can combine QUASR with real time monitoring with intercalating dyes, such as SYTO 9, 62, or 82, to achieve uninhibited real time detection with a subsequent screen for false positives by end point detection with QUASR. This makes QUASR particularly useful for surveillance of rare viruses, for which true positive rates are similar to rates of false positives by SYTO dye detection.

The origins of nonspecific amplification in LAMP are complex, and different primer sets are susceptible to this phenomenon to different degrees. We cannot rule out that the labeled primer could participate in nonspecific amplification reactions in some circumstances, which could prevent post-reaction quenching by QUASR. However, we have observed that primer sets that occasionally give rise to positive signals in no-template control reactions monitored with an intercalating dye (which include the WNV and MS2 primer sets used in this study) rarely give rise to false positives with the QUASR technique (Table S4). A detailed examination of this phenomenon, across many primer sets, is beyond the scope of this report, but we note cautiously that the QUASR technique appears more resistant to false positive detection than nonspecific techniques such as intercalating dyes,[5] turbidity,[3] quenched calcein,[6] or pH-sensitive dyes.[8] We found that in extra-long incubations of no-template control WNV LAMP reactions, QUASR had a false positive rate of 1/197, whereas SYTO had a false positive rate of 67/145. More information is provided in the Supporting Information.

The acronym "QUASR" suggests a relationship to black holes and, by connotation, to darkness. Indeed, the previously described DARQ[13] technique exists on a continuum with our new QUASR technique (Figure 5). In both the QUASR and

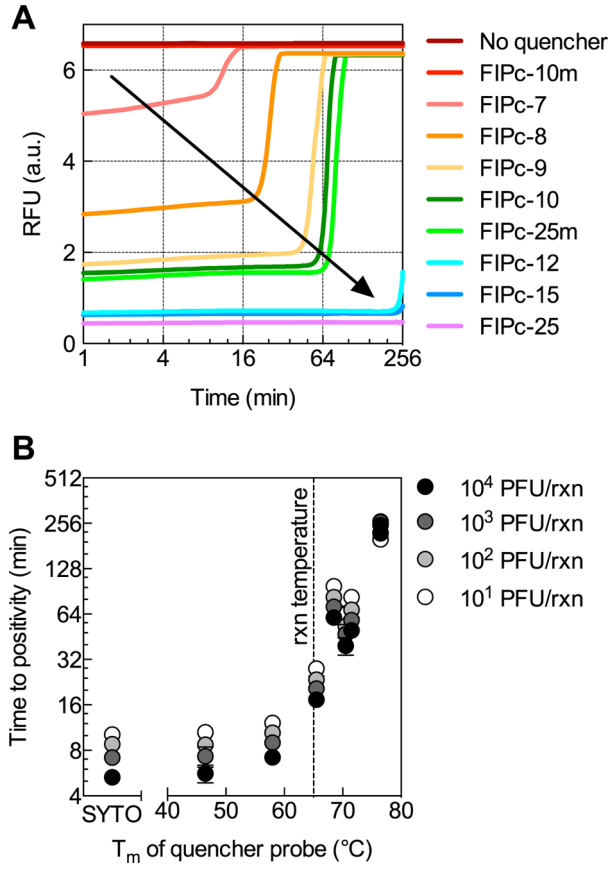

Figure 5. QUASR LAMP and DARQ LAMP exist on a continuum. (A) Real-time fluorescence detection of 10 000 PFU equivalent WNV RNA per 10 μL of reaction by RT-LAMP using FIP-ROX primer. Increasing the melting temperature of the FIP-complementary quencher probe decreases background fluorescence but dramatically slows amplification time. Quencher probes with internal base pair mismatches are denoted with the letter "m" at the end of their name. A full list of quencher probes is provided in Table S2. The arrow demonstrates the transition from QUASR RT-LAMP to DARQ RT-LAMP, represented by the full-length quenching probe FIPc-25. (B) The time to positivity, determined by real time monitoring with SYTO dye in a separate fluorescence channel, increases dramatically as the FIP/FIPc complex melting temperature approaches and surpasses the reaction temperature for RT-LAMP. Melting temperature is far more important than even a 1 000-fold change in WNV template RNA concentration.

DARQ techniques, fluorescent signal arises from a dye-labeled incorporating primer and complementary quencher (the converse arrangement is also reported for DARQ).[13] In contrast to DARQ LAMP, however, QUASR LAMP is noninhibitory and brighter but provides end point detection only. The key difference is that in the DARQ technique, a *full-length* complementary quencher is used, which is hybridized to the incorporating primer prior to the start of the reaction and must be displaced during the course of amplification to generate a signal. Although this approach allows real-time monitoring of the reaction, the presence of the bound quencher dramatically inhibits the reaction.

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As we demonstrate in Figure 5, QUASR and DARQ exist on a continuum determined by quenching probe Tm. We performed QUASR RT-LAMP for WNV using a ROX-labeled FIP primer and nine different quencher probes with increasing Tm. We monitored the fluorescent signal over time (Figure 5A) in the ROX channel and saw that noninhibitory quencher probes initially appear as bright or nearly as bright as reactions without quencher probes at 65 °C (Figure 5A). Consequently, minimal real time fluorescence change is observed in these reactions. In contrast, quencher probes with high Tm values led to reactions with low initial fluorescence and clear fluorescence increases upon delayed amplification. Using a separate fluorescence channel, we monitored the real-time progress of these RT-LAMP reactions with SYTO dye. This allowed us to observe that amplification times increased nonlinearly with quencher probe Tm. We plot this relationship between quencher probe Tm and time to positivity in Figure 5B. RT-LAMP was performed for all quencher probes using 10-fold dilutions of target WNV RNA, ranging from 10 000 to 10 PFU equivalent per reaction. We found that a 1000-fold dilution of target RNA was far less impactful on time to positivity than was an increase in quencher probe Tm to a value equal to or in excess of the reaction temperature. Thus, when designing quencher probes for QUASR, one should design the probes to anneal to the labeled primer at least 5−10 °C below the LAMP reaction temperature to avoid entering the regime of the DARQ LAMP technique.

QUASR also generates greater signal discrimination than DARQ LAMP. In ref 13, the authors typically use a 50:50 mixture of labeled and unlabeled primer to reduce the degree of inhibition seen in DARQ LAMP, but that approach also diminishes the intensity of the real time signal developed. In our hands, using the DARQ technique with several targets requires an even lower ratio of labeled to unlabeled primer for optimal speed, but this results in further reduced signal intensity (data not shown). Because QUASR utilizes shorter quenching probes that are dissociated at the temperature of the reaction, inhibition is negligible, and the fluorescently labeled primers can be used at full strength. If desired, simultaneous real time monitoring is easily facilitated with the addition of a spectrally distinct SYTO dye.

We also note that Curtis et al. reported use of full-length quenchers complementary to a labeled loop primer for RT-LAMP detection of HIV.[22] Their technique was recently replicated by Dauner et al. for RT-LAMP detection of dengue virus.[23] As noted above, use of a full-length quencher significantly inhibits the amplification and thus the technique of Curtis et al. and Dauner et al., while similar in principle to QUASR requires opening the tube to add the quencher at the conclusion of the reaction. This adds an extra step to the procedure and (like any open-tube method) adds the risk of amplicon contamination of the laboratory. Curtis et al. and Dauner et al. do note that their approach, like QUASR, eliminates false positives resulting from the nonspecific amplification that occasionally occurs in LAMP, supporting the observation that the dye-labeled primers are not incorporated to a high degree in nonspecific amplification products.

In a recent paper by Rudolph et al., Curtis et al. improved their previous technique by utilizing a truncated quenching probe for closed-tube detection of HIV in a fashion quite similar to the QUASR method.[24] From the data presented, however, it is hard to tell where the truncated quenching probe lay along the QUASR-DARQ spectrum. In addition, the focus of their work was on HIV detection in extracted RNA samples and did not explore the potential for multiplexing or crude sample analysis.

The typical cost of RT-LAMP is around $0.57 per 10 μL reaction assuming reagents are purchased at list price, with the majority of the cost attributable to the RT and DNA polymerases. In our experience, the additional cost of QUASR in LAMP or RT-LAMP ranged from $0.02 to $0.30 per reaction, about 3−10 times less than estimated maximum costs when ordering from the oligo supplier IDT. Higher costs per reaction were associated with small order sizes, lower yields from some fluorophore labeling reactions, and HPLC purification.

CONCLUSION

QUASR enables noninhibitory, bright, single-step, closed-tube, and multiplexed detection of DNA and RNA targets with LAMP and RT-LAMP. The specific duplex demonstrated here would offer, for example, the opportunity for a portable "field test kit" to detect the presence of both WNV (currently endemic across the continental USA) and CHIKV (currently a worldwide epidemic and currently emerging in the southeastern USA) in field-caught mosquitoes. Furthermore, QUASR is compatible with complex sample matrixes, such as blood, and requires no specialized equipment to observe reaction end points. We have applied the QUASR technique to numerous other bacterial and viral targets, with similar performance to that described here for MS2, WNV, and CHIKV. Combined with the general tolerance of LAMP and RT-LAMP to crude samples, we anticipate that QUASR will be an enabling technology for simple, rapid detection of nucleic acid targets.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04054.

Primer and quencher sequences; viral culture methods; characterization of RT-LAMP primer set for MS2 phage; reduction of false positives by QUASR RT-LAMP; and additional fluorescence images of duplex QUASR RT-LAMP (PDF)

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Author Contributions

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C.S.B., Y.K.L., C.-Y.K., and R.J.M. designed the study; C.S.B., Y.K.L., S.S.W., and R.J.M. performed the experiments; C.S.B., Y.K.L., C.-Y.K., and R.J.M. analyzed the data; and S.S.W. and L.L.C. provided the materials. All authors wrote the paper, and all authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Anal. Chem. 2016, 88, 3562−3568

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