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Development of advanced control material for reverse transcription-mediated bacterial nucleic acid amplification tests

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ABSTRACT Detection of bacterial RNA by nucleic acid amplification tests (NAATs), such as reverse transcription PCR (RT-PCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP), offers distinct advantages over DNA-based methods. However, such assays also present challenges in ascertaining positive and internal control material that can reliably monitor success over all phases of testing (bacterial lysis, nucleic acid recovery, reverse transcription, amplification, and signal detection): since they are unable to distinguish between amplification of bacterial RNA transcripts and the DNA templates that encode them, using intact organisms as controls can inform cell lysis but not successful detection of RNA. We developed a control strategy for RNA-based bacterial NAATs that allows ready discrimination of RNA from DNA templates using self-splicing bacterial introns, such that those nucleic acids ultimately encode different sequences. We engineered two vectors encoding synthetic transgenes based on this principle, one that is active in the Gram-negative bacterium *Escherichia coli* and one that functions in both *E. coli* and the Gram-positive organism *Staphylococcus aureus*. We subsequently designed RT-LAMP assays that either target RNA and DNA from transgenic organisms or target RNA exclusively and demonstrated the specificity of amplification using purified nucleic acids. Using multiplex fluorescent RT-LAMP of heat-lysed specimens, we showed the practicality of deploying such transgenic organisms as an internal control to ascertain sample integrity and assay performance during clinical diagnostic testing. Our approach has broad utility for RNA-based bacterial NAATs, especially point-of-care assays and other applications where nucleic acids are nonspecifically liberated for testing.

KEYWORDS molecular methods, diagnostics

Although DNA-based nucleic acid amplification tests (NAATs) presently comprise the dominant molecular detection methods for bacteria, RNA-based NAATs are beginning to be developed and adopted (1–3). RNA-based microbial NAATs have demonstrated advantages over DNA-based methods in their specificity for live bacteria (1, 4–8) and enhanced sensitivity resulting from signal amplification of highly transcribed bacterial genes (1, 2, 4, 5, 9, 10). Few such assays are able to directly recognize and amplify RNA templates using transcription-mediated amplification (TMA) (2, 3, 11, 12), while most depend on the action of reverse transcriptases to convert RNA to DNA templates prior to their amplification by DNA polymerases, as in reverse transcription PCR (RT-PCR) (10, 13, 14) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) (5). Of particular appeal are RT-LAMP assays, which integrate reverse transcription and isothermal template amplification into a single, one-step reaction (15). RT-LAMP provides a low-cost, reliable testing approach that can be performed without specialized instrumentation and can be made compatible with point-of-care or at-home testing (16–20). Already widely used for the detection of RNA viruses (15, 21), RT-LAMP assays for pathogenetic bacteria are rapidly emerging (4, 5, 22–27).

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When performing NAATs for clinical molecular diagnosis, it is critical to include an appropriate combination of negative, positive, and internal controls to assess successful assay performance (28–30). Negative controls allow the exclusion of false-positive results from contamination or other artifacts, while positive controls ensure that false-negative results from assay technical failures can be recognized. In contrast, internal controls allow verification of assay performance for each individual sample that is subjected to testing, permitting the recognition of inhibitors from specimen matrices that may impair testing and yield false-negative outcomes. These well-accepted paradigms for assay controls, however, pose a challenge for most RNA-based NAATs. A comprehensive positive or internal control for such assays must allow the user to ascertain success through all phases of testing, from bacterial lysis and recovery of intact cellular RNA through reverse transcription, amplification, and the ultimate detection of RNA templates. Yet, in RT-PCR and RT-LAMP, one cannot distinguish between amplification of bacterial RNA transcripts and the DNA that encodes them because DNA is recognized by the polymerases used in such assays and because RNA bears the same sequence as the DNA from which it is derived. Consequently, using intact bacteria as controls can identify successful release of nucleic acids but does not have specificity for the recovery or detection of RNA itself. This limitation is especially problematic for qualitative tests where the degree of amplification cannot be accurately measured and for applications where nucleic acid recovery is achieved via nonselective approaches like thermal bacterial lysis, where both DNA and RNA templates are present in the testing matrix.

Here, we have developed an approach that enables users to readily distinguish an expressed RNA transcript from its template DNA sequence in bacteria and have used it to construct transgenic bacteria with value as positive and internal control material for RNA-based molecular diagnostic assays. This strategy (Fig. 1) takes advantage of autocatalytic, self-splicing bacterial introns (31, 32), which result in RNA transcripts that differ in sequence from the DNA that encodes them. An intron with activity in the target organism, including its catalytic exonic components, is inserted into a marker gene and expressed. Primers that specifically amplify the spliced RNA transcript can be designed, either by virtue of their binding across exon junctions that are absent from template DNA or because the target length is prohibitively large prior to excision of the intron. We demonstrate the feasibility of this approach in two different organisms (*Escherichia coli* and *Staphylococcus aureus*) and show that transgenic strains can be used to provide comprehensive positive and internal controls in RT-LAMP assays.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. aureus strain JE2 and *E. coli* IM08B were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources). *E. coli* DH5 alpha was obtained from New England Biolabs. *Pseudomonas aeruginosa* PAO1 was generously gifted by Dr. Colin Manoil (University of Washington). All bacteria were cultured at 37°C in Luria–Bertani (LB) medium containing antibiotics if appropriate to select for plasmid vector maintenance (100 µg/mL ampicillin for *E. coli* and 10 µg/mL chloramphenicol for *S. aureus*).

Vector design and construction

The general design of expression cassettes used in this study was the same in both engineered vectors. Cassettes incorporated a T7 promoter sequence for possible *in vitro* transcription of the cloned transgenes, followed by a broad host range *Pcap* promoter and ribosome-binding site (33) to drive *in vivo* transcription. Downstream of the transgene, cassettes incorporated the T7hyb10 terminator sequence, which has strong activity against both T7 RNA polymerase and native bacterial RNA polymerases (34).

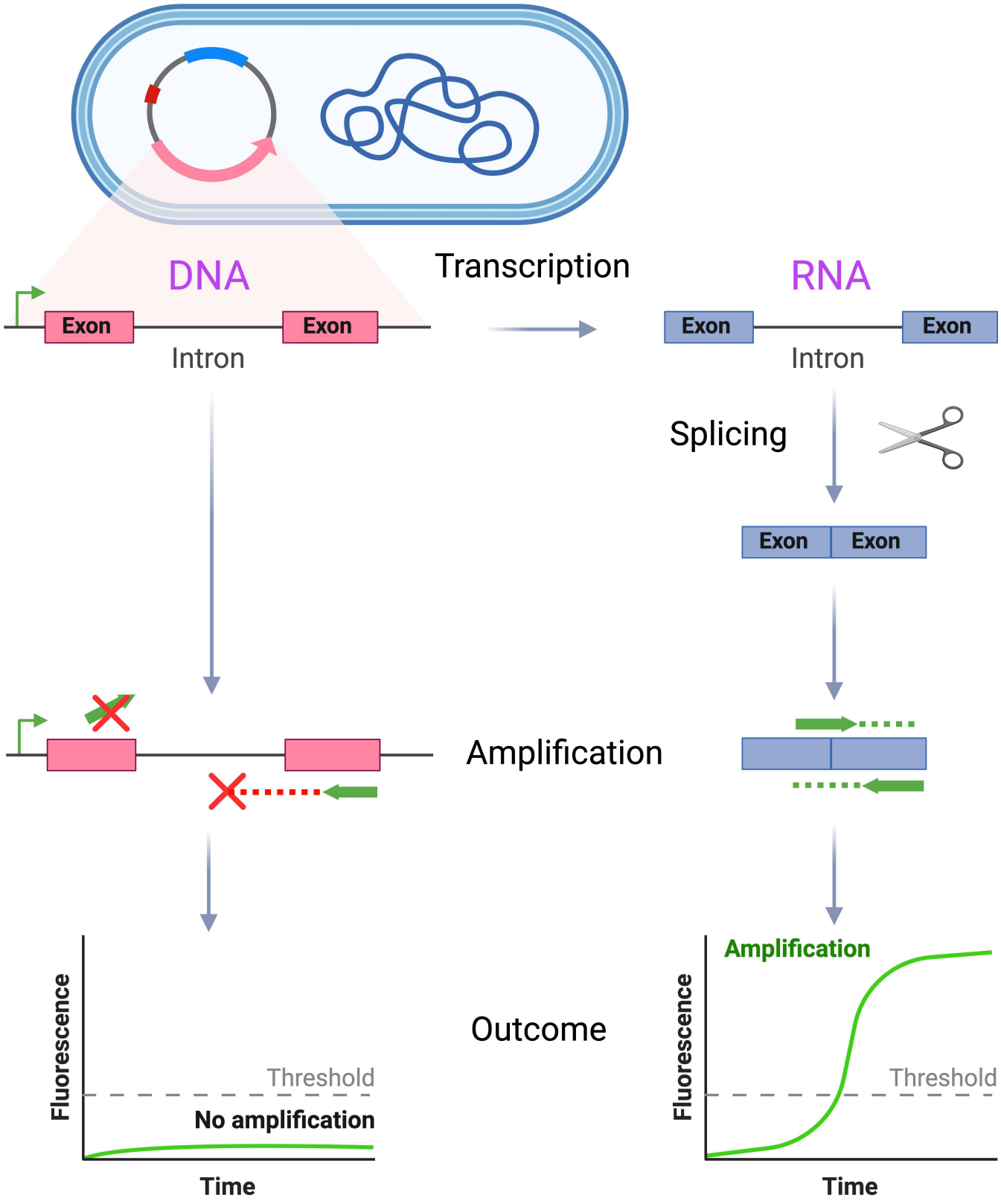

FIG 1 The engineered control material allows differential amplification of RNA and DNA from transgenic organisms. The bacterium of interest is transformed with a plasmid vector containing a target gene construct, wherein the target gene is disrupted by an autocatalytic, self-splicing intron that has activity in the chosen organism. Primers are designed that specifically amplify the spliced RNA transcript, rather than the template DNA, either by binding across the

(Continued on next page)

FIG 1 (Continued)

exon–exon junction (top primer) or because the amplicon length is prohibitively large, unless the intron has first been successfully excised (bottom primer). This schema allows selective amplification of RNA from the transgenic organism, which can be used to monitor successful assay performance through all phases of testing (bacterial lysis, nucleic acid recovery, reverse transcription, template amplification, and signal detection).

The *E. coli* splicing vector (pKTM_IAC) was synthesized by IDT into the pUC19-derived pUCIDT (Amp) Golden Gate vector. The KTM_IAC transgene (Fig. S1) contained the Tet-119-derivative of the *Tetrahymena* group I intron (wild-type sequence from Genbank JN547815.1) inserted into a version of *KTM* (Genbank HM151400.1), as previously described (35), except that *KTM* was re-encoded using *E. coli* codon optimization (Fig. S2) through the IDT codon optimization tool (https://www.idtdna.com/pages/tools/codon-optimization-tool), while preserving exonic residues deemed critical to splicing.

The *S. aureus* splicing cassette (Fig. S3) was initially synthesized by GenScript into pUC19. The transgene comprised the first exon, intron, and initial 22 bp of the second exon from bacteriophage Twort *orf142* (Genbank AF132670.1) with a short spacer sequence added at the 5′ end to improve primer design characteristics and followed by an in-frame superfolder green fluorescent protein (GFP) (36) codon optimized for *S. aureus* as above and from which the start codon was removed. We generated a temperature-stable derivative of the *E. coli–S. aureus* shuttle vector pCN50-BSAI (37) by replacing the temperature-sensitive pT181 *cop–634ts repC4* replicon with the wild-type pT181 *cop-wt repC* replicon from pCN33 (38), yielding pCN50wt. The expression cassette was subsequently cloned into pCN50wt to yield the final *S. aureus* splicing vector (pOrf142+gfp_mod). Vectors were transformed into *S. aureus* JE2 after plasmid conditioning in IM08B, as previously described (39).

Nucleic acid extraction

Total RNA was extracted from *E. coli* after lysis with lysozyme (Sigma) using the Monarch Total RNA Miniprep Kit (NEB), with on-column DNase I (NEB) treatment. *S. aureus* total RNA was similarly extracted after lysis with lysostaphin (Sigma) using the RNeasy Kit (Qiagen), followed by an in-solution DNase I digestion and secondary cleanup with the RNeasy Kit. Total DNA was extracted from both organisms using the DNeasy UltraClean Microbial Kit (Qiagen), followed by removal of residual RNA from the eluate by RNase A digestion (Thermo Fisher) and secondary purification using AMPure XP beads (Beckman Coulter).

RT-LAMP assays

Primers, probes, and quenchers were synthesized by IDT (Table S1). LAMP primers were designed using NEB LAMP Primer Design tool v 1.4.1 (https://lamp.neb.com/), with LB primers subsequently modified to carry 5′ adapter sequences for interaction with primer-specific fluorescent probes and quenchers (21). Fluorescent probes and quencher sequences were synthesized as previously described (21).

Cell equivalents of DNA used as the template for RT-LAMP were calculated, as elsewhere (40), based on the genomic sizes of *E. coli* DH5alpha and *S. aureus* JE2. Cell equivalents of RNA used were based on an estimate of 100 fg total RNA per bacterial cell (41).

All RT-LAMP reactions were conducted at 65°C using the WarmStart LAMP Kit (DNA & RNA) (NEB) following the manufacturer's instructions. Primer and probe concentrations were as specified elsewhere (21). Reaction fluorescence was measured using QuantStudio 3 (Applied Biosystems) in 30-second intervals over the course of a 1-hr incubation.

Bacterial thermal lysis experiments used aliquots of 10,000 cells per RT-LAMP reaction, based on spectrophotometric readings of overnight cultures (42, 43). Cells were diluted in TE buffer (pH 8.0), heated to 65°C for 10 m, followed by cooling on ice. Varying concentrations of RNase A were then added to lysed cell aliquots prior to RT-LAMP.

Fully deidentified residual stool specimens (~20–100 mg) were resuspended in 500 µL molecular-grade water by vortexing. Serial dilutions were prepared using molecular-grade water. *E. coli* carrying pKTM_IAC was added to each specimen at a final concentration of 1,140 cells per microliter. Aliquots were spiked with *P. aeruginosa* to a final concentration of 57 cells per microliter. Bacterial thermal lysis followed by RT-LAMP was conducted as before, using 8.75 µL specimen volume per reaction.

RESULTS

Design of a self-splicing marker gene in *E. coli* and RNA-specific RT-LAMP

We initially developed a system for enabling specific detection of RNA in the canonical Gram-negative bacterium *E. coli*. We repurposed a previously described, self-splicing construct comprising a bacterial antibiotic resistance gene (kanamycin nucleotidyltransferase, *KNT*) into which was inserted a 413-bp self-splicing intron derived from *Tetrahymena* (35) (Fig. S1). It is theoretically possible that a native form of the *KNT* gene could be present in test specimens, for example, if carried by other organisms in a patient microbiome or contaminating organisms from the environment and that this could lead to false-positive amplification from exogenous DNA sequences. To ensure specificity for the synthetic, spliced RNA target, we, therefore, re-encoded the sequence of this resistance gene to a form that is not found in nature (Fig. S2).

E. coli transformed with the transgene-containing vector (pKTM_IAC), but not the parental vector backbone (pUC19), proved resistant to kanamycin at a level of 50 µg/mL, indicating successful splicing of the synthetic construct into an active form of the RNA transcript.

We designed RNA-specific RT-LAMP assay primers that spanned the target gene's intron such that it fell between both the FIP and BIP primers and also between the F1 and F2 domains targeted by the FIP primer itself (8, 20). This configuration disrupts the binding of FIP primer to DNA templates and simultaneously makes the target region too large (621 bp from the ends of the outermost F3 and B3 primers) to be effectively amplified by RT-LAMP (44), unless splicing has successfully occurred (208 bp from the ends of F3 and B3 primers). RT-LAMP primers were additionally designed to leverage sites of sequence divergence between the native and re-encoded forms of the gene, with discrimination for the synthetic construct further enhanced by incorporating locked nucleic acid (LNA) bases at mismatched sites overlying the 3′ termini of assay primers (45).

We separately developed an RT-LAMP primer set for the ampicillin resistance gene (amp^R) encoded by the pUC19 plasmid vector backbone itself, which is expected to amplify both the DNA that encodes the gene and the RNA that is constitutively expressed from it.

RT-LAMP assays can specifically identify RNA templates in *E. coli*

To test our strategy for differential detection of nucleic acids in RNA-based NAATs, we selectively purified RNA and DNA from transgenic bacteria and conducted RT-LAMP reactions with primer sets targeting either plasmid DNA and RNA (amp^R primers) or those designed to be specific for spliced RNA from the plasmid-encoded target gene (transgene primers). RT-LAMP was performed using a commercially available cocktail of *Bst* polymerase and reverse transcriptase. We tested tenfold serial dilutions of purified RNA and DNA templates spanning 10,000 to 100 cell equivalents of *E. coli* that carried either transgene-expressing pKTM_IAC or the precursor pUC19 vector (Fig. 2A).

As expected, RT-LAMP primers targeting pUC19-encoded amp^R amplified both DNA and RNA fractions purified from bacteria proportionately to the amount of those templates provided. Amplification was achieved from *E. coli* carrying either pUC19 or pKTM_IAC, indicating the presence of intact nucleic acids for each condition tested. In contrast, amplification using the RNA-specific transgene primers occurred only for RNA purified from *E. coli* expressing the pKTM_IAC construct, with no measurable signal

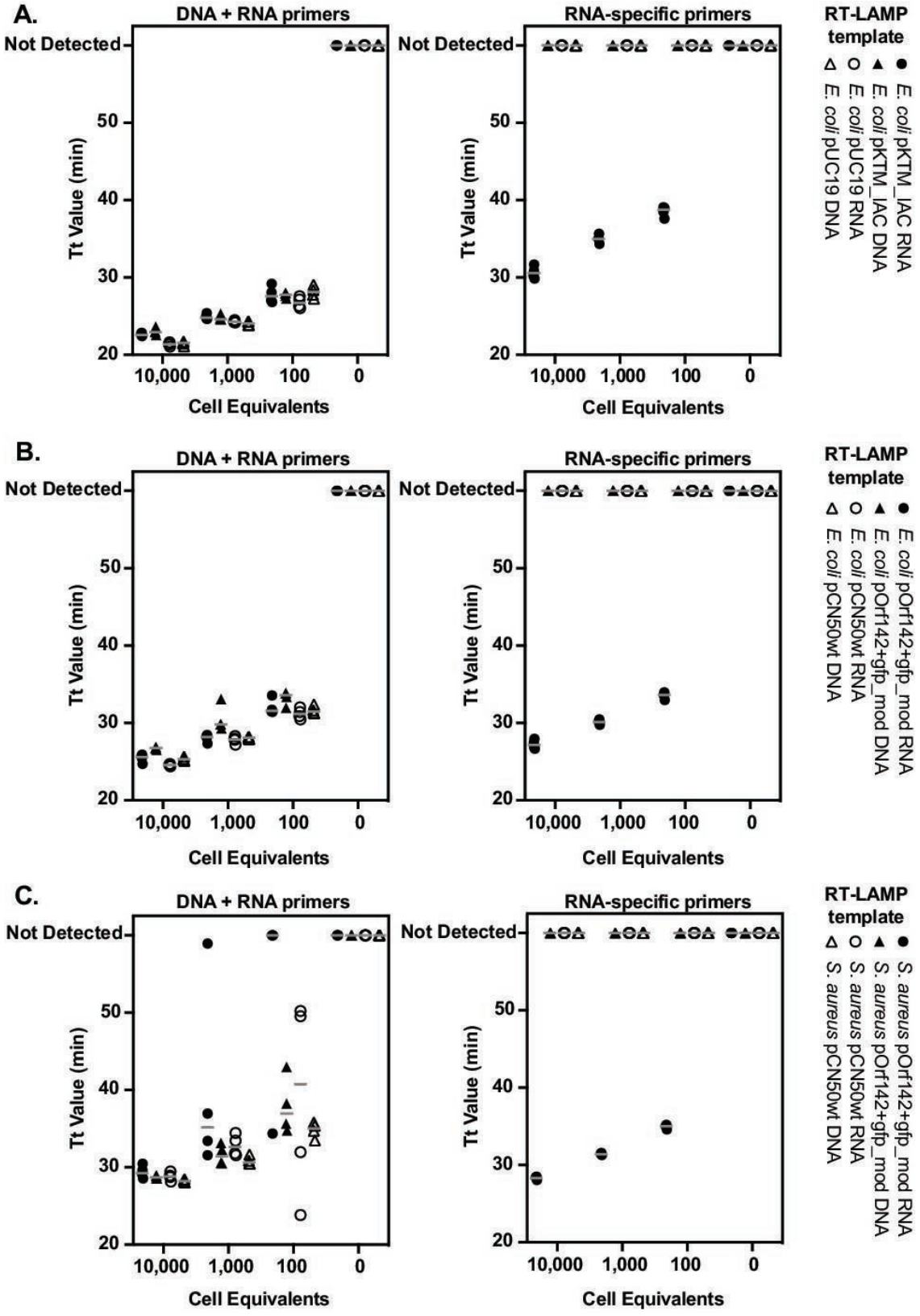

FIG 2 Selective amplification of RNA from self-splicing transgenes by RT-LAMP. Time to threshold (Tt) values are shown for RT-LAMP reactions targeting both RNA and DNA or spliced, transgenic RNA. Organisms carrying transgenic expression vectors and precursor vectors lacking the transgene were tested: (A) *E. coli* carrying pKTM_IAC or the pUC19 precursor vector, (B) *E. coli* carrying pOrf142+gfp_mod or the pCN50wt precursor vector, and (C) *S. aureus* carrying pOrf142+gfp_mod or the pCN50wt precursor vector. Various cell equivalents of purified RNA and DNA templates were tested in triplicate, with results of four technical replicates from each test superimposed. Gray horizontal bars represent the mean for replicates.

generated from the DNA fraction of those cells. RNA-specific RT-LAMP similarly failed to amplify either RNA or DNA of *E. coli* transformed with pUC19, which lacks the spliced transgene.

We conclude that RT-LAMP targeted against the spliced *KTM* target is highly specific for RNA templates and does not amplify the DNA template that encodes it.

Design of a self-splicing transcript with activity in both *E. coli* and *S. aureus*

To expand our strategy to a harder-to-lyse, Gram-positive organism, we next designed an analogous system that would function in *S. aureus*. We constructed a second vector (pOrf142+gfp_mod) housing a synthetic fusion of the self-splicing *orf142* gene from bacteriophage Twort (46, 47) and a start-loss mutated *GFP* (36) that was codon-optimized for *S. aureus* and cloned in-frame with the second exon of *orf142* (Fig. S3). Since in this implementation the *GFP* target gene is both synthetic and exogenous to bacteria, the design does not pose the risk of contamination from organisms in the environment or in tested specimens. The construct was built into an *E. coli-S. aureus* shuttle vector (pCN50wt) that carried pUC19- and pT181-derived replicons (38) in order to facilitate its construction and ultimate transformation into *S. aureus* (39).

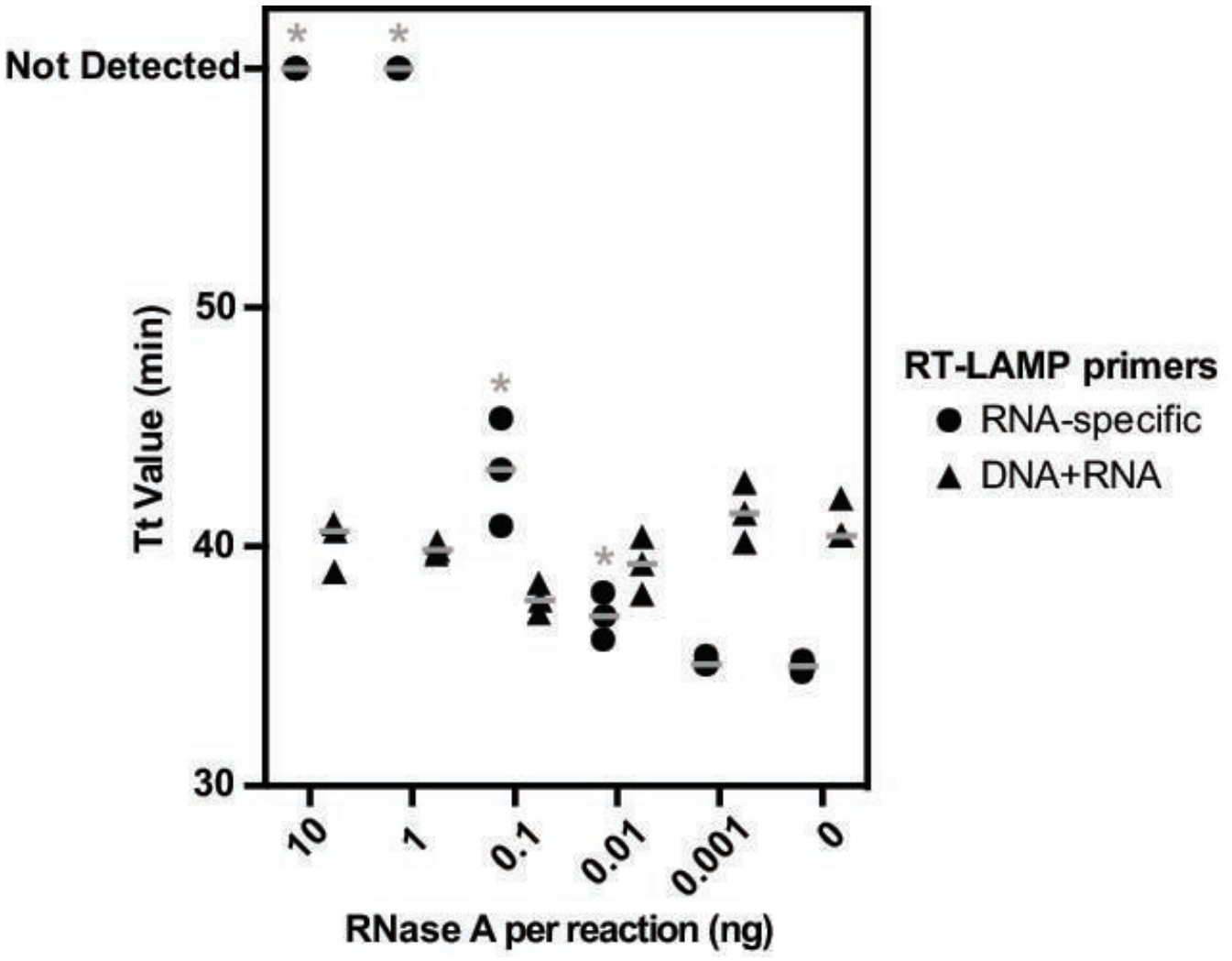

FIG 3 Self-splicing transgenes serve as internal assay controls. Time to threshold (Tt) values are shown for multiplex RT-LAMP reactions incorporating primer sets that target both RNA and DNA and those that are specific for spliced RNA from transgenic expression vectors. Heat-lysed specimens of *E. coli* carrying pKTM_IAC were combined with the indicated quantity of RNase A prior to RT-LAMP. Results from three technical replicates are superimposed, with Tt values significantly ($P \leq 0.05$, two-tailed T test) higher than controls lacking RNase A, indicated with an asterisk (*). Gray horizontal bars express the mean for replicates.

The pOrf142+gfp_mod transcript underwent successful splicing in *S. aureus*, as evidenced by visible GFP fluorescence in transgenic bacteria, but this phenotype was unexpectedly also observed for *E. coli* carrying the vector. This result indicated that autocatalytic activity in the intron was conserved across these two distantly related bacteria and that the vector construct could be used in both organisms.

We therefore tested the ability of pOrf142+gfp_mod to act as a control in both *E. coli* and *S. aureus*. We designed primers for RT-LAMP of the spliced target gene using the same schema as in our earlier construct and again performed serial dilutions of purified nucleic acids from transgenic *E. coli* (Fig. 2B) and *S. aureus* (Fig. 2C) that harbored either pOrf142+gfp_mod or the precursor pCN50wt vector, which lacks the transgene construct.

As in previous experiments, amplification of DNA and RNA fractions using *amp*^{R}-targeted primers confirmed the presence of both nucleic acids in specimens derived from *E. coli* and *S. aureus*. We noted that RT-LAMP of *amp*^{R} was less robust from *S. aureus* RNA fractions at 1,000 and 100 cell equivalents than from corresponding cell equivalents of *S. aureus* DNA, *E. coli* DNA, and *E. coli* RNA, evidenced both by increased time to threshold (Tt) values and greater variability between replicates. These outcomes (48) are consistent with less efficient transcription being driven by the Gram-negative *amp*^{R} promoter when transferred from *E. coli* to *S. aureus*, resulting in fewer RNA template molecules per organism. Regardless of this, we again observed that amplification with transgene-specific primers was specific for purified RNA from both *E. coli* and *S. aureus* expressing the Orf142+gfp_mod cassette. No product was detected from the DNA of those organisms or from RNA or DNA of bacteria carrying the empty pCN50wt vector backbone. These findings indicate that selective amplification of the spliced RNA transcript was achieved in both *E. coli and S. aureus*, without measurable amplification from plasmid DNA that encodes the expression cassette.

Multiplex RT-LAMP provides an internal control for monitoring assay performance

We next determined whether our approach could be used to provide an internal control for monitoring RT-LAMP assay performance when deployed alongside testing for a diagnostic target. To allow for simultaneous detection of the signals from separate primer sets within the same reaction, we adopted a strategy for multiplex RT-LAMP employing fluorescently labeled probes and quencher complexes that differentially bind to nucleotide tails on assay loop primers (21), allowing distinct amplification products to be fluorometrically distinguished.

We initially ascertained the viability of this multiplex assay strategy by ascertaining nucleic acid recovery and detection after thermal lysis of *E. coli* carrying pKTM_IAC, a crude method for total nucleic acid extraction that is compatible with point-of-care testing (49–51). In order to test a range of conditions under which RNA would be variably degraded, aliquots of the cell lysate were spiked with varying amounts of RNase A prior to multiplex RT-LAMP containing primers targeting pUC19 *amp*^{R} (serving as a diagnostic target) and the spliced *KTM* transgene (acting as an internal control) (Fig. 3).

Amplification of *amp*^{R} and the spliced *KTM* transgene was readily achieved by multiplex RT-LAMP after heat lysis, demonstrating robust signal detection from both targets simultaneously. Signals from the spliced *KTM* internal control decreased inversely with the amount of RNase A added to reactions, indicating that the assay's ability to detect RNA had been compromised. Significantly ($P \leq 0.05$, two-tailed T test) delayed Tt values relative to a lysed cell control were observed when using ≥0.01 ng RNase A per reaction, and complete signal loss from RNA-specific primers occurred with inclusion of 1 ng or more of RNase A. In contrast, the signal from the *amp*^{R} target was not measurably decreased under any test condition, consistent both with amplification occurring off the DNA present in those specimens and with the insensitivity of the pan-nucleic acid primer set to identify reduced RT-LAMP assay performance for RNA templates.

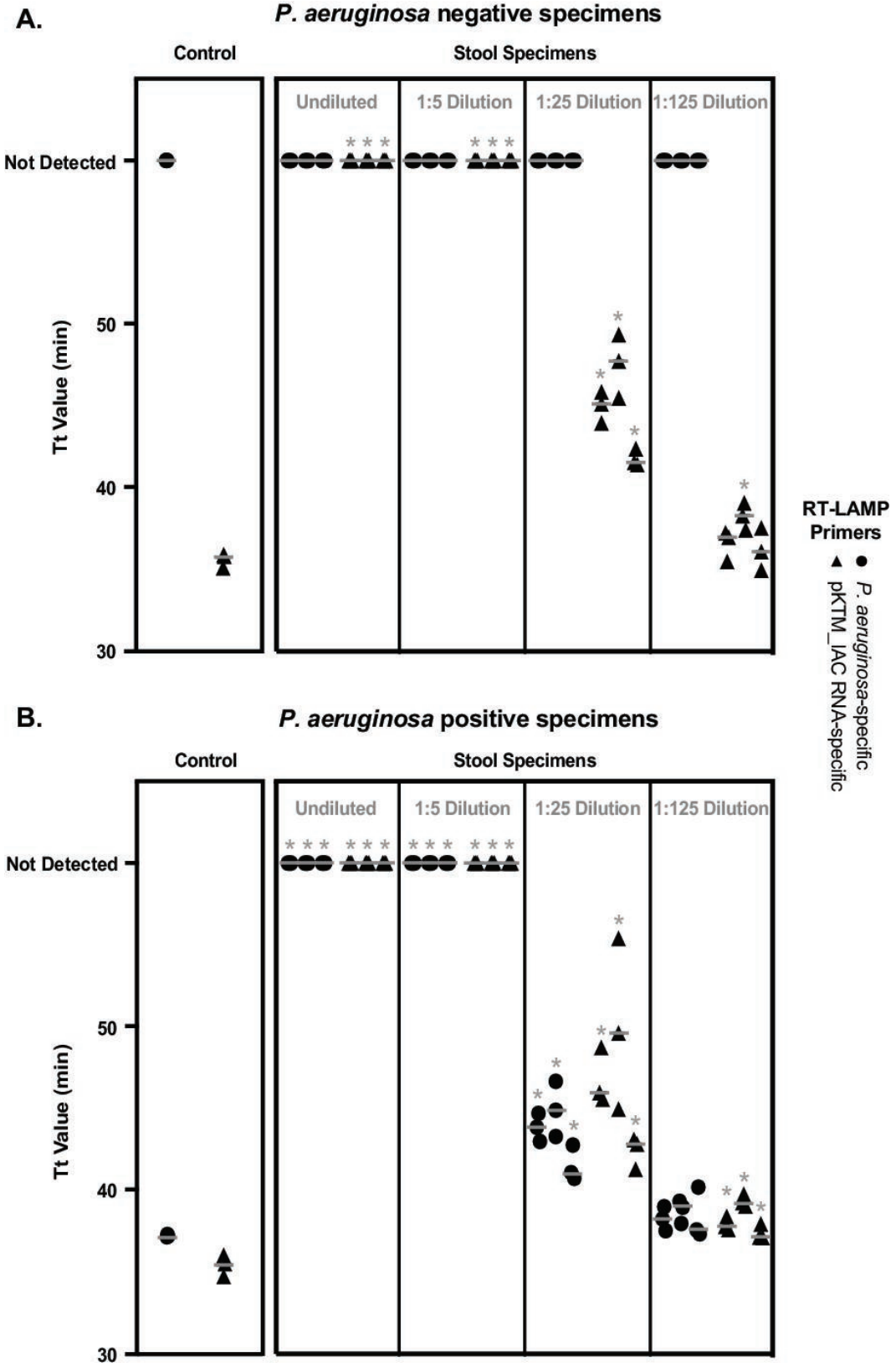

FIG 4 Diagnostic RT-LAMP assay for *P. aeruginosa* using transgenic *E. coli* as an internal assay control. Time to threshold (Tt) values are shown for multiplex RT-LAMP reactions incorporating diagnostic primer sets for *P. aeruginosa* and RNA-specific primers for the transgenic expression vector pKTM_IAC to serve as an internal assay control. Results are shown for control reactions lacking the clinical specimen matrix and for a series of three separate stool specimens. All

(Continued on next page)

FIG 4 (Continued)

specimens were tested in the presence of *E. coli* carrying pKTM_IAC, (A) without or (B) with the introduction of exogenous *P. aeruginosa*. Dilutions of stool specimens are indicated. Results from three technical replicates for each specimen are superimposed, with Tt values significantly ($P \leq 0.05$, two-tailed T test) higher than those of controls lacking clinical matrices indicated with an asterisk (*). Gray horizontal bars express the mean for replicates.

Finally, we explored the feasibility of using transgenic organisms to monitor assay performance in a clinical context. To this end, we tested an independent diagnostic target within clinical matrices that could contain additional microorganisms and inhibitory substances capable of confounding testing. We designed organism-specific RT-LAMP primers targeting a gene that identifies *Pseudomonas aeruginosa*, (52) an opportunistic pathogen able to seed hematogenous and respiratory infections after colonizing the gut of critically ill patients (53), and multiplexed this diagnostic assay with RNA-specific primers for the pKTM_IAC control. For these studies, we evaluated stool specimens as a relevant and challenging clinical matrix containing a high amount of molecular inhibitors (54, 55) and resident microbiota (56). *E. coli* carrying pKTM_IAC was added to stool specimens as an internal control, and aliquots were spiked with exogenous, cultured *P. aeruginosa* to generate positive reference material. Thermal bacterial lysis and multiplex RT-LAMP testing were then performed (Fig. 4).

In control specimens tested without clinical matrices, multiplex amplification from *P. aeruginosa* and transgenic control targets yielded similar Tt when both templates were present. Stool specimens showed complete inhibition of amplification from the internal control target, indicating that assay performance had been compromised by using that sample type. We therefore evaluated serial dilutions of the stool specimens that had been similarly spiked with control and target organisms to identify conditions under which valid testing results could be achieved. Amplification from the internal control and spiked *P. aeruginosa*, if present, could be detected using 1:25 dilutions of specimens, although the Tt from both targets was significantly delayed, relative to controls ($P \leq 0.05$, two-tailed T test). Assay performance was more fully restored with 1:125 dilution, although some specimens continued to show inhibition, as indicated by nominally higher Tt for internal controls ($P \leq 0.05$, two-tailed T test).

These experiments demonstrate the feasibility of using autocatalytic splicing constructs as an internal control for monitoring the RNA-dependent functions and diagnostic performance of RT-LAMP reactions, as well as the compatibility of such an approach with point-of-care testing procedures used in clinical diagnostic assays.

DISCUSSION

We have developed a robust strategy that enables users to specifically detect a targeted RNA transcript in bacteria using synthetic genes containing autocatalytic introns (Fig. 1). This system can be leveraged to design NAATs that are able to amplify templates only after self-splicing has occurred, achieving specificity for successfully reverse-transcribed RNA (Fig. 2). Although this concept has long been applied to the design of RT-qPCR assays for monitoring human gene expression (57), its application in prokaryotes represents a novel means to provide control material suitable for RNA-based bacterial NAATs, including RT-PCR and RT-LAMP. Because the system is self-contained within transgenic bacteria, the intact organisms themselves can be used as a control for all phases of testing: cell lysis, nucleic acid extraction, reverse transcription, template amplification, and detection. We have shown that control-encoded RNA transcripts and an experimental target can be simultaneously detected in multiplex RT-LAMP assays, which can be leveraged in clinical molecular diagnostic assays to provide an internal control for assessing test performance (21) (Fig. 3 and 4). This internal control schema is expected to provide an especially high value to applications such as point-of-care testing, where nucleic acid release is typically achieved by nonspecific methods and inhibitors present in the testing matrix are not removed prior to testing. Indeed, several *P. aeruginosa*-containing clinical matrices subjected to diagnostic RT-LAMP in this study would have been falsely reported as negative for that organism, rather than

indeterminate due to assay inhibition, without the interpretive framework enabled by the internal control. Inhibited tests could be validly interpreted after dilution of the specimen matrix was sufficient to allow amplification from the internal control (Fig. 4), illustrating how this schema could be used to assess assay performance in clinical practice.

We generated two versions of a transgenic control vector in order to facilitate its use in bacteria having different physical and genetic properties: One encodes an antibiotic resistance gene and is active in *E. coli*, while the other contains a synthetic GFP gene and functions in both *E. coli* and *S. aureus*. The functionality of the latter construct in Gram-positive and Gram-negative species alike was surprising, as prior work has suggested that the autocatalytic activity of such sequences requires species-specific optimization (35). This finding instead suggests that the synthetic target gene developed here may have even broader range generalizability outside of the bacteria tested in this study and could potentially be utilized in other species after integration with vector backbones and/or promoters that are compatible with those organisms.

Future improvements to this system can be envisioned. Transgenic bacteria expressing the target genes could be lyophilized or otherwise preserved to be rendered as stable control reagents for use in molecular assays (58). Inducible or repressible promoters could be introduced to tailor the expression of the target gene to specific levels. Separately, although the phenotypes encoded by our target genes were convenient for confirming proper splicing *in vivo*, they are not relevant to the control schema during molecular testing. Transgenes could consequently be altered to exhibit a particular GC content or amplicon length, making control assays targeting them better-matched to the primary diagnostic targets they are intended to be used with. Relatedly, the fluorescence of the GFP does not interfere with our molecular detection assays, but the function of that gene could be ablated through inactivating mutations if deemed necessary for some applications.

The systems presented in this work enable a novel form of internal and positive control material that is useful for RT-LAMP, RT-PCR, and other RNA-based bacterial NAATs. The strategy enables testing to simultaneously control for the full complement of pre-analytic and analytic points of failure possible with such assays. We project that this approach will be applicable for both laboratory-based and point-of-care molecular diagnostics and may be generalizable or adaptable to other bacterial species that are targeted for testing or are selected to emulate key properties of such targeted organisms.

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S.J.S. discloses a patent application based on the approach described in this work.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (JCM00243-24-s0001.pdf). Fig. S1 to S3 and Table S1.

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