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Fluorogenic Templated Reaction Cascades for RNA Detection

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Abstract

Nucleic acids detection is essential to the study of biological processes and to diagnosis of pathological states. Although PCR is highly effective in vitro, methods that can function without prior sample preparation, thermal cycling, or enzymes are of interest due to their simplicity. Most current non-PCR detection methods rely on linear signal amplification, which hinders the detection of small amounts of genetic material. To address this limitation, we tested a new strategy for attaining higher-order signal amplification, in which a target sequence templates a chemical ligation, and the product of this reaction is in turn detected with a second templated reaction. The method is nonenzymatic, isothermal, and fluorogenic, allowing the direct detection of nucleic acids in complex matrices. Using this approach, as little as 500 attomoles (10 pM) could be detected with single nucleotide resolution. In a test of selectivity, single nucleotide substitutions and deletions could successfully be detected, including a deletion that is associated with tetracycline resistance in *Helicobacter pylori*. Compatibility with biological matrices was demonstrated by the direct detection of rRNA in bacterial lysate. Imaging and detection of target sequences on a solid support further illustrates the potential of the new approach for high-throughput analysis.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b00466. Further experimental details and spectra, Figure S1–S4 and oligonucleotide structures (PDF)

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Notes

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INTRODUCTION

The rapid detection and imaging of minute amounts of nucleic acids is an ongoing challenge that holds great promise for application in biology and medicine.^{1–6} DNAs and RNAs contain valuable information about pathological conditions and disease progression and can be powerful biomarkers for diagnostics including cancer pathogenesis,³ identifying infections² and drug resistance.⁷ However, the direct detection of nucleic acids in biological samples without sample preparation and target isolation remains challenging.⁸

Recent technologies like nanoflakes,⁹ hybridization chain reactions,¹⁰ molecular beacons,¹¹ strand displacement,^{12,13} and nucleic acid-templated reactions^{14,15} are aimed at addressing these issues, and offer turn-on signaling without enzymes. However, the linear amplification of most of these strategies results in limited signal gain. In particular, fluorogenic RNA- and DNA-templated reactions have gained popularity,^{16–20} because the bio-orthogonality and isothermal nature of these approaches allow for the direct detection of nucleic acids in biological samples without the need for any workup or target isolation. To date, however, the linear amplification of such methods limits their sensitivity.⁸

RNA and DNA in nature evolved to support amplification beyond linear dimensions. The extraordinary molecular recognition properties of nucleic acids can be exploited to propagate higher-order replication and signal generation.^{8,21–23} Several in vitro self-replicating and cross-replicating systems that rely on base-pairing have been developed, including RNA enzymes^{24–26} and nucleic acid-templated ligation reactions.^{27–29} For example, in a study by Gibbs-Davis,^{29,30} a target sequence templates an enzymatic ligation reaction. The product of this ligation can itself template a second reaction, resulting in exponential amplification. To date, such quadratic and exponential amplifying systems have not been developed for the nonenzymatic and direct detection of cellular nucleic acids. We hypothesized that use of carefully coupled templated chemistries together with fluorogenic signaling might be applied to detect small quantities of genetic material.

Here we describe the coupling of two nucleic acid-templated reactions to attain quadratic signal formation. Both templated reactions are nonenzymatic and isothermal, and the initiation of the replication process depends solely on the presence of a target sequence. The fluorogenic amplified cascaded templated reactions (FACTR) enable direct detection of nucleic acids in complex matrices. Using this approach, as little as 500 attomoles of a target could be detected with single nucleotide resolution.

By exploiting inherent selectivity of one of the templated reactions we could detect a single nucleotide deletion that is associated with tetracycline resistance in *Helicobacter pylori* (*H. pylori*). Moreover, a single nucleotide substitution was efficiently detected by employing a second set of probes that complement the substitution. The compatibility of the coupled templated reactions with biological matrices was established by targeting 16S rRNA in bacterial lysate, which resulted in a selective and robust fluorescence signal. Furthermore, on-bead detection of target sequences was achieved, demonstrating the potential for high-throughput analysis by bead imaging.

MATERIALS AND METHODS

Synthesis

Details on the synthesis of the DNA modifying groups are given in the Supporting Information (SI). QSTAR and QUAL modifying groups were prepared as previously reported.^{31,32}

Oligonucleotide Probes

Oligonucleotides and DNA-conjugates were prepared on an ABI 392 synthesizer on a 1 μ mol scale using standard phosphoramidite chemistry. All reagents and columns were purchased from Glen Research Corporation. DNA strands were deprotected and cleaved with concentrated ammonium hydroxide at 55 °C for 16 h. Ultramild protected DNA strands were deprotected and cleaved with 0.05 M K₂CO₃ in methanol at room temperature for 5 h. Oligonucleotides were purified using RP-HPLC and eluted with MeCN/0.1 M TEAA buffer. Unmodified DNAs were purchased from Integrated DNA Technologies, Inc. Universal linker probes and 3' thiophosphate oligodeoxynucleotides were prepared as described previously.³² QSTAR and TPP-probes were obtained by post-DNA synthesis modification as reported.³¹

QUAL Probes Turnover

Turnover of the QUAL probes in the first amplification step was determined with analytical RP-HPLC (Shimadzu LC-10AD and SPD-M10A Diode Array Detector equipped with an XBridge Oligonucleotide BEH C18 column) and eluted with MeCN/0.1 M TEAA buffer. The peak area was determined with Shimadzu EZstart 7.4 software and was converted into the amount of product formed using a calibration curve (Figure S2). The turnover number was defined as the number of formed products per template strand.

Coupled Templated Reactions

The coupled templated reactions in series were carried out with 5 μ M Universal Linker DNA (UL-DNA) and Thio-DNA and template concentrations as indicated in 70 mM Tris-Borate buffer pH = 7.0, 10 mM MgCl₂ at 30 °C for 20 h unless indicated differently. This was added to 200 nM QSTAR-DNA, 400 nM TPP-DNA in buffer. The parallel reactions were carried out with 1 μ M UL-DNA and Thio-DNA, 200 nM QSTAR-DNA, 400 nM TPP-DNA and template concentrations as indicated in buffer at 30 °C. Fluorescence emission was observed on a Fluorolog 3-11 instrument (Jobin Yvon-SPEX). λ_{ex} = 497 nm and λ_{em} = 519 nm. Graphs were smoothed by adjacent averaging.

Detection in Lysate

Escherichia coli (*E. coli*) K12 (ATCC 107988) cells were grown overnight at 37 °C in LB media (10 mL) to an OD₆₀₀ of ~2.5. Cultures were centrifuged and the supernatant discarded. The pellet was resuspended in lysis buffer and RNase OUT (Invitrogen) was added to a final concentration of 1u/ μ L. Cell disruption beads (0.1 mm) were added and the suspension was vortexed at 4 °C for 10 min. The suspension was centrifuged, and the supernatant was diluted 100 $\times$ in tris-borate buffer and stored at -80 °C until use. Five μ M

UL-DNA and Thio-DNA were incubated in lysate for 16 h at 30 °C. This was added to 200 nM QSTAR-DNA and 400 nM TPP-DNA in buffer and fluorescence emission was observed.

On-Bead Detection

Q-STAR probes were immobilized on Oligo-Affinity Support (PS) (Glen Research) and a hexaethylene glycol linker was incorporated between the support and oligonucleotides to ensure spacing. QSTAR probes were synthesized on the support and included a 5' amino modifier 5 (Glen Research). QSTAR probes were modified on the support postsynthesis to attach the azido-ether dabsyl quencher and washed thoroughly. Five μM UL-DNA and Thio-DNA and 100 nM WT template in buffer were incubated for 20 h at 30 °C. This was added to a solution containing QSTAR-modified beads and 5 μM TPP-DNA and incubated for 2 h. The beads were imaged on a Nikon Eclipse 80i epifluorescence microscope equipped with a Nikon Plan Fluor 10 $\times$ objective and a QIClick digital CCD camera.

RESULTS

Design of Coupled Templated Reactions

To potentially achieve quadratic signal formation, we explored whether two templated reactions could be combined (Figure 1). The challenge of our two reaction design is to avoid interference and self-inhibition. The first reaction chosen was a chemical autoligation³² (QUAL) that results in the formation of a linked but hybridization-destabilized product (Figure 2A). Upon binding of the two QUAL probes to a target sequence, the nucleophilic phosphorothioate group of one probe (Thio-DNA) attacks the electrophilic carbon of a butyl group on the neighboring probe (UL-DNA). The resulting butyl linker was shown to be destabilizing and enhances dissociation of the ligated product from the target, enabling turnover.³² We envisioned that this first linked product might then act as the template for a second reaction, which we chose to be a Quenched Staudinger Azidoether Reduction (QSTAR, Figure 3A).³¹ One QSTAR probe bears a triphenylphosphine (TPP) group that will reduce an azide on the neighboring probe when bound adjacent on the template. This results in the release of a dabsyl quencher, causing an increase in fluorescence emission of the fluorescein label also attached to the probe (Figure 1).

Both reactions are nonenzymatic, isothermal, and are expected to be chemically orthogonal to each other. During the course of the first reaction, increasing amounts of ligated product strands will be formed that will then be available, in principle, as templates for the QSTAR reaction. If successful, this is expected to result in a quadratic conversion of quenched substrate probes into fluorescent products, since the QSTAR reaction itself was shown to exhibit substantial turnover.³¹ One unknown in this FACTR strategy was whether the QUAL ligation product could serve as an effective template for QSTAR, since the two halves of the ligated probe are separated by an unnatural butyl linker, thus potentially interfering with the subsequent templated reaction by separating them with unnatural structure.

Additionally, we envisioned a potential mechanism for achieving high selectivity for target detection: in previous work it was shown that the QUAL reaction exhibits significant turnover only when a nucleobase is positioned opposite to the destabilizing linker on the

template strand (Figure 2a).³² This could potentially be exploited to detect deletions: when the QUAL reaction does not turn over, there will be no template available to catalyze the QSTAR reaction and no fluorescence increase will be observed. A single nucleotide deletion at position 733 of *Helicobacter pylori* (*H. pylori*) 16S rRNA is associated with tetracycline resistance.³³ Probe sequences were chosen to flank this position allowing for discrimination between wild-type and tetracycline-resistant *H. pylori* 16S rRNA (Figure 2) with the FACTR. All probes were synthesized as previously described^{31,32} using phosphoramidite chemistry and postsynthesis modification for TPP-DNA and QSTAR-DNA probes.

Performance of Individual QUAL and QSTAR Reactions

To assess the performance of the first-stage templated reaction (QUAL) in the *H. pylori* sequence context, product formation was monitored by RP-HPLC (see Figure S1). QUAL probes were incubated with wild-type (WT) and resistant (MUT) template in tris-borate buffer. Reactions proceeded in high yields, and a significantly higher turnover number of 20 was found for the WT template, compared to only 4 for the MUT template at a concentration of 1 nM (Figure 2B). This is in concurrence with earlier work that showed a nucleobase opposite to the universal linker is required for enhanced turnover and supports the hypothesis that this approach might be exploited to detect single nucleotide deletions. At a higher template concentration of 100 nM the turnover number drops significantly as expected due to the relative smaller excess of QUAL probes.^{32,34} Turnover numbers are typically low to moderate for ligation reactions,^{32,34} but the overall turnover is expected to be significantly improved when combined with a second templated reaction.

The probes for the QSTAR reaction consist of a 3' TPP bearing ODN (TPP-DNA) and an ODN that is equipped with a fluorescein label and a 5' azidoether linker that carries a dabsyl quencher. The QSTAR probes are designed to target the product of the QUAL reaction. As mentioned above, this product contains a butyl linker that could potentially interfere with the efficiency of the QSTAR reaction, as the reacting probes are separated by the added intervening bonds. To examine this, a synthetically prepared (see SI) butyl linker-bearing ODN was tested as a template for the QSTAR reaction, alongside the analogous natural ODN. The data showed clearly (Figure 3B) that the presence of the butyl linker has no deleterious effect on the rate of the QSTAR reaction despite the intervening linker.

Coupled Nucleic Acid Templated Reactions

Initial studies on the performance of the FACTR were conducted by running the two templated reactions in series. Relatively high concentrations of QUAL probes (5 μ M) were incubated with 100 nM of WT or no template in tris-borate buffer for 20 h at room temperature. When TPP-DNA (400 nM) and QSTAR-DNA (200 nM) were then subsequently added to this solution, a robust increase in fluorescence emission was observed in the WT template sample and not in the control (see Figure S3), indicating that product template had formed during the QUAL reaction and could be detected with QSTAR probes. To optimize the conditions, the reactions were carried out at varied temperatures and the initial rates of fluorescent increase were compared (Figure 4A). Reactions at 30 °C were found to have the highest rate and therefore this temperature was chosen for all further experiments. The calculated melting temperatures (T_m 's) of UL-DNA and Thio-DNA to the

target are around 37 °C. The ligation reaction is relatively slow, which is possibly why temperatures below the T_m result in a higher initial rate. This is in accordance with earlier observations.³²

One outstanding challenge is to be able to detect small amounts of nucleic acid. 10-fold dilutions of WT template were subjected to the nucleic-acid templated network to determine the limit of detection. At a concentration of 10 pM (500 amol) WT template, a significant signal could still be detected over background. At lower concentrations, signal was not distinguishable from background. The results show that the rate of fluorescence increase is dependent on the amount of target template. Therefore, a calibration curve may allow quantification of target template between 1 and 10 pM.

To test the utility of this approach for the detection of single nucleotide deletions, probes were incubated with WT template, representing *H. pylori* 16S rRNA, and MUT template that has a single point deletion that is associated with tetracycline resistance in *H. pylori*.³³ As is apparent from the data (Figure 4C), there is a large difference in fluorescence increase between WT and MUT template. This can be attributed to decreased destabilization when there is no nucleobase opposite to the butyl linker in duplex DNA. These results show that the FACTR is efficient in the discrimination of single point deletions. Although QSTAR probes have been shown to be highly sensitive to point mutations,³¹ we know of no previous use of templated reactions to detect single nucleotide deletions. To broaden the applicability of the method to other single nucleotide polymorphisms (SNPs) we tested the possibility of detecting substitutions. When template **3** containing a single base mutation was used in the FACTR, a considerably lower fluorescence signal was obtained compared to the WT template **1** (Figure 4C). QUAL and QSTAR probes were then prepared that complement the single nucleotide substitution template **3**; when applied in the FACTR, fluorescence was fully restored (Figure 4C). This indicates that the new method can be applied for detection of multiple classes of SNPs.

It might be advantageous in certain situations to run the two templated reactions in parallel, i.e., simultaneously in one tube. All probes and target are then added at the same time, which simplifies sample handling. To assess the performance of the FACTR when conducted in parallel, target template (100 nM), UL-DNA, Thio-DNA (both 1 μ M), QSTAR-DNA (200 nM), and TPP-DNA (400 nM) were incubated simultaneously for 20 h at 30 °C. Next, fluorescence emission spectra of the samples with and without template were taken. A clear difference in emission was observed between the sample with and without WT template (Figure 5A), showing that both templated reactions can be performed simultaneously in the same solution. When the fluorescence emission was monitored over time, a sigmoidal-shaped curve was observed (Figure 5B), as expected for quadratically amplifying systems.⁸ The limit of detection (Figure S4) was relatively high when compared to the FACTR in series. This is possibly due to background signal from untemplated hydrolysis of QSTAR-DNA. This might be overcome in future experiments by employing doubly quenched QSTAR-DNA.³⁵

Detection of rRNA in Biological Samples

We set out to target 16S rRNA in bacterial lysate to explore the utility of this method for detection of genetic sequences in biological samples. Applying nucleic acid-templated reactions for the detection of certain genetic sequences holds great promise for diagnosing infections⁷ and other pathological conditions³ as well as studying biological processes such as cell differentiation.³⁶

Our initial set of probes target 16S rRNA in *H. pylori*, which is homologous to *Escherichia coli* (*E. coli*) 16S rRNA at the targeted site.³⁷ Therefore, experiments were conducted with *E. coli* as a safer alternative to *H. pylori*. Bacteria were grown overnight to an OD₆₀₀ of ~2.5 and lysed by bead beating (see SI). QUAL probes were added to the supernatant and incubated for 20 h at 30 °C. Next, the QSTAR probes were added to the solution and a robust increase in fluorescence was observed (Figure 6A). As a control, Thio-DNA and QSTAR-DNA that target 16S rRNA 50 nucleotides downstream from the initial sequence were synthesized (see SI). In this case, control UL-DNA and control Thio-DNA are spatially separated and should therefore not ligate to form the template for the QSTAR reaction. Indeed, an absence of fluorescence was observed when these probes were employed in bacterial lysate (Figure 6A). This strongly indicates that the increase in emission when the neighboring probes are used stems from the interaction between these probes and the 16S rRNA target sequence. Thus, we conclude that FACTR is efficient in detecting specific rRNA sequences in bacterial lysate.

Solid Support

For convenient and high-throughput analysis, many genetic detection methods rely on probes attached to a surface.^{38,39} Therefore, we tested if the FACTR nucleic acid-templated reaction network would be suitable for detection of templates on a solid support, by immobilizing the final signaling QSTAR probe. In principle, a target nucleic acid could catalyze the ligation reaction between UL-DNA and Thio-DNA in solution, and this ligated product could template the reaction on the bead surface between TPP-DNA and immobilized QSTAR-DNA. The fluorescent product of this reaction cascade is retained on the surface and would result in a green emissive bead.

To test this, the fluorogenic QSTAR-DNA probes were immobilized on 130- μ m PEG-polystyrene beads (see SI for methods). Reactions were carried out with 5 μ M UL-DNA and Thio-DNA and 100 nM WT template in buffer and were incubated for 20 h. Next, the solution was added to the QSTAR-modified beads and 5 μ M TPP-DNA and the results were monitored by fluorescence microscopy. A clear difference in fluorescence signal was observed between the reactions that were conducted with and without WT template (Figure 6B,C). These preliminary experiments show that the nucleic acid-templated network can indeed be used to detect genetic sequences on a solid support and can potentially be employed for high-throughput genetic detection.

CONCLUSIONS

Our data demonstrate that the molecular recognition properties of nucleic acids can be exploited to devise a network of nonenzymatic templated reactions that can achieve quadratic signal formation. Two nucleic-acid templated reactions were combined, in which the first reaction yielded a ligated product that could catalyze the second reaction, which in turn produced a fluorescent product. Our results show that we could employ the nucleic-acid templated network to detect target templates in a concentration-dependent manner and a target quantity of 500 amol (10 pM) could still be detected significantly over background. We know of no previous reports of such cascaded nonenzymatic and fluorogenic templated methods for nucleic acid detection with single nucleotide resolution. In recent work by the group of Gibbs-Davis,²⁹ a self-replicating nucleic-acid templated reaction was developed that relied on destabilization by an abasic lesion. This elegant approach showed sigmoidal amplification, but relied on enzymatic ligation and was not assessed on direct detection in complex matrices. Recent work by our laboratory⁴⁰ combined rolling circle amplification (RCA) with a nucleic-acid templated reaction to attain quadratic amplification. That method contained a convenient fluorescent readout, but still relied on enzyme activity to perform RCA, and also included a denaturing step.

By exploiting the destabilizing properties of autoligated probes in our FACTR approach, a single-point deletion that is associated with tetracycline resistance in *H. pylori* could be detected. A considerable difference in fluorescence increase was observed over the wild-type sequence. This could potentially be useful for the diagnosis of pathological conditions that are associated with single point deletions such as antibiotic-resistant infections³³ and genetic disorders like Tay-Sachs disease.⁴¹ Although previous template chemistry approaches have shown good selectivity against point mutations^{18,31,42,43} we know of no previous examples of detecting deletions. Additionally, we show that the FACTR approach can be successfully employed for the detection of single nucleotide substitutions and therefore has a potentially wide applicability for detection and discrimination of mutations.

The current approach is isothermal and nonenzymatic and solely depends on the presence of target sequence. The fluorogenic nature of the method ensures a convenient readout and renders this approach suitable for the detection of genetic sequences without sample purification and gel electrophoresis. The possibility of using various fluorophores potentially allows for multicolor detection of several RNAs at the same time.⁴⁴ Moreover, since the reactions do not rely on enzyme activity and thermocycling, they can easily be performed in a biological context. Indeed, our results show that the coupled nucleic-acid templated reactions perform well to detect rRNA directly in bacterial lysate. This drastically reduces sample handling and purification and increases accuracy, because the target sequence can be detected directly in lysate without the need of isolation.

The combination of FACTR with bead-supported detection potentially allows for high-throughput analysis of samples.³⁹ The fluorescent product of the reaction is captured on the solid surface, keeping background levels low and eliminating the need for wash steps, rendering this an exceptionally simple method for the detection of genetic sequences.

Future experiments will be directed at decreasing the reaction time and lowering background signal for even greater sensitivity. The QUAL reaction exhibits moderate reaction rates that may be increased by using stronger nucleophiles such as phosphorotrithioates⁴⁵ or by fine-tuning the leaving group ability. Furthermore, by employing doubly quenched QSTAR-DNA probe designs,³⁵ the background signal can likely be reduced, which is expected to increase the limit of detection of FACTR yet further.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic representation of the fluorogenic amplified cascaded templated reaction (FACTR). The first reaction (QUAL, red probes) is a chemical ligation that forms a destabilized product, which dissociates and in turn templates the second reaction (QSTAR, green probes). A TPP-bearing probe reduces an azido-ether linker, causing the release of a fluorescent quencher, resulting in an increase in emission. During the course of the reactions, more product template is being formed in the QUAL reaction, which is available to catalyze more QSTAR reaction, resulting in quadratic signal generation. LG = leaving group; TPP = triphenylphosphine; QUAL = quenched autoligation; and QSTAR = quenched Staudinger azidoether release.

Figure 2.

Performance of the QUAL reaction, which ligates reactive probes on an RNA or DNA template. (A) Probe design and target sequences. MUT template has a single point deletion compared to WT template. (B) Effect of target sequence and concentration on turnover. Reactions were carried out in 70 mM Tris-Borate buffer pH = 7.0, 10 mM MgCl₂, at 30 °C, with 5 μM UL-DNA and Thio-DNA with varying amounts of target template for 20 h. Error bars show standard deviations from triplicate measurements. LG = leaving group.

Figure 3.

Performance of QSTAR reaction with natural and butyl linker-containing DNA. (A) QSTAR probe design and template sequence with and without butyl-linker. When in close proximity, the TPP will reduce the azide functionality, resulting in cleavage of the azidoether linker and release of the quencher. F = fluorescein; Q = quencher (dabsyl); TPP = triphenylphosphine. (B) Time course of fluorescent increase with both templates under equimolar conditions. 200 nM template **3** or **4**, 200 nM QSTAR-DNA and 400 nM TPP-DNA in 70 mM Tris-Borate buffer pH = 7.0, 10 mM MgCl₂ at 30 °C. λ_{ex} = 497 nm and λ_{em} = 519 nm.

Figure 4.

Sequential coupling of templated reactions. (A) Analysis of initial fluorescence rate at different temperatures with 100 nM WT target. The rate was determined by averaging the slope from 15 to 30 min. Error bars represent measurements in triplicate. (B) Amplified fluorescent signal under sub stoichiometric amounts of WT template. (C) Time course of fluorescence with 100 nM of template **1**, **2**, **3**, or no template. The coupled templated reactions were carried out with 5 μ M UL-DNA and Thio-DNA and template concentrations as indicated for 20 h in 70 mM Tris-Borate buffer pH = 7.0 10 mM MgCl₂ at 30 °C. This was added to 200 nM QSTAR-DNA and 400 nM TPP-DNA in buffer. λ_{ex} = 497 nm and λ_{em} = 519 nm.

Figure 5.

Simultaneous coupling of templated reactions. (A) Fluorescence emission spectra of the coupled templated reactions when carried out simultaneously after 20 h incubation with 100 nM of WT, MUT, or no template. (B) Sigmoidal amplification by the simultaneous templated reactions with 100 nM of WT template. The graph is background corrected by subtracting the fluorescence from a blank reaction lacking template. The coupled templated reactions were carried out with 1 μ M UL-DNA and Thio-DNA, 200 nM QSTAR-DNA, 400 nM TPP-DNA, and template concentrations as indicated in 70 mM Tris-Borate buffer pH = 7.0 10 mM MgCl₂ at 30 °C. λ_{ex} = 497 nm and λ_{em} = 519 nm.

Figure 6.

Detection of 16S rRNA in bacterial lysate in solution and on beads. (A) Fluorescence time course of probes targeting 16S rRNA in *E. coli* lysate and controls. Five μM UL-DNA and Thio-DNA were incubated in lysate for at 30 $^{\circ}\text{C}$. This was added to 200 nM QSTAR-DNA and 400 nM TPP-DNA in buffer. $\lambda_{\text{ex}} = 497 \text{ nm}$ and $\lambda_{\text{em}} = 519 \text{ nm}$. (B) On-bead detection of target sequence. Five μM UL-DNA and Thio-DNA and 100 nM WT template were incubated for 20 h in 70 mM Tris-Borate buffer pH = 7.0, 10 mM MgCl_2 at 30 $^{\circ}\text{C}$. This was added to a buffered solution containing QSTAR DNA-modified beads and 5 μM TPP-DNA and imaged without washing after incubating for 3 h at 30 $^{\circ}\text{C}$. (C) Control bead incubated under the same conditions with probes but without template.