# Efficient Nucleic Acid Detection by Templated Reductive Quencher Release

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The detection of nucleic acids directly in living cells[1] holds considerable promise for bioanalytical and clinical assays, as it bypasses time-consuming isolation and amplification of target strands. Particularly appealing are fluorescence approaches that shorten and simplify the detection protocol by obviating cell fixation and washing steps.[2−5] Both molecular beacon-based probes[3] and nucleic acid template reactive probes[4] have recently been investigated for this purpose. DNA/RNA templated fluorescence activation, in particular, offers very high selectivity, allowing for the discrimination of single nucleotide differences by a straightforward fluorescence readout.[5] Recent studies have established the use of templated fluorogenic reactions to detect RNAs both in bacterial[6] and mammalian cells.[7]

Evaluation of multiple chemical transformations for templated fluorescence activation[8] has to date revealed two types of reactive probes suitable for cellular RNA detection. First, quenched auto-ligation (QUAL) probes rely on an $S_N2$ displacement of a fluorescence quencher to generate a fluorescence turn-on signal.[9] Although QUAL probes allow sensing of highly expressed RNAs inside cells and have been used to distinguish several closely related bacteria, they can be limited by slow ligation and undesired reactions with endogenous nucleophiles.[6,7] A second promising class of templated fluorescence activation probes uses the Staudinger reduction; such probes exhibit rapid kinetics and a high degree of bioorthogonality, which are beneficial for RNA sensing in cells.[10] However, the reported templated Staudinger schemes have involved the reduction of individually designed profluorophores, thus limiting their versatility and simplicity.

Here we present a novel and versatile probe design for templated fluorescence activation that combines the strong fluorescence enhancement and generality of quencher-release probes with the kinetic benefits and bioorthogonality of templated Staudinger reductions. The described quenched Staudinger-triggered α-azido-ether release (Q-STAR) probes are fluorophore-containing DNA probes whose fluorescence is deactivated by a quencher attached through an α-azidoether linker. Reduction of the azide functionality, for example by triphenylphosphine (TPP), triggers cleavage of the linker and release of the quencher, eliciting a robust fluorescence turn-on signal (Figure 1).

To prepare Q-STAR probes, we designed the α-azidoether linker **1**, which contains an amino functionality suitable for chemical derivatization. The synthesis of **1** was achieved in five steps (Scheme 1). The α-azidoether linker was obtained by the iron(III)-catalyzed coupling of trimethylsilyl ether **3** and trifluoroacetamide-protected aldehyde **4**,[11] followed by the selective, hydrolytic removal of the amine protecting group. Modification of **1** with the dabsyl quencher and hydrolysis of the ester provided **2** as a phosphine-responsive quencher-release linker that is amenable to bioconjugation. Attachment of **2** to DNA proceeded readily using solid-phase amide coupling to 5′-amino-modified DNA prior to deprotection/cleavage. Q-STAR probes were obtained with >98% purity after HPLC purification. Triarylphosphine−DNA conjugates
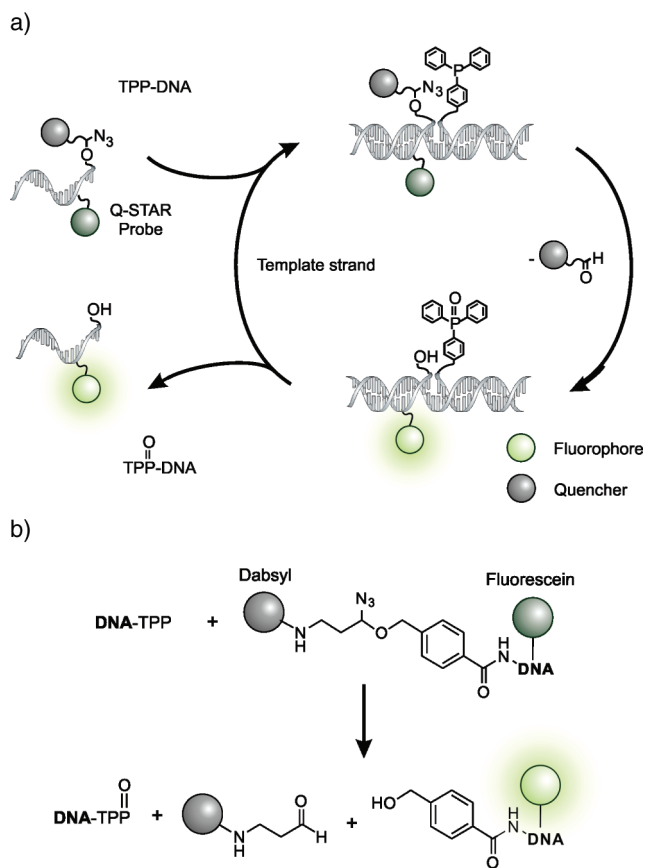


**Figure 1.** Detection of nucleic acids by templated fluorescence activation of Q-STAR probes. (a) A Q-STAR probe and a TPP-modified DNA bind to a common target strand (the template). Proximity-induced reduction of the Q-STAR azide functionality results in cleavage of the α-azidoether linker and release of the quencher, yielding a fluorescence turn-on signal. Subsequent probe exchange on the template allows for multiple turnovers, isothermally amplifying the signal. (b) Molecular structures of reactants, including (top) the quencher-conjugated release linker and (bottom) the products after reduction, cleavage, and dissociation from the template.

(TPP−DNA) were prepared as previously described[10e] using 3′-amino-modified DNA synthesized in the 5′ → 3′ direction.

To assess the performance of Q-STAR probes in a template-dependent configuration, we prepared a fluorescein-labeled probe (green STAR) (Figure 2a) complementary to a sequence element of *Escherichia coli* 16S rRNA.[6b] To trigger the release of its quencher, we prepared a TPP−DNA conjugate designed to bind directly adjacent to green STAR on the 16S rRNA target sequence. For solution studies, we used as a target a synthetic DNA (EC DNA) homologous to the 16S rRNA sequence.

Upon addition of TPP−DNA (600 nM) to a solution containing green STAR (200 nM) and EC DNA (200 nM), a strong

**Scheme 1**
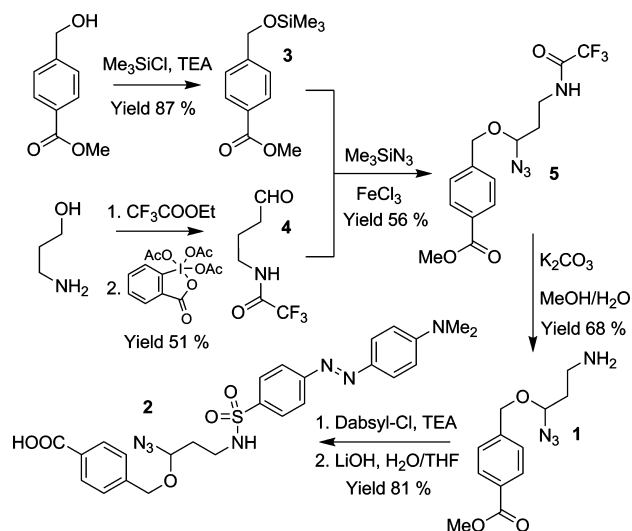


fluorescence signal emerged (Figure 2b, red trace). Fluorescence activation at 37 °C was rapid, reaching 90% conversion within 32 min, and substantial, with a 61-fold fluorescence increase after 115 min. A single mismatch in the target strand reduced the rate of reaction dramatically, as illustrated for the SE DNA template (Figure 2b, blue trace). The relative kinetic mismatch discrimination between these closely related sequences, as estimated from the initial rate of reaction, was 120 ± 20. Omitting either the EC DNA template or TPP–DNA further reduced the rate of reaction, suggesting insignificant background signal and establishing substantial rate acceleration induced by the matched template.

In a templated detection scheme, each DNA/RNA analyte in principle can mediate multiple reactions and provide an amplified fluorescence signal[9c,12] unless long probe sequences or bond formation hinders product dissociation. To evaluate the signal
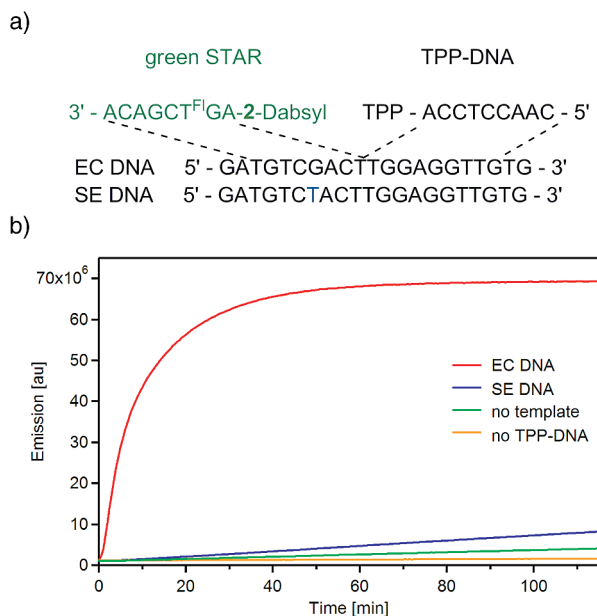


**Figure 2.** Template dependence of Q-STAR activation for the detection of complementary EC DNA. (a) Sequences of DNA probes and targets ($T^{Fl}$ = fluorescein-labeled dT; the blue letter indicates a mismatch position). (b) Time courses of fluorescence activation with various targets. Conditions: 200 nM green STAR, 200 nM EC DNA or SE DNA, 600 nM TPP–DNA, 70 mM tris borate buffer (pH 7.55) containing 10 mM $MgCl_2$, 37 °C; $\lambda_{ex}$ = 494 nm, $\lambda_{em}$ = 520 nm.

amplification of Q-STAR probes, we investigated the template-mediated activation of green STAR in the presence of substoichiometric amounts of the complementary target EC DNA (Figure 3). The fluorescence intensity of green STAR increased rapidly and significantly exceeded the emission expected for stoichiometric conversion. For example, the fluorescence emission of a sample containing only 2 nM EC DNA, corresponding to 1% of the green STAR probe, approached the level of complete fluorescence activation within a few hours. This outcome demonstrates that template turnover is efficient for Q-STAR probes, providing a robustly amplified signal under isothermal conditions.
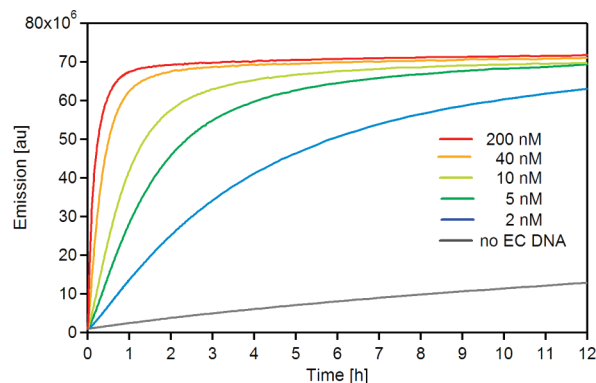


**Figure 3.** Amplified fluorescence signal in the presence of substoichiometric amounts of EC DNA. Conditions: 200 nM green STAR, 600 nM TPP–DNA, 70 mM tris borate buffer (pH 7.55) containing 10 mM $MgCl_2$, 37 °C; $\lambda_{ex}$ = 494 nm, $\lambda_{em}$ = 520 nm; concentration of EC DNA is indicated in figure.

Next, we assessed the potential of the designed Q-STAR probes to discriminate between two bacterial species, *E. coli* and *Salmonella enterica*. We chose as the target the aforementioned polymorphic sequence element on the 16S rRNA that contains a single nucleotide difference between *E. coli* and *S. enterica*.[6b] We designed a two-color system for distinguishing these microorganisms: the *E. coli*-specific green STAR probe contained an internal fluorescein label, while the *S. enterica* complementary probe (red STAR) contained both a fluorescein label and a terminal TAMRA fluorophore (Figure 4a and Table S1 in the Supporting Information). The latter probe was designed to yield a red signal upon loss of quencher as a result of Förster resonance energy transfer (FRET) from the fluorescein donor to the TAMRA acceptor.[6c] This FRET design allows the green and red signals to be observed simultaneously using a single excitation and a long-pass emission filter.

In an in vitro experiment, EC DNA selectively activated the green STAR probe with an emission maximum at $\lambda_{em}$ = 517 nm (Figure S2 in the Supporting Information), whereas the red STAR probe was responsive to the SE DNA target and had a different emission maximum ($\lambda_{em}$ = 580 nm) (Figures S1 and S3). A mixture of green STAR and red STAR probes yielded distinct emission spectra after incubation in the presence of TPP–DNA depending on which target sequence was present (Figure 4b).

To test the probes in a cellular context, we incubated *E. coli* or *S. enterica* cells with a combination of green STAR and red STAR probes (both 200 nM) and TPP-DNA* (2 μM) at 37 °C in hybridization buffer containing 0.05% SDS to aid probe delivery. Two unmodified helper DNAs[6b] (3 μM each) were added to increase the accessibility of the rRNA target (see Table S1). Notably, this detection protocol requires neither cell fixation steps nor post-hybridization washes. Within 4 h, strong fluorescein (green) emission emerged in the *E. coli* cells (Figure 5a), whereas *S. enterica* bacteria exhibited a distinct red fluorescence (Figure 5c).
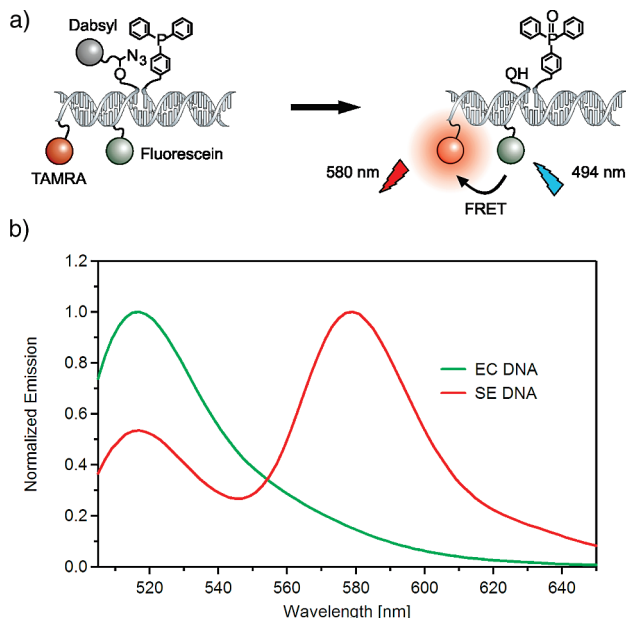
**Figure 4.** FRET probes for the two-color detection scheme. (a) Conceptual design of the red STAR probe. (b) Normalized fluorescence emission spectra of a mixture of green STAR and red STAR probes incubated with TPP−DNA in the presence of either EC DNA or SE DNA. Conditions: 200 nM green STAR, 200 nM red STAR, 200 nM EC DNA or SE DNA, 600 nM TPP−DNA; 1 h incubation at 37 °C in 70 mM tris borate buffer (pH 7.55) containing 10 mM MgCl$_2$; $\lambda_{ex}$ = 494 nm.

With the use of a single excitation filter and a long-pass emission filter ($\lambda_{ex}$ = 450−490 nm, $\lambda_{ex}$ > 515 nm), it was possible to assign single bacteria to either species on the basis of fluorescence color when the two bacterial types were present as a mixture (Figure 5b). Thus, the data confirm that Q-STAR probes allow the discrimination of these two microorganisms by a single nucleotide difference. Furthermore, fluorescence activation was negligible in the absence of TPP−DNA (Figure S4), suggesting that Q-STAR probes are stable with respect to cellular constituents, in particular to adventitious reduction by thiols.
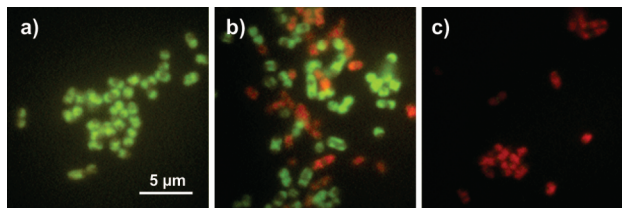


**Figure 5.** Two-color discrimination of bacterial species based on a single nucleotide polymorphism on the 16S rRNA: (a) *E. coli* cells; (b) *E. coli* and *S. enterica* cells; (c) *S. enterica* cells. Bacteria were incubated for 4 h at 37 °C in hybridization buffer (6× SSC + 0.05% SDS) containing green STAR and red STAR (both 200 nM), TPP−DNA (2 $\mu$M), and helper DNAs (3 $\mu$M); a B-2A filter set was used for imaging.

The present results demonstrate that the described Q-STAR probes can sequence-selectively report on nucleic acids both in vitro and directly in prokaryotic cells. The cellular detection protocol is exceedingly simple, requiring only a single experimental step, and thus offers strong benefits over PCR-based methods for distinguish-

ing sequence polymorphisms. Q-STAR probes, like previous S$_N$2-based QUAL probes,[9] rely on a quencher release strategy for fluorescence turn-on and share the same beneficial fluorescence properties. Additionally, Q-STAR probes offer a number of potential advantages, including faster reaction kinetics and improved signal amplification. Importantly, Q-STAR probes also appear to be more stable to cellular constituents (including water), reducing background fluorescence in vivo, which is a limiting factor in many approaches to cellular RNA detection. Finally, the quencher-release approach is versatile compared to other templated reduction schemes;[10] the use of alternative quencher molecules could allow the design of Q-STAR probes with a wide spectral range. The unmatched performance of Q-STAR probes makes them attractive for widespread application in nucleic acid detection assays.

**Supporting Information Available:** Experimental details, additional data, and characterization of synthetic intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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