Supplementary Information (SI) for Nanoscale.

Supplementary Information

Nucleic acid detection with single-base specificity integrating isothermal amplification and light-up aptamer probes

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Materials

Auramine O (AO) was purchased from MP Biomedicals. 1M Tris-HCl (pH 7.5) was purchased from SeouLin Bioscience. Magnesium chloride solution and potassium chloride were purchased from Sigma-Aldrich. DEPC-treated water was purchased from Thermo Fisher Scientific. TwistAmp® Basic kit was purchased from TwistDx limited. T7 exonuclease was purchased from New England Biolabs. PCR tubes were purchased from Corning. 384-well microplates for fluorescence measurements were purchased from SPL Life Sciences. Synthetic dsDNA targets were purchased from IDT, while all other oligonucleotide strands were purchased from Bioneer. Genomic DNA of *Mycobacterium tuberculosis* H37Ra strain was purchased from ATCC.

Validation of UHP and DHP in synthetic single-stranded target recognition

50 μL samples containing – 500 nM of each UHP and DHP, 2 μM AO, 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 25 mM $MgCl_2$, and different concentrations of target (ss_*katG*, ss_WWW, or ss_G>C) – were prepared in a 384 -well plate. Then, AO fluorescence of the mixtures was measured in a Hidex Sense microplate reader (Hidex) at 544 nm upon excitation at 460 nm following incubation at room temperature for 20 minutes. The normalized fluorescence values were calculated by subtracting the AO fluorescence of blank samples (2 μM AO, 20 mM Tris-HCl (pH 7.5), 20 mM KCl, and 25 mM $MgCl_2$) from that of analyte samples.

CLASSIC assay protocol

(1) Recombinase Polymerase Amplification (RPA)

RPA was carried out, according to the user manual from TwistDx. An RPA reaction mixture contained 2.5 μL each of 10 μM forward and reverse primers, 29.5 μL of RPA rehydration buffer, and 5.5 μL of DEPC-treated water, and 5 μL of target samples of (ds_*katG* or gDNA of *M*. *tb* strain H37Ra) or DEPC-water for negative control. A blank RPA sample contained 29.5 μL of RPA rehydration buffer and 15.5 μL of DEPC-treated water. Each reaction was commenced by adding 5 μL of 210.5 mM Magnesium acetate. Immediately after brief vortex-mixing and spin-down, each 50 μL sample was heated for 20 minutes at 42°C (i.e., amplification) and for 5 minutes at 95°C (i.e., reaction termination) in a CronoSTAR™ PCR instrument (Takara).

(2) T7 exonuclease Digestion

A digestion reaction was prepared by mixing the RPA amplicon mixture (reaction terminated, as described

above) and T7 exonuclease in a 13:1 ratio. Each digestion mixture was then processed at 25°C for 5 minutes in the PCR machine.

(3) SDA detection

50 μL samples containing 500 nM of each UHP and DHP, 2 μM AO, 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 25 mM $MgCl_2$, and 20% v/v (e.g., 10 μL) of digested amplicon mixture were prepared in a 384 -well plate. Then, AO fluorescence of the mixtures was measured in the microplate reader at 544 nm upon excitation at 460 nm following incubation at room temperature for 15 minutes. The normalized fluorescence values were calculated by subtracting the AO fluorescence of blank samples (2 μM AO, 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 25 mM $MgCl_2$, and 20% v/v of T7 exonuclease-treated blank RPA sample) from that of analyte samples.

Determination of the optimal CLASSIC buffer ratio

A positive target sample (10 pM ds_*katG*) or a negative sample (DEPC-H_2O) was subjected to RPA and T7 digestion according to the CLASSIC assay protocol. Then, samples (50 μL) containing 500 nM of each UHP and DHP, 2 μM AO, 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 25 mM $MgCl_2$, and different volumetric ratios (X% v/v) of digested amplicon mixture were arranged in a 384-well plate, followed by AO fluorescence recording at 544 nm upon excitation at 460 nm after 15 minutes of incubation at room temperature. The signal-to-nose ratio for each CLASSIC buffer ratio was calculated by dividing the fluorescence values from the positive sample by the mean fluorescence value of the negative sample.

Data Processing

The Limit of Detection (LoD) value was calculated as per MacDougall's guideline[1] in Microsoft Excel. The Calibration curve and R^2 value were obtained via Microsoft Excel. All graphs in the manuscript were generated using OriginLab. Two-tailed t-tests were performed using Microsoft Excel.

All fluorescence data (with normalized values) were processed via subtracting the AO fluorescence of a blank sample from that of an analyte sample.

Sequence information of oligonucleotides used in this work

The asterisk (*) mark represents a phosphorothioate (pt) bond modification within the sequence. Complementary nucleotide sequences between targets and binding arms are marked in red (UHP) or blue (DHP). Unmatched nucleotide substitutions in the UHP-targeted region are colored in orange.

Name	Sequence (5' → 3')
Primer (Forward)	G*T*A*T*G*GCACCGGAACCGGTAAGGACGCGATCA
Primer (Reverse)	/Phosphate/CGAGGAAACTGTTGTCCCATTTCGTCGGGGT
Cy5-Primer (Forward)	/Cy5/G*T*A*TG*G*CACCGGAACCGGTAAGGACGCGATCA
Cy3-Primer (Reverse)	/Cy3/CGAGGAAACTGTTGTCCCATTTCGTCGGGGT
ss_*katG* (single strand)	GTATGGCACCGGAACCGGTAAGGACGCGATCACCAGCGGCATCGAGGTCGTATG GACGAACACCCCGACGAAATGGGACAACAGTTTCCTCG
ss_G>C (single strand)	GTATGGCACCGGAACCGGTAAGGACGCGATCACCACCGGCATCGAGGTCGTATG GACGAACACCCCGACGAAATGGGACAACAGTTTCCTCG
ss_WWW (single strand)	GTATGGCACCGGAACCGGTAAGGACGCGATCACCAWWWGCATCGAGGTCGTAT GGACGAACACCCCGACGAAATGGGACAACAGTTTCCTCG
ds_*katG* (double strand)	GTATGGCACCGGAACCGGTAAGGACGCGATCACCAGCGGCATCGAGGTCGTATG GACGAACACCCCGACGAAATGGGACAACAGTTTCCTCG
ds_G>C (double strand)	GTATGGCACCGGAACCGGTAAGGACGCGATCACCACCGGCATCGAGGTCGTATG GACGAACACCCCGACGAAATGGGACAACAGTTTCCTCG
ds_WWW (double strand)	GTATGGCACCGGAACCGGTAAGGACGCGATCACCAWWWGCATCGAGGTCGTAT GGACGAACACCCCGACGAAATGGGACAACAGTTTCCTCG
UHP	A*T*G*A*C*CTTGGTTCGTAGTTCCGCTGGTG
DHP	T*T*C*G*T*CCATACGACCTCGATGTTCTACGGGGGAGGGTGTGTGGTTTTGGTCA T

Supplementary Figures

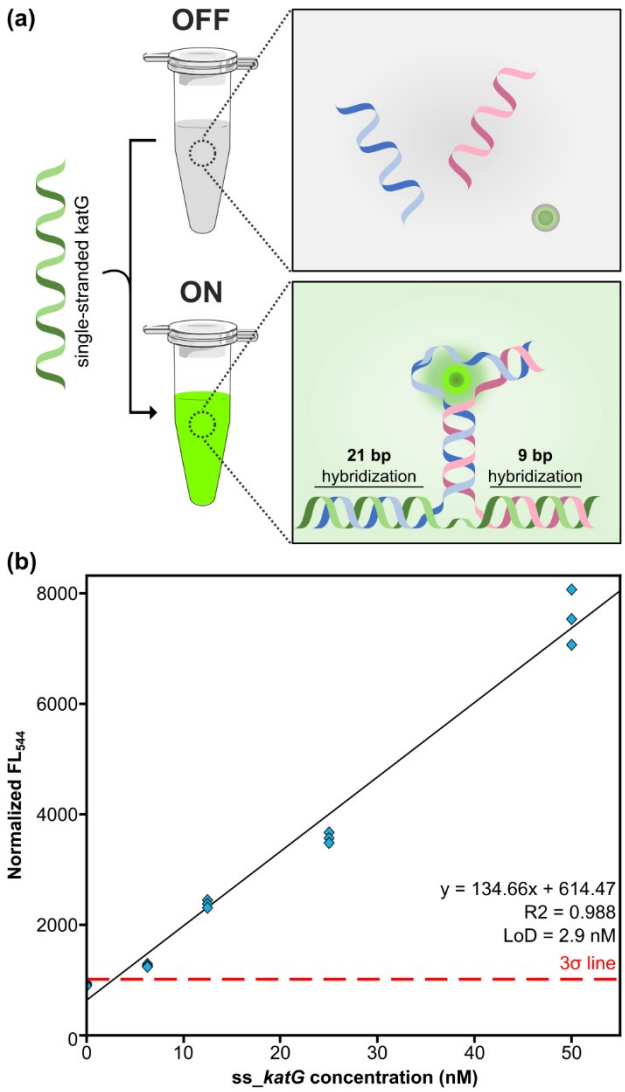

Figure S1 **(a)** Schematic illustration of SDA detection to study the effect of pt modifications on the performance of SDA probes. In the presence of a synthetic single-stranded DNA target, the dapoxyl aptameric core is assembled, lighting-up AO fluorescence. **(b)** Calibration curve of the engineered SDA probe pair. The curve represents a trend line of normalized AO fluorescence values (cyan rhombus) of the samples at specified concentrations. Based on the 3-sigma calculation (red dotted line), The Limit of Detection value (LoD) for the engineered probes was calculated to be approximately 2.9 nM. The data were obtained using 2-fold serial dilutions of synthetic single-stranded *katG* (ss_*katG*) samples and negative control (DEPC-H_2O). The results indicated that pt modifications on the SDA probes had no effect on their functionality.

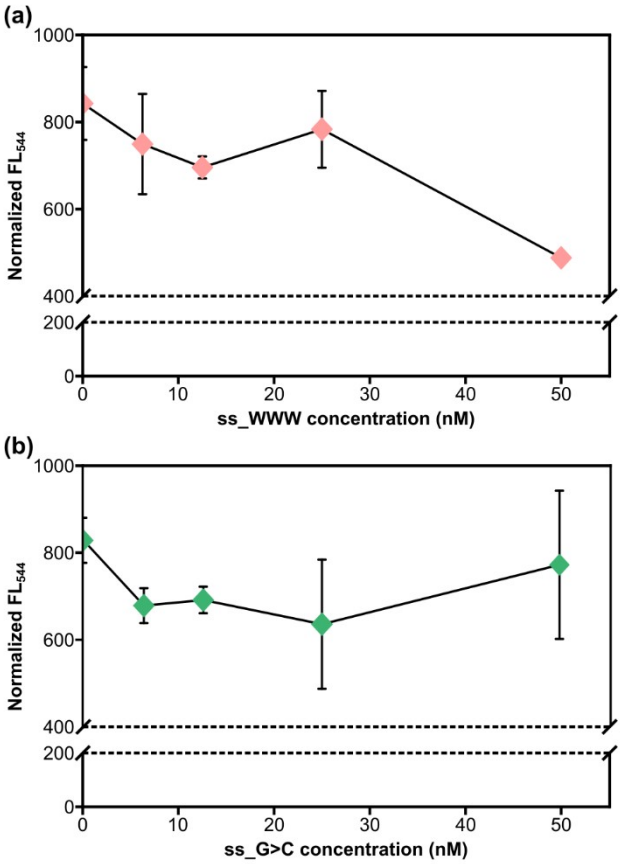

Figure S2 (a) Response of the engineered probe pair and AO to synthetic single-stranded WWW (ss_WWW), where the UHP-binding region contains consecutive three-base mismatches. The scatter plot displays the normalized mean values of AO fluorescence (pink rhombus) ± standard deviation (error bar) at specified concentrations of ss_WWW. Data were collected from three independent measurements. **(b)** Response of the engineered probe pair and AO to synthetic single-stranded G>C (ss_G>C), where the UHP-binding region has a single-nucleotide mismatch. The scatter plot shows the normalized mean values of AO fluorescence (green rhombus) ± standard deviation (error bar) at specified concentrations of ss_G>C. Data were collected from three independent measurements.

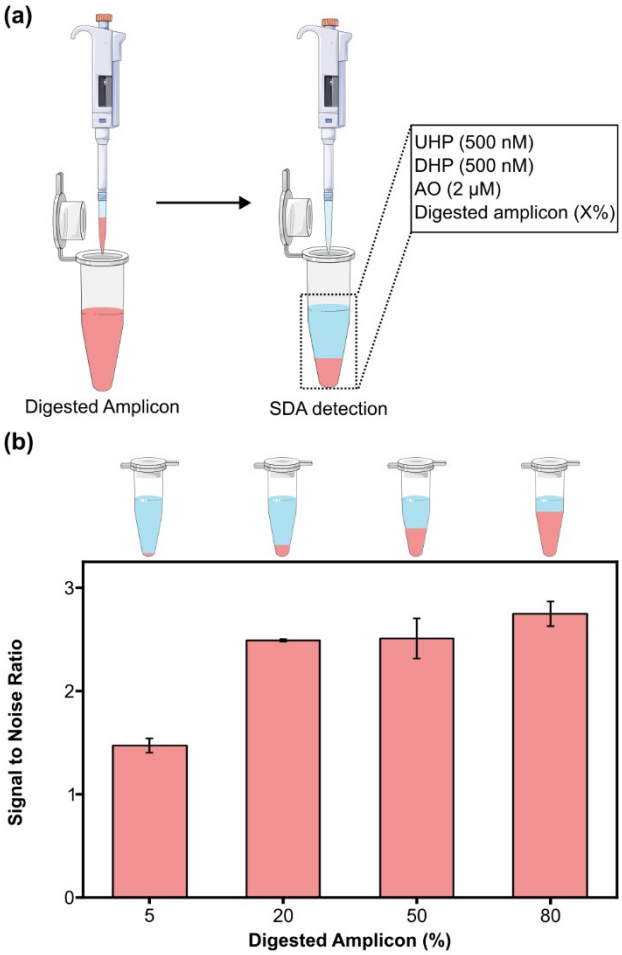

Figure S3 (a) Investigation of the signal-to-noise ratio (S/N) to determine the optimal CLASSIC buffer ratio. For example, a 10 pM positive sample (with ds_*katG*) or a negative sample (with DEPC-H_2O) was subjected to RPA and T7 digestion steps, then different volume ratios (X% v/v) of the positive (or negative) samples were prepared to a final volume of 50 µL. All 50 µL samples included 500 nM of each UHP and DHP, 2 µM of AO, 20 mM Tris-HCl, 20 mM KCl, and 25mM $MgCl_2$. **(b)** The signal (AO fluorescence with 10 pM target) to noise (AO fluorescence with DEPC-H_2O) ratio was calculated and plotted. Each red bar represents the mean ± standard deviation (error bar) of the signal-to-noise ratio at specified volume ratios of the amplified then digested samples. The tubes above each bar graphically represent the different volume portions (X% v/v) of the digested amplicon mixture in each 50 µL sample. A volume ratio of 80% digested amplicon mixture had the largest S/N, yet volume ratios above 20% had similar values. In order to maximize the sample usage efficiency (i.e., less use of digested amplicons for similar amount of fluorescent signal) and to minimize liquid handling errors, a 20% ratio was chosen as the CLASSIC buffer ratio for all CLASSIC experiments.

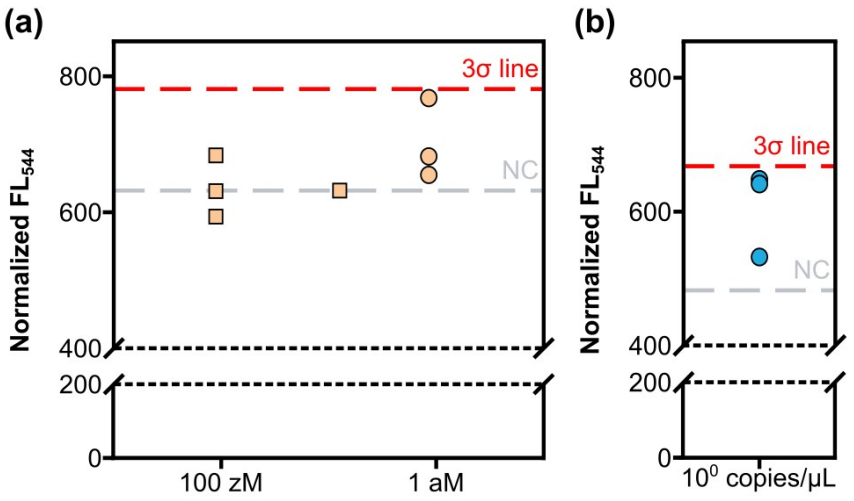

Figure S4 (a) Additional experiments for determining the sensitivity of CLASSIC. AO fluorescence values were measured for samples with ds_*katG* at 100 zM (orange square) and 1 aM (orange circle). All signals were recorded below the 3σ line, indicating that ds_*katG* at concentrations below 1 aM is undetectable via CLASSIC. **(b)** Similar experiment was performed using H37Ra gDNA with a copy number of 50 (e.g., 1 copy/µL, cyan circle). All signals were measured below the 3σ line, implying that H37Ra gDNA was only successfully detectable at approximately 10 copies per µL or higher.

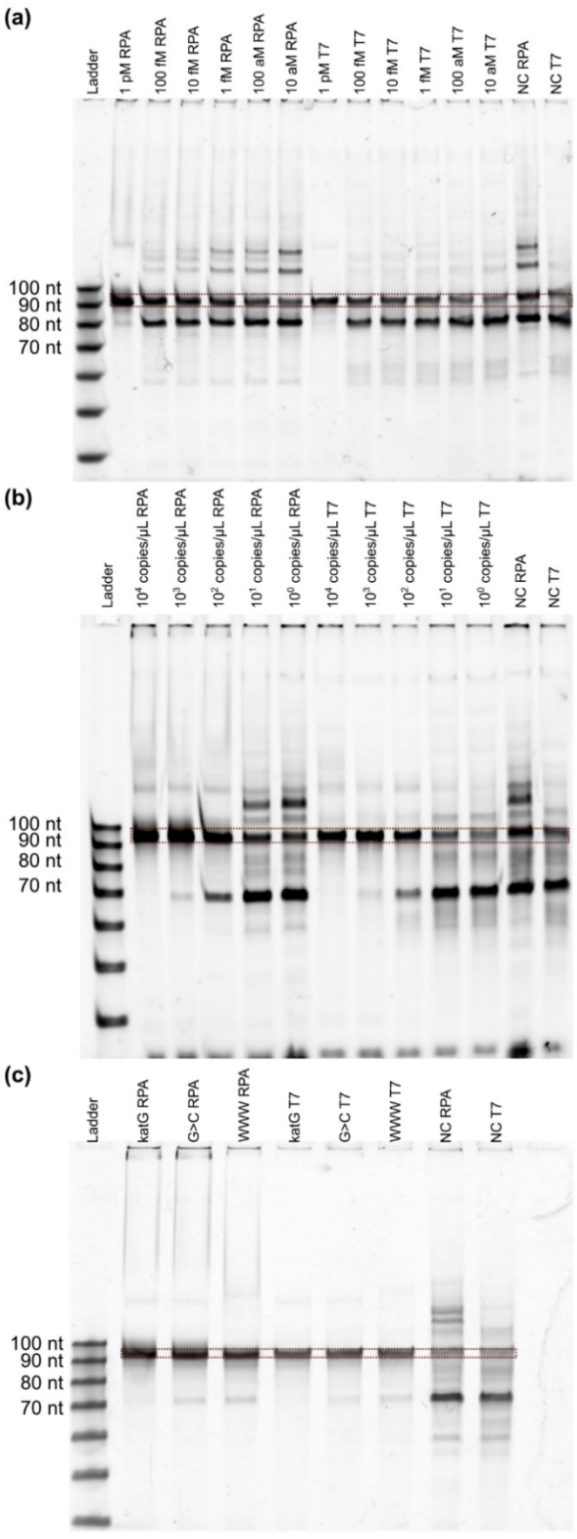

Figure S5 10% denaturing urea-PAGE images for **(a)** Figure 3(c), **(b)** Figure 3(d), and **(c)** Figure 4(b).

Dotted rectangle represents the expected length of amplicons produced from amplification and/or digestion step(s).

References

1. MacDougall, D. and W.B. Crummett, *Guidelines for data acquisition and data quality evaluation in environmental chemistry.* Analytical Chemistry, 1980. **52**(14): p. 2242-2249.