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Split Dapoxyl Aptamer for Sequence-Selective Analysis of Nucleic Acid Sequence Based Amplification Amplicons

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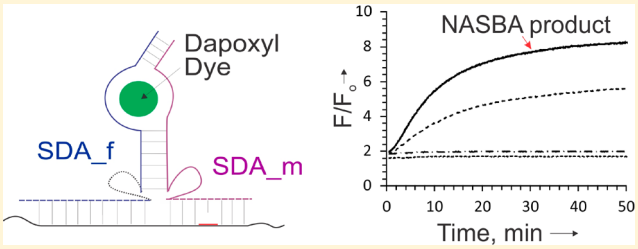
Supporting Information

ABSTRACT: Hybridization probes have been used for the detection of single nucleotide variations (SNV) in DNA and RNA sequences in the mix-and-read formats. Among the most conventional are Taqman probes, which require expensive quantitative polymerase chain reaction (qPCR) instruments with melting capabilities. More affordable isothermal amplification format requires hybridization probes that can selectively detect SNVs isothermally. Here we designed a split DNA aptamer (SDA) hybridization probe based on a recently reported DNA sequence that binds a dapoxyl dye and increases its fluorescence (Kato, T.; Shimada, I.; Kimura, R.; Hyuga, M., Light-up fluorophore-DNA aptamer pair for label-free turn-on aptamer sensors. *Chem. Commun.* **2016**, *52*, 4041−4044). SDA uses two DNA strands that have low affinity to the dapoxyl dye unless hybridized to abutting positions at a specific analyte and form a dye-binding site, which is accompanied by up to a 120-fold increase in fluorescence. SDA differentiates SNV in the *inhA* gene of *Mycobacterium tuberculosis* at ambient temperatures and detects a conserved region of the Zika virus after isothermal nucleic acid sequence based amplification (NASBA) reaction. The approach reported here can be used for detection of isothermal amplification products in the mix-and-read format as an alternative to qPCR.



Sequence-selective fluorescence sensors, such as molecular beacon (MB)[1−4] and Taqman probes,[5] have been widely used in quantitative real time polymerase chain reaction (qPCR) assays[2,6,7] for diagnosis of infectious diseases and cancer, among other applications.[8−17] The use of these expensive hybridization probes is justified by the need to detect single nucleotide variations (SNV) in nucleic acids. However, Taqman and MB probes can distinguish SNVs only if melting temperature profiles are recorded, which requires expensive qPCR instruments with melting capabilities.[18] Moreover, qPCR-based technologies require expensive maintenance, software, and reagents as well as trained personnel. These are the reasons why molecular diagnostics is shifting toward adopting isothermal amplification reactions,[19−23] which do not require expensive instrumentation and thus are potentially affordable not only by specialized diagnostic laboratories but also by health care providers and even individual patients.[19−25] The genotyping potential of such affordable diagnostics depends on development of hybridization probes selective toward SNVs at constant temperatures and preferably label-free to further lower the assay costs. Here, we designed such a probe and demonstrated that it is suitable for the analysis of an amplicon after isothermal amplification.

Earlier, we have introduced the concept of split aptameric probes for nucleic acid analysis (Scheme 1A).[26−28] The approach takes advantage of the aptamers that can bind a low fluorescence dye and thereby increase its fluorescence. Split aptameric probes consist of two unlabeled nucleic acid strands that bind a complementary DNA or RNA analyte sequence and form a dye-binding site. Binding of the dye to the aptamer increases its fluorescence.

Previously published constructs used RNA-based aptamers including malachite green[26] and spinach.[27,28] However, RNA is less chemically stable than DNA. Moreover, as a commercial product, synthetic RNA is about 20 times more expensive than DNA. The only example of a split probe based on a DNA aptamer requires a Hoechst derivative as a fluorophore,[29] which can be obtained only via a lengthy synthetic scheme and is not commercially available.

Recently a DNA aptamer, **DAP-10** (Figure S1A, Table S1), was selected to bind dapoxyl dyes.[30] In this study, we took advantage of **DAP-10** structure to design a split dapoxyl aptamer (SDA) probe for highly selective nucleic acid analysis at room temperature. Further, we demonstrate that SDA can selectively detect a sample of Zika virus RNA after isothermal nucleic acid sequence based amplification (NASBA).[22,23]

EXPERIMENTAL SECTION

Materials and Instruments. All oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Dapoxyl fluorine (dapoxyl-F, Scheme 1B) was synthesized as described by Diwu et al.[31] Fluorescent spectra

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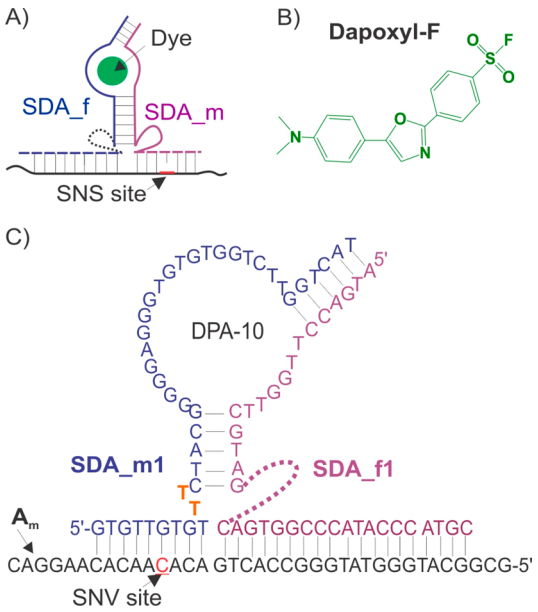
Scheme 1. Design of SDA Probes for the Analysis of Single Nucleotide Substitutions (SNS)[a]



[a](A) General scheme of a split aptameric probe: two RNA or DNA oligonucleotides bind an analyte and form a dye-binding site. Bound dye produces a fluorescent signal.[26−28] (B) Structure of **dapoxyl-F** dye. (C) Detailed sequence of SDA in complex with fully matched analyte ($\mathbf{A_m}$), a fragment of inhA gene of Mycobacterium tuberculosis. SNS site is red underlined. **SDA_f1** contains a triethylene glycol linker (dashed line). **SDA_m1** contains dithymidine (TT) linker (orange).

were recorded using fluorescence spectrometer LS55 (PerkinElmer) and Cary Eclipse fluorescence spectrophotometer (Agilent). Unless otherwise noted, the excitation wavelength was set to 390 nm and emission was taken at 505 nm. Time dependence experiments were performed using a Cary Eclipse fluorescence spectrophotometer (Agilent). Zika virus (strain 1840) was obtained from World Reference Center for Emerging Viruses and Arboviruses.

Source of Zika Virus RNA. Zika virus (strain 1840) was obtained from the World Reference Center for Emerging Viruses and Arboviruses as grown in Vero cells in DMEM media containing 10% fetal bovine serum. Viral RNA was extracted using Trizol LS (Life Technologies) and quantified via absorbance at 260 nm using a NanoDrop One[c] (Thermo Scientific).

General Fluorescent Assay for DAP-10. Dapoxyl-F (0.5 μM) and **DAP-10** (0.58 μM) or **SDA_m** and **SDA_f1** (at indicated concentrations) were added to 30 μL of buffer 1 (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM $MgCl_2$). Total volume was adjusted to 60 μL by water. Control samples contained only dapoxyl-F (0.5 μM). All samples were incubated at room (22.5 °C) temperature. Fluorescent spectra were recorded after indicated incubation times. Data of three independent experiments were processed using Microsoft Excel.

NASBA Assay. Viral RNA, was added to 1× NASBA reaction buffer (TrisHCl, pH 8.5 at 25 °C, $MgCl_2$, KCl, DTT, and DMSO; Life Sciences Advanced Technologies Inc.), nucleotide mix (NECN-1-24) 250 nM reverse and forward primers (Table S1) to the final concentration of 50 fg/μL (total volume 12 μL with water). Samples were incubated at 65 °C for 2 min followed by cooling to 41 °C for 10 min. A volume of 3 μL of NASBA enzyme cocktail (AMV RT, RNase H, T7 RNAP, BSA, Life Sciences Advanced Technologies Inc.) was added and samples were incubated 90 min at 41 °C. Samples were analyzed in 2% agarose gel (Figure 2B). The concentration of **Z-147** amplicon was estimated based on the intensity of the correspondent band in gel and comparison with the intensity of the 200 nt band of RiboRuler Low Range RNA Ladder (ThermoFisher Scientific).

Analysis of NASBA Amplicon by SDA Probe. Time drive experiments were performed in NASBA buffer at room temperature (~22.5 °C) using a Cary Eclipse fluorescence spectrophotometer in a kinetic mode. The samples contained 0.5 μM. **Dapoxyl-F** (0.5 μM), **SDA_m2** (0.5 μM), **SDA_f2** (4 μM), and either 10% NASBA amplicon Z-147 or 10% NASBA no-target control. Blank samples contained Dapoxyl-F (0.5 μM), **SDA_m2** (0.5 μM), and **SDA_f2** (4 μM) in the absence of an analyte. As a positive control, a sample containing 100 nM **Z-64** (Table S1) synthetic DNA analyte instead of NASBA amplicon was used.

RESULTS AND DISCUSSION

Dapoxyl-F dye (Scheme 1B) was synthesized according to the procedure described earlier.[31] The compound was characterized by H^1 NMR (Figure S2). **Dapoxyl-F** was then tested for its ability to fluoresce upon binding to **DAP-10** aptamer. The presence of a full **DAP-10** aptamer resulted in 228-fold increase in the **dapoxyl-F** fluorescence (Figure S2C).

As a model analyte, we chose a fragment of *inhA* gene of *Mycobacterium tuberculosis* (Mtb). Point mutations in this gene fragment are associated with Mtb resistance to one of the key drug used for tuberculosis treatment, isoniazid.[32] For optimization of SDA design, we used two model *inhA*-related analytes, a fully matched DNA analyte $\mathbf{A_m}$ (5′-GCG GCA TGG GTA TGG GCC ACT GAC A **C** A ACA CAA GGA C) and a mismatched analyte $\mathbf{A_{mm}}$ (5′-GCG GCA TGG GTA TGG GCC ACT GAC A **T** A ACA CAA GGA C) containing a C → T single nucleotide substitution (underlined nucleotides).

To construct a split aptameric sensor, **DAP-10** structure was divided into two strands (**SDA_f1** and **SDA_m1**, Scheme 1C and Figure S1), each of which was equipped with an analyte-binding arm. Splitting of the dye-binding aptamer prevented dapoxyl dye from binding to the two separated strands, which maintained low background signal (Figure 1A, compare bars 1 and 2). Sequence of stem 1 was chosen based on the constructs reported by Kato et. al; two terminal A/T base pairs were added to increase stem stability. The hexanucleotide stem was, however, short enough (T_m = 15.6 °C) to pervert **SDA_f1** and **SDA_m1** association in the absence of analyte. When the analyte-binding arms of **SDA_f1** and **SDA_m1** hybridized to their complementary $\mathbf{A_m}$ fragments next to each other, the dapoxyl binding site was reformed, and the dye's fluorescence increased thus reporting the presence of the analyte (Figure 1A, bar 4). Each analyte-binding arm was connected to the aptamer half with a linker, either a trietyhylene glycol (teg) spacer or a dinucleotide bridge. These linkers impart flexibility to the aptameric structure, which is presumably required to ensure simultaneous formation of the **dapoxyl-F**-binding site and tight binding of the analyte.[26−28] Indeed, in the absence of the linkers, the SDA probe demonstrates either reduced signal-to-background ratio (F/F_0) or inability to differentiate $\mathbf{A_m}$ from $\mathbf{A_{mm}}$ analyte (Figures S3 and S4). SDA1 consisting of **SDA_f1** and **SDA_m1** produced F/F_0 = 63 after 60 min of hybridization reaction (Figure 1A). However, longer incuba-

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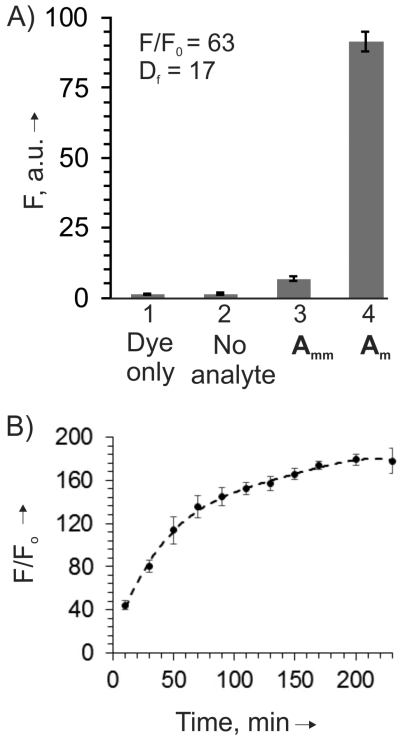

Figure 1. Analysis of $\mathbf{A_m}$ and $\mathbf{A_{mm}}$ by SDA1 probe. (A) Selectivity of SDA toward matched analyte. All samples contained 0.5 μM **dapoxyl-F** in Buffer 1. In addition, Samples 2–4 contained 1 μM **SDA_m1** and 0.2 μM **SDA_f1** in the absence (bar 2, ~1.44 au) or presence of 0.1 μM mismatched ($\mathbf{A_{mm}}$, bar 3, 6.7 au) or 0.1 μM matched ($\mathbf{A_m}$, bar 4, ~91.5 au) analytes. Fluorescent measurements were taken after 60 min of incubation. The data is the average of three independent experiments with standard deviations as error bars. (B) Fluorescence time dependence. Measurements were taken in the presence of 0.5 μM **dapoxyl-F**, 1 μM **SDA_m1**, 0.2 μM **SDA_f1**, and 1 μM of $\mathbf{A_m}$ in Buffer 1 (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM $MgCl_2$). The background fluorescence for SDA (no analyte) was ~1.5 (panel A, no analyte). Therefore, after 10 min F/F_0 was ~27 and after 200 min F/F_0 reached a s mach as 120.

tion time further increased F/F_o to ~120, reaching a plateau after ~200 min of incubation (Figure 1B). Generally, higher analyte concentration also increases signal (Figure S7). Despite this long time required to achieve maximum signal, up to 30-fold fluorescent increase was observed within the first 10 min of the hybridization reaction (Figure 1B), which is above or comparable to the fluorescent hybridization assays with conventional probes.[1−3] Split probe produced a lower turn on ratio ($F/F_0 \sim 120$) than full **DAP-10** aptamer ($F/F_0 \sim 228$) due to possibly lower stability of the aptamer core. Indeed, in DAP-10 the core is formed within single DNA molecule, while formation of aptameric core for SDA hybridization probe requires association of 3 strands: SDA_f1, SDA_m1, and the analyte. In this later association (Scheme 1C), the aptameric core could be slightly disturbed by the presence of the anatyte.

SDA_ m1 was designed short enough to enable differentiation of the fully matched $\mathbf{A_m}$ from a single-base mismatched $\mathbf{A_{mm}}$ at room temperature. SDA1 demonstrated remarkable selectivity in recognition of fully matched $\mathbf{A_m}$. The differentiation factor (D_f) of 17 was calculated from the data presented in Figure 1A according to the formula:

$$D_f = \frac{F_m - F_0}{F_{mm} - F_0}$$

where F_m is the fluorescence in the presence of $\mathbf{A_m}$; F_{mm} is the fluorescence in the presence of $\mathbf{A_{mm}}$, and F_0 is the fluorescence of SDA in the absence of the analytes. Importantly, this differentiation was achieved at room temperature, which is difficult to obtain using the most common TaqMan and molecular beacon probes.[2]

Next, we determined the limit of detection (LOD) for the SDA1 probe based on the data shown in Figure S5. The found LOD of 0.44 nM is lower than or comparable to that of MB probes (typical LOD is ~1 nM)[2] and is acceptable for nucleic acid analysis after amplification.

To confirm that SDA design is universal for the detection of other analyte sequences, an additional DNA analyte, **Z-64**, was selected. **Z-64** (Table S1) is a sequence corresponding to a highly conserved fragment of the Zika virus (ZIKV) genome. New f and m strands, **SDA_f2** and **SDA_m2** (Table S1) were designed to be complementary to the **Z-64**) analyte; otherwise, the design of the probes was as shown in Scheme 1. Up to ~54 for **Z-64** F/F_0 values were observed (Figure S6). D_f for this sensor was not measured, due to the absence of the practically significant mutations that require differentiation. Even though the maximum fluorescence intensity was the same for both $\mathbf{A_m}$ and **Z-64**, the difference in the background fluorescence, which depends on analyte-independent association of strands SDA_m and SDA_f, accounted for the variation in F/F_0.

It is important that for each of the buffer conditions, we optimized concentrations of dapoxyl dye and SDA_f and SDA_m strands using the following criteria: (1) highest F/F_0 and (2) highest differentiation factor. The optimization was done similar to that shown in Figure S3 for optimization of linker design (data not shown). Our typical setup for split probes employs higher concentrations of strands with longer analyte binding arm (SDA_f in this case) and lower concentration of strand with shorter arm (SDA_m). The long arm of SDA_f binds the analyte with high affinity and unwinds the analyte secondary structure, and a low concentration of SDA_m with short analyte-binding arm ensures high sequence selectivity, as was discussed in details in our earlier work.[33]

Most hybridization probes require higher concentrations of a nucleic acid analyte than can be obtained in clinical samples. Therefore, nucleic acid amplification step is required prior to the analysis. We, therefore, turned our attention to NASBA, which is known to produce high concentrations of a single-stranded RNA amplicon,[22,23] and is considered one of the most promising isothermal amplification techniques.[34−36] A fragment of the ZIKV envelope gene was amplified by NASBA using RNA ZIKV (Brazil 1840 isolate) isolated from Vero cells to produce a 147-nt RNA amplicon (**Z-147**, Table S1). The production of **Z-147** fragment by NASBA was confirmed by 2% agarose gel electrophoresis (Figure 2A). In comparison with Buffer 1, NASBA buffer increased the intensity of background dye fluorescence, possibly due to the presence of dimethyl sulfoxide.[31] However, SDA2 was able to detect the NASBA product directly in NASBA buffer (Figure 2B) with the detection limit of 10 nM (Figure S5B). Next we tested the performance of SDA2 in NASBA buffer at elevated temperatures. It was found that the sensor responded at 37 °C with F/F_0 up to 5. This turn-on ratio was about 2 times lower than at 22.5 °C but still acceptable for NASBA product analysis

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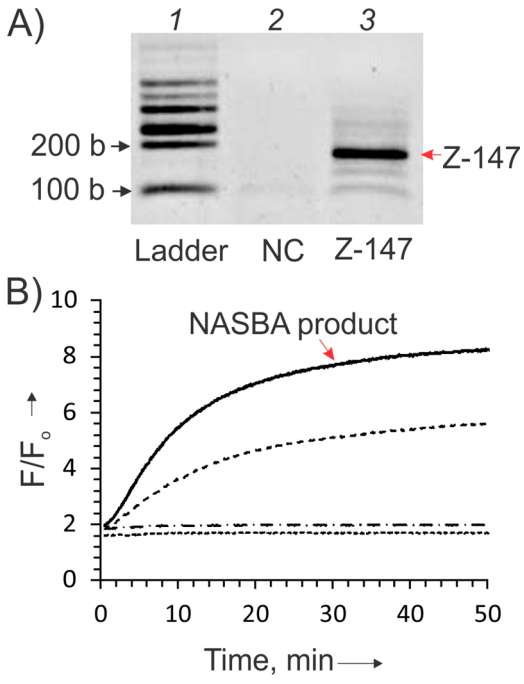

Figure 2. SDA2 can detect RNA product of nucleic acid sequence based amplification (NASBA). (A) Agarose gel analysis of Zika viral RNA amplicon (**Z-147**) obtained by NASBA amplification of a vial RNA sample as described in the Supporting Information. Lane 1, 100−1000 nucleotide Low Range RNA Ladder. Lane 2, negative control (NC, no ZIKV RNA added for NASBA reaction). Lane 3, 147-nucleotide NASBA RNA amplicon **Z-147**. (B) Time dependence for the fluorescent signal of SDA2 in the presence of **Z-147** amplicon (10%) in NASBA buffer (solid line). As negative controls, SDA2 in the absence of the analyte (dotted line) or in the presence of NASBA no-target control (dashed-dotted line) was used. Positive control (dashed line) contained SDA2 in the presence of 100 nM Z-64 synthetic DNA analyte. All samples contained 0.5 μM **dapoxyl-F**, 4 μM **SDA_f2**, and 0.5 μM **SDA _m2** strands in 60 μL of NASBA buffer. Measurements were taken at room temperature (22.5 °C).

(Figure S7). There was no signal above the background observed at 41 °C, the temperature recommended for NASBA (data not shown).

We designed a new fluorescent hybridization probe, SDA which detected complementary analyte with high selectivity, turn-on ratio up to 120 and LOD in the subnanomolar range. It was capable of analyzing 147 nucleotide RNA **Z-147**. SDA maintained its function in complex NASBA buffer which contains a cocktail of enzymes and high concentration of DTT among other proprietary components. SDA maintained fluorescence at temperatures up to 37 °C (Figure S7). NASBA reaction, however, requires 41 °C. At this temperature, SDA was found to lose its ability to produce appreciable signal, most likely due to lower affinity of **dapoxyl-F** to the aptamer core at this temperature. Lowering NASBA reaction temperature or reselecting the **dapoxyl-F**-binding aptamer would enable development of real time NASBA isothermal alternative to qPCR. However, the presented analytical format allows NASBA product detection after cooling samples to room temperature. In this nonoptimized in terms of practical utility format, the assay requires two thermostats for 65 and 41 °C (primer annealing and NASBA reaction temperatures) and ∼15−25 min of total assay time (without accounting RNA purification time). The signal can be registered by a portable fluorimeter. Therefore, time and equipment requirement satisfy the ASSURED criteria of a point-of-care detection techniques[37] and could be adopted by low-resources laboratories or doctor offices.

The LOD of SDA was in subnanomolar range similar to other aptameric sensors that do not used signal amplification strategies.[38] For SDA, the LOD could be limited by an aptamer-dye dissociation constant. Typical dissociation constants for an aptameric complex is from picomolar to mid nanomolar range.[26−30] For dapoxyl dye binding aptamers, K_d values are in the range of 7−25 nM.[30] Despite this limitation, the LOD is lower than that for MB probes (∼1 nM)[2] and, therefore, is acceptable for the detection of nucleic acids after amplification. The LOD for NASBA product was found to be 10 nM, which is high than that of synthetic analyte most likely due to the folded structure of the RNA NASBA product, which reduces association of SDA probe with the analyte.

The probe required up to 200 min to reach maximum fluorescent response. However, the resulting repose maximum was higher than that of conventional fluorescent probes (e.g., MB probe).[2] Importantly, $F/F_0 \sim 27$ appears in the first 10 min of incubation with a fully complementary probe (Figure 1B), which is acceptable for analytical purposes. While the delay in response should be investigated in details, we speculate that intimate slow structural adjustments of the dapoxyl dye in the aptameric site may be responsible for additional fluorescent increase after initial aptamer-dye binding. Indeed, some long-lasting fluorescent adjustments have been described for fluorophore-labeled DNA probes.[39]

SDA offers high selectivity at ambient temperatures, as was well-documented for other representatives of split probes[40] but not for conventional Taqman and molecular beacon probes.[2] Another SDA advantage is the straightforward design: adjustments of analyte binding arms of SDA_f and m strands is the only change required for tailoring SDA to each new analyte. Finally, each SDA probe requires synthesis of a pair of inexpensive label-free DNA oligonucleotides, which together have about 10−20 times lower cost than a typical MB or Taqman probe obtained from commercial vendors. Therefore, SDA probes promise to lower the cost of designing and mass producing SNV-selective sensors.

■ CONCLUSION

In this study we divided the **DAP-10** into two separate parts and equipped each part with an analyte-binding arm for hybridization to specific DNA or RNA analytes. The resultant SDA probe was able to fluorescently report the presence of a specific nucleic acid at 22.5 and 37 °C with high selectivity toward SNV in analytes. The novelty of this manuscript is 2-fold: (1) DNA split aptameric probe was used as inexpensive label free and highly selective tool for nucleic acid analysis. (ii) Split probe was for the first time used to detect a biological samples straight after NASBA amplification without downstream processing of the sample, and this is an unprecedented example that can eventually lead to real time NASBA reaction with a potential to replace real time PCR at least in some applications. SDA, therefore, is a cost efficient hybridization probe in a mix-and-read format that can be used with NASBA amplification and potentially in other isothermal amplification formats.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b03964.

List of oligonucleotides used in this study, NMR spectrum of **Dapoxyl-F** dye, detailed design of SDA contracts, original data for calculation of LODs of SDA, selectivity data for SDA, and SDA performance at 37 °C (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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