METHOD

RNA detection with high specificity and sensitivity using nested fluorogenic Mango NASBA

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ABSTRACT

There is a pressing need for nucleic acid–based assays that are capable of rapidly and reliably detecting pathogenic organisms. Many of the techniques available for the detection of pathogenic RNA possess one or more limiting factors that make the detection of low-copy RNA challenging. Although RT-PCR is the most commonly used method for detecting pathogen-related RNA, it requires expensive thermocycling equipment and is comparatively slow. Isothermal methods promise procedural simplicity but have traditionally suffered from amplification artifacts that tend to preclude easy identification of target nucleic acids. Recently, the isothermal SHERLOCK system overcame this problem by using CRISPR to distinguish amplified target sequences from artifactual background signal. However, this system comes at the cost of introducing considerable enzymatic complexity and a corresponding increase in total assay time. Therefore, simpler and less expensive strategies are highly desirable. Here, we demonstrate that by nesting NASBA primers and modifying the NASBA inner primers to encode an RNA Mango aptamer sequence we can dramatically increase the sensitivity of NASBA to 1.5 RNA molecules per microliter. As this isothermal nucleic acid detection scheme directly produces a fluorescent reporter, real-time detection is intrinsic to the assay. Nested Mango NASBA is highly specific and, in contrast to existing RNA detection systems, offers a cheap, simple, and specific way to rapidly detect single-molecule amounts of pathogenic RNA.

Keywords: RNA; Mango; TO1 fluorophore; isothermal amplification; nucleic acid detection; nested NASBA; single-molecule detection

INTRODUCTION

Pathogens, such as viruses, bacteria, and eukaryotic parasites, are among the most serious sources of public health concerns worldwide (Law et al. 2015). Successful methods for disease diagnosis and treatment, food safety control, and environmental monitoring, therefore, require rapid and specific identification of infectious agents (Zhao et al. 2014; Law et al. 2015). Simplicity and low cost are equally important. For example, methods that do not rely on high-end instrumentation and skilled personnel can be used in settings where measles, influenza, HIV and Ebola viruses, malaria, gonorrhea, and salmonella-based food poisoning pose human health risks. By using these methods, their diagnosis could be facilitated when advanced diagnostic technologies are limited or nonexistent because of economic constraints (Niemz et al. 2011; Wang et al. 2016). Despite the tremendous efforts that have been made in biochemical engineering and nanotechnology, few pathogen detection methods currently meet all five criteria of affordability, sensitivity, specificity, rapidity, and robustness.

Traditional methods for bacterial detection involve culturing of organisms on agar plates and are laborious and time-consuming. They typically require 2–3 d of culturing and more than a week for the identification to be confirmed, greatly slowing the rate of effective diagnosis (Zhao et al. 2014; Law et al. 2015). Misdiagnosis is significant, and inappropriate therapies have been shown to reduce survival for serious infections by fivefold (Craw and Balachandran 2012). Significantly, culture methods can only identify organisms that are capable of growing on culture and thus fail to identify viable but unculturable pathogens (Ramamurthy et al. 2014; Law et al. 2015). These problems can be overcome by nucleic acid testing (NAT) methodologies, which fundamentally involve three steps: nucleic acid isolation, amplification, and detection. NAT methods are extensively used for pathogen detection (Niemz et al. 2011; Zhao et al. 2014; Law et al. 2015). Between DNA and RNA, RNA detection is of particular interest, as many pathogens express higher initial template

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Nevertheless, amplifying very low concentrations of nucleic acid presents the increased possibility for nonspecific primer hybridization allowing for the amplification of incorrect products. Recently SHERLOCK was developed to address this problem by using a second CRISPR-mediated step to effectively screen the products of isothermal ampli-

fication (RPA was demonstrated) for template specific amplification products (Gootenberg et al. 2017, 2018). This additional validation step allows the artifactual background of the preceding isothermal step to be selectively overcome. Although having an impressive sensitivity down to the aM level and although it enables SNP-based specificity, SHERLOCK-based detection requires a total of six enzymes (reverse transcriptase, RPA: recombinase/polymerase/strand displacement proteins, T7 RNA polymerase, and Cas13a). This, together with a requirement for a synthetic fluorescent reporter construct, adds significant complexity to the method.

Fluorogenic RNA aptamers offer an exciting opportunity to report the successful amplification of target RNA sequences. Although they have been previously used to monitor the course of a transcription in vitro by producing a fluorescent signal (Sando et al. 2008; Höfer et al. 2013; Autour et al. 2016; Alam et al. 2019; Shi et al. 2019), coupling their synthesis to the amplification provided by an isothermal assay has, to our knowledge, not been described prior. The RNA Mango aptamer series has nanomolar binding affinity to a thiazole orange–based ligand (TO1-Biotin) and enhances the brightness of the fluorogenic ligand up to 4000 times upon binding, which makes them useful in vitro fluorescent reporters (Dolgosheina et al. 2014; Dolgosheina and Unrau 2016; Autour et al. 2018). Of particular note, the second generation of RNA Mango aptamers (Mango II, III, and IV) are highly resistant to the magnesium concentrations and work in a broad range of monovalent metal ion concentrations that are typically found in in vitro assays (Autour et al. 2018; Trachman et al. 2019).

Here, by nesting NASBA primers and modifying the inner set of NASBA primers to encode a fluorogenic aptamer, we demonstrate that we can detect attoMolar (aM, 1.5 RNA molecules per μL) concentrations of RNA template. In the absence of nesting, we show that Mango NASBA (Fig. 1B) has a sensitivity in the picoMolar (pM) template range but such an unnested method is highly susceptible to background amplification artifacts. Nested Mango NASBA (NMN, Fig. 1C; Supplemental Fig. S1) in contrast is much more robust and even in the presence of significant amounts of competitor template can reliably detect low concentrations of RNA template.

RESULTS AND DISCUSSION

Sensitivity of Mango NASBA

We modified previously published NASBA primers for *Escherichia coli* *ClpB* (Heijnen and Medema 2009) to include a Mango III sequence variant allowing us to detect ~ 25 pM (1.5×10^7 RNA/ μL) of *E. coli* *ClpB* RNA template. However, detection was hampered by a high artifactual background fluorescent signal that amplified rapidly even in the complete absence of RNA template and allowed only a twofold distinction between background and signal (primers P1/P2A; Table 1; Fig. 2A; Supplemental Fig. S2). This relatively poor intrinsic sensitivity was not primer- nor template-specific, as *Pseudomonas fluorescens* *ClpB* reverse-complement RNA template could be detected with similar sensitivity using primers designed to hybridize a shorter distance apart than for the *E. coli* *ClpB* target. Although these primers had considerably less intrinsic background fluorescence signal, they too could only demonstrate an RNA detection sensitivity of pM (primers P7/P8; Table 1; Supplemental Fig. S3). To explore the source of the artifactual signal found with the *E. coli* primers, RNA

TABLE 1. Sequences of primers and targets

Identifier	Sequence
ClpB Short Target <i>E. coli</i> (Ec)	GGA CGU CUG GAA GAA CGU GGU UAU GAA AUC CAC AUU UCU GAC GAG GCG CUG AAA CUG CUG AGC GAG AAC GGU UAC GAU CCG GUC UAU GGU GCA CGU CCU CUG AAA CGU GCA AUU CAG CAG CAG AUC GAA AAC CCG CUG GCA CAG CAA AUA CUG UCU GGU GAA UUG GUU CCG GGU AAA GUG AUU CGC CUG GAA GUU AAU GAA GAC CGG AUU GUC GCC GUC CAG UAA AUG AUA AAA CGA GCC CUU CGG G
Ec PCR Forward Primer	CTT TAA TAC GAC TCA CTA TAG GAC GTC TGG AAG AAC GTG GTT ATG
Ec PCR Reverse Primer	CCC GAA GGG CTC GTT TTA TCA TTT A
P1: Ec T7 Outer NASBA Top Primer	AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GCT GGA CGG CGA CAA TCC GGT CTT CA
P2A: Ec Mango III A10U Outer NASBA Bottom Primer	GGC ACG TAC GAA TAT ACC ACA TAC CAA ACC TTC CTT CGT ACG TGC CAA ATC CAC ATT TCT GAC GAG G
P2B: Ec Outer NASBA Bottom Primer (- Mango)	AAA TCC ACA TTT CTG ACG AGG
P2C: Ec Mango III Outer NASBA Bottom Primer	GGC ACG TAC GAA TAT ACC ACA TAC CAA TCC TTC CTT CGT ACG TGC CAA ATC CAC ATT TCT GAC GAG G
P3: Ec Inner T7 NASBA Top Primer	AAT TCT AAT ACG ACT CAC TAT AGG GAA GGA AGT CTG GTG AAT TGG TTC CGG
P4: Ec Inner Mango III A10U NASBA Bottom Primer	GGC ACG TAC GAA TAT ACC ACA TAC CAA ACC TTC CTT CGT ACG TGC CTT CCA GGC GAA TCA CTT TAC
ClpB Short Target <i>P. fluorescens</i> (Pf)	GGU CGC CUG GCC GAG CGU GAG CUU GAC CUG GAG CUG AGC AGC GAG GCG UUG GAC AAG CUG AUU GCG GUC GGU UAC GAC CCG GUG UAU GGC GCA CGG CCA CUU AAA CGU GCG AUC CAG CGC UGG AUC GAA AAC CCA CUG GCA CAG UUG AUC CUG UCG GGC AGC UUC AUG CCA GGC ACC CGC GUG ACG GCC ACG GUG GAA AAC GAC GAA AUC GUC UUC CAC UAA GCC CAG CCU GUA GGG UUA UUA GAG A
Pf PCR Forward Primer	CTT TAA TAC GAC TCA CTA TAG GTC GCC TGG CCG AGC GTG AGC TTG
Pf PCR Reverse Primer	TCT CTA ATA ACC CTA CAG GCT GGG C
P5: Pf T7 Outer NASBA Top Primer	CTT TAA TAC GAC TCA CTA TAG GGA GGC TGG GCT TAG TGG AAG A
P6: Pf Outer NASBA Bottom Primer	GAG CAG CGA GGC GTT GGA CA
P7: Pf Inner T7 NASBA Top Primer	CTT TAA TAC GAC TCA CTA TAG GGC GAA AAC CCA CTG GCA CAG T
P8: Pf Inner Mango III A10U NASBA Bottom Primer	GGC ACG TAC GAA TAT ACC ACA TAC CAA ACC TTC CTT CGT ACG TGC CCG CGG GTG CCT GGC ATG AAG

Sequence in bold corresponds to bolded identifier information.

products were analyzed after 2 h of NASBA incubation in a denaturing gel. Small RNA products were produced for all template concentrations between 0 and 25 fM as judged by TO1-Biotin gel staining (Supplemental Fig. S4A). These gel results suggest that primer mishybridization results

FIGURE 2. Nested Mango NASBA is sensitive and specific to target RNA sequence and is robust even when an unrelated nucleic acid sample is added. (A) Un-nested outer (*E. coli ClpB* RNA, *E. coli* primers P1/P2A) sensitivity. (B) Nested RNA Mango dramatically improves sensitivity. *E. coli* primers (P1/P2B and P3/P4) with *E. coli ClpB* RNA (Ec/Ec) and is shown in blue. Using the same *E. coli* primers, *P. fluorescens ClpB* RNA was added (Ec/Pf) instead of *E. coli*; target is shown in purple. *P. fluorescens* primers (P5/P6 and P7/P8) with *P. fluorescens ClpB* RNA (Pf/Pf) is shown in red. (C) Nested Mango NASBA using *E. coli* primers as in panel B was performed with the inner NASBA time courses shown. (Blue) 150 *E. coli* target molecules/ μL , (red) 150 *E. coli* target molecules/ μL in the presence of 5 ng/ μL of A549 Human Lung Carcinoma total nucleic acid, (black) no template added, (gray) 5 ng/ μL of A549 Human Lung Carcinoma cell total nucleic acid. Error bars reflect the standard deviation of three replicates for all panels.

in transcriptional artifacts able to produce short Mango aptamer containing RNA. Although such artifacts could in principle be avoided by careful primer design, based on these results the intrinsic sensitivity of Mango NASBA remains highly limited for the detection of target RNAs at concentrations lower than pM. As similar problems are observed during PCR amplification of low-copy-number templates and because nesting PCR primers is a viable solution, we decided to test the nesting of NASBA primers (Fig 1C; Supplemental Fig. S1), which to our knowledge, has not been previously implemented.

Sensitivity and specificity of nested Mango NASBA

Analogous to nested PCR, nesting NASBA primers avoids artifacts by selectively amplifying only RNA sequences that

are recognized by not one set of primers, but by two (outer: PA/PB; inner: PC/PD; Fig. 1C). To optimize the transition to the inner primer NASBA reaction, we first tested how little NASBA primer is needed to result in isothermal amplification, based on the hypothesis that dilution below this concentration would preclude the activity of the outer primers in the inner NASBA reaction. Using an *E. coli* template concentration of 25 pM, it was observed that primer concentrations of <5 nM resulted in negligible amplification product even after 120 min (Supplemental Fig. S4B). Therefore, we decided that after quenching the outer NASBA reaction by the addition of EDTA, we would dilute the stopped reaction 100-fold into the inner NASBA reaction, as our NASBA primer concentrations are used at a concentration of 250 nM.

In our first test of NMN, we used *E. coli* outer primers lacking the Mango tag but otherwise identical in sequence to those used in the previously discussed Mango NASBA (P1/P2B; Table 1). The outer NASBA reaction was incubated for 40 min at 41°C. We then diluted this EDTA quenched outer reaction 100-fold into an inner NASBA reaction in which nested inner NASBA Mango primers designed to contain a Mango III tag were now added (P3/P4; Table 1). In contrast to un-nested Mango NASBA, in which amplification artifacts emerged even in the zero template control, we could

now easily detect 25 aM (15 RNA/ μL) (Ec/Ec, Fig. 2B). Even though the outer primers were presumably not optimal in design, nesting these primers with inner Mango NASBA primers provided a more than six orders of magnitude increase in sensitivity. Examining the time-dependent emergence of the inner Mango NASBA fluorescence indicated that a robust time-dependent signal emerged in <60 min of incubation (Supplemental Fig. S5), indicating that NMN can be performed in as little as 100 min.

Using this NMN strategy we then designed a NMN *P. fluorescens* primer set. A new outer primer pair was designed while we utilized the previously designed outer Mango NASBA primers as the inner primer pair RNA (outer: P5/P6; inner: P7/P8 Table 1). Here we could detect 2.5 aM (1.5 RNA/ μL) of *P. fluorescens ClpB* target (Pf/Pf, Fig. 2B; Supplemental Fig. S5). These *P. fluorescens* primers

yielded higher sensitivity, possibly because of the more stringent design rules we used to minimize both primer dimerization and primer self-extension. Specifically, we designed the 3' ends of each primer so as to preclude hybridization to either itself or its partner by any more than 3 nt where possible. Nesting NASBA primers, together with these simple design constraints, sensitivity dramatically increased by eight orders of magnitude relative to the unnested Mango NASBA (Supplemental Fig. S3).

The *E. coli* and *P. fluorescens* *ClpB* templates tested here differ by 78 nt in the nested primer binding sequence region, 65 nt of which are in primer hybridization regions (see alignment in Supplemental Fig. S6). When the NMN primers used to target *E. coli* *ClpB* were used against the *P. fluorescens* target, fluorescence remained within the same error as the 0 RNA/ μ L reaction control for all ranges of template concentrations tested (Ec/Pf; Fig. 2B; Supplemental Fig. S5). This suggests that even nonoptimal primers, when used in a nested fashion, can be specific for their targets and do not suffer from the artifactual amplification products we had observed in unnested NASBA.

To further explore the specificity of NMN, a large amount of human total nucleic acid was mixed with a low concentration of *E. coli* *ClpB* template. *E. coli* *ClpB* RNA target was mixed with or without an excess of human total nucleic acid (150 RNA molecules/ μ L final *E. coli* *ClpB*, 5 ng/ μ L human total nucleic acid) and NMN was performed using the Ec primers (Fig 2C; outer: P1/P2B; inner: P3/P4; Table 1). Although this amount of human nucleic acid definitely had an effect, decreasing the amplification of the *E. coli* positive signal and enhancing the emergence of the negative template sample, the assay was still remarkably robust and was able to detect the specific *E. coli* target in ~80 min of total inner and outer reaction time, as signal was clearly differentiated from the negative controls after as little as 40 min of inner Mango NASBA incubation.

To further test the applicability of NMN, we explored the ability of the system to detect *E. coli* cells in human tissue culture. Exponentially growing cells were quantified by OD, pelleted, and then added to 50 μ L of depleted tissue culture media. Using a standard RNA column purification kit, serial dilution of this sample allowed us to detect by NMN (Table 1; outer primer: P1/P2B; inner primer: P3/P4; Supplemental Fig. S7) what we calculate to be the equivalent of 32 to 1000 cells of *E. coli*. As expected, amplification artifacts from the outer reactions were observed, with amplification products visible by SYBR Safe in the highest bacterial concentrations tested (Supplemental Fig. S8A). After a longer 4 h inner Mango NASBA amplification, so as to maximize the accumulation of possible artifacts, the resultant RNA products were loaded into denaturing gels and stained with either TO1-Biotin (to visualize Mango containing RNA transcripts) or SYBR Safe (to visualize total RNA transcribed). In contrast to the outer NASBA products, the inner RNA products show the ex-

pected RNA size when stained with TO1-Biotin, down to the 32- to 1000-cell concentration range, at which point a smaller product was also formed that was present in the depleted media negative control lane (Supplemental Figure S8B). Staining the inner NASBA RNA products with SYBR Safe indicated that in addition to these minor artifactual species, other RNAs had been produced that were both longer and shorter (Supplemental Fig. S7C); however, these bands did not encode a Mango tag as they were not observed to be significantly stained in the TO1-Biotin gel.

The ability to detect aM concentrations of RNA using NMN in a fashion that is intrinsically resistant to the presence of high levels of competing nucleic acids makes this assay generally useful as it can detect single molecules. New technologies, such as SHERLOCK, also report ~2 aM sensitivity (Gootenberg et al. 2017). Comparatively, NMN was able to reliably detect a concentration of 2.5 aM or 1.5 RNA molecules per μ L of reaction. Notably in this regard, at very low concentrations of template the large error bars we observe for 0.15 mol/ μ L (three molecules in this 20 μ L reaction) and 0.5 mol/ μ L (10 molecules per reaction) *P. fluorescens* time series (Supplemental Fig. S5) result from averaging a very digital all-or-nothing response in the triplicate data (Supplemental Fig. S9). Therefore, we are perhaps being conservative in setting our limits of detection, which may in actuality be lower than stated here, but that would require more precise methods of single-molecule analysis to fully resolve.

Although these systems are both very sensitive to RNA detection, NMN can be performed using only a reverse transcriptase, RNase H, and T7 RNA polymerase (three enzymes) in two simple sequential reactions that differ only in primer composition and that provide real-time detection by the addition of commercially available TO1-Biotin fluorophore. This may well make NMN more robust than SHERLOCK, which by comparison requires the use of a reverse transcriptase, a three-enzyme isothermal amplification system (RPA), T7 RNA polymerase, and a Cas13a (six enzymes total) along with a cleavable fluorescent reporter (Gootenberg et al. 2017). However, SHERLOCK, with orthogonal CRISPR enzymes, can be multiplexed for the detection of multiple targets and can be performed in a single reaction tube (Gootenberg et al. 2018). Although NMN has not been demonstrated in a multiplexed form at this time, it appears entirely possible that given the high ligand specificity of the Mango aptamer (Jeng et al. 2016), NMN multiplexing will be possible in the future via the generation of orthogonal RNA aptamers for same tube multiplexing. Further, it is also entirely possible to multiplex by first performing an outer Mango NASBA reaction across a conserved region of RNA that contains variable interior sequence regions. Parallel inner NASBA reactions could then be run to identify individual sequence variants. Work using NMN should ideally be performed in a clean and

sterile environment to prevent cross-contamination during transfer from the outer reaction into the inner reaction (a common point of care for nested PCR, as the tube containing amplified target must be opened for transfer).

In conclusion, the Mango NASBA approach offers a simple real-time method for the fluorescent method for detection of RNA. By nesting NASBA and achieving a real-time readout with Mango aptamers, NMN displays dramatically improved sensitivity capable of single-molecule detection as demonstrated here. NMN has high specificity and allows for detection of target RNA even in the presence of a large background of nucleic acid. As the inner and outer reactions used in NMN are identical except for their primer composition, the system remains minimally complex, making such assays highly scalable.

MATERIALS AND METHODS

ClpB target RNA generation

ClpB DNA template was first generated by colony PCR reactions performed by picking either *E. coli* (strain DH5 α) or *P. fluorescens* (strain ATCC 13525) colonies directly into PCR reactions consisting of *Taq* (NEB, 1 U/10 μ L final), 0.2 mM each dNTP, 10 mM TRIS buffer pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin and the relevant primers (IDT) from Table 1. The resulting DNA was cloned into a pGEM-T Easy Vector (Promega) and sequences confirmed by Eurofins tube sequencing. Using plasmid as template, PCR reactions were then carried out and followed by ethanol precipitation in 300 mM NaCl and 70% ethanol. Pellets were resuspended in water to generate a 10 $\times$ DNA template stock. Transcriptions were carried out using 2 $\times$ template, T7 RNA polymerase (Applied Biological Materials), in 8 mM GTP, 5 mM CTP and ATP, 2 mM UTP, 40 mM Tris buffer pH 7.9, 2.5 mM spermidine, 26 mM MgCl₂, and 0.01% Triton X-100. RNA was purified via 5% PAGE (19:1 Acrylamide:bis). The resolved RNA band excised and placed on a rotator overnight in 300 mM NaCl, before recovery by ethanol precipitation and resuspension in water. Concentrations were determined using a SHIMADZU dual beam spectrophotometer and IDT's on-line nearest neighbor estimate for extinction coefficient.

Mango NASBA

NASBA primers (IDT) were designed to target RNA amplification to the *E. coli* or *P. fluorescens* *ClpB* mRNA templates generated above (shown as "*ClpB* short target *E. coli*" and "*ClpB* short target *P. fluorescens*," respectively, Table 1; Supplemental Fig. S6). Reactions were carried out using NASBA buffer mix (Life Sciences, NECB-1-24), nucleotide mix (Life Sciences, NECN-1-24), 250 nM of each primer described in the text, 480 nM TO1-Biotin (Applied Biological Materials), and NASBA enzyme mix (Life Sciences, NEC-1-24) mixed to a final volume of 14 μ L. NASBA reactions were mixed excluding the enzyme mix and RNA target. RNA template was heated for 2 min to 65°C and brought down for 5 min to 41°C in a MJ research PTC-100 thermocycler before adding 1 μ L of the template to each reaction

mix (final template concentrations of either 0, 25 aM, 25 fM, 25 pM). To initiate the reaction, 5 μ L of enzyme mix was added to the reactions and mixed. Reaction tubes were then sealed and incubated at 41°C in eight-tube strips with optical caps (Applied Biosystems, catalog # 4358293, 4323032) on a StepOne Real-Time PCR System (Applied Biosystems). This instrument was set to read SYBR Green reagent in the following program: (i) Ramp to 41°C, read; (ii) hold for 30 sec at 41°C, read; and (iii) repeat step 2 until 480 cycles complete. Experiments shown in Supplemental Figure S4 were carried out with primer P2C (Table 1) using the WT Mango III that did not carry the A10U mutation. This mutation is known to make Mango III 14% brighter (Trachman et al. 2019).

Nested Mango NASBA

Outer amplification reactions were performed as just described above and using the primers in the Results and Discussion. Outer reactions were stopped at 40 min by the addition of 5 μ L of 50 mM EDTA to a final concentration of 10 mM and flash frozen in either liquid nitrogen or ethanol on dry ice. After thawing, 5 μ L of these reactions were diluted with 15 μ L ddH₂O, and 1 μ L of this dilution was promptly added into the inner-nested Mango NASBA reactions for a net 100-fold dilution. The inner primers are as described in the text (Table 1). Reactions were monitored for fluorescence of TO1-Biotin in real time again using the StepOne Real-Time PCR System and settings as just described.

Detection of *E. coli* RNA in the presence of human nucleic acid

Total human nucleic acid was extracted from A549 Human Lung Carcinoma cells via phenol-chloroform extraction and concentrations were measured on a NanoDrop 2000 by OD. Reactions were performed as above; however, reactions either had 150 *E. coli* target molecules/ μ L or not, with 5 ng/ μ L of A549 human lung carcinoma cell total nucleic acid or not.

Detection of *E. coli* in the presence of conditioned mammalian cell culture media

To test the validity of the use of nested Mango NASBA, LB media was inoculated with *E. coli* and concentration was monitored by absorbance at 600 nm (cell number calculated using Agilent on-line tool; OD₆₀₀ = 0.5 implying 4 $\times$ 10⁸ cells/mL). An aliquot of 10⁸ cells exposed to a heat shock for 10 min at 41°C to induce *ClpB* RNA was then promptly pelleted at 4000 g for 4 min. The cell pellet was resuspended in 50 μ L of depleted cell culture media (MCF7 waste media generated during cell passaging) and incubated further for 3 min at 41°C. Samples were pelleted for 4 min at 4000g before subject to a Nucleospin RNA kit (Macherey-Nagel) using their recommended protocol with the exceptions of avoiding the DNase step and performing elution using 2 mM EDTA. Total nucleic acid samples were used for nested Mango NASBA reactions as described above. A negative control sample for NMN was an extraction of nucleic acid from depleted media containing no *E. coli* cells treated to the same extraction procedure.

PAGE Mango visualization

Samples to be visualized on PAGE were mixed with three volumes formamide supplemented with 20 mM EDTA and then heated for 5 min to 90°C. Samples were then loaded and run into 8% PAGE (19:1 Acrylamide:bis). Post staining of the gels was performed using 100 mL of 1× WB (140 mM KCl, 1 mM MgCl₂, 10 mM NaH₂PO₄ pH 7.2, 0.05% Tween-20) supplemented with 20 nM TO1-Biotin, and Mango NASBA bands were imaged on a GE AI600RGB imager as described elsewhere (Yaseen et al. 2019). Alternatively, gels were stained with 1× SYBR Safe stain otherwise under the same conditions.

Sequence alignment

Sequences were aligned using Geneious software and aligned using the Clustal method (Geneious version 11.0 created by Biomatters; available from <https://www.geneious.com>).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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