Electronic Supplementary Information (ESI)

for

Rapid detection of a Dengue virus RNA sequence with single molecule sensitivity using tandem toehold-mediated displacement reactions

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Materials and Methods

Materials and Reagents. All DNA and RNA samples were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The sequences were listed in Table S1. Magnesium chloride hexahydrate was purchased from Mallinckrodt Pharmaceuticals (St. Louis, MO), Tris base was purchased from Geno Technology, Inc. (St. Louis, MO), ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich Co. (St. Louis, MO), and 40% acrylamide/bis-acrylamide solution was purchased from Thermo Fisher Scientific Inc. (Ward Hill, MA). All reagents are of analytical grade unless otherwise statements. Analog vortex mixer (VWR, Radnor, PA) was used to mix the solutions and 18.2 MΩ·cm H_2O was used for all experiments.

Table S1. Sequences of the nucleic acids used in this study. The toeholds are shown in *italics*. Recognition regions are shown in **boldface**. Amplification regions are underlined.

Name	Nucleic Acid Type	Sequence (5' to 3')	Modification	Base
DNA 1	DNA	TGC TCT TCC CGA ***AAA CAG*** **CAT ATT GAC GCT GG***C AAC TCC C*AC TCA ACT GCC TGG TGA TAC GAG GAT GGG CA	N/A	71
DNA 2	DNA	GGT GAT AAA ACG TGT AGC AAG CTG TAA TCG ACG GGA AGA GCA TGC CCA TCC ACT ACT ATG GCG	N/A	63
DNA 3	DNA	AGG CAG TTG AGA CGA ACA TTC CTA AGT CTG AAA TTT ATC ACC CGC CAT AGT AGA CGT ATC ACC	N/A	63
DNA 4	DNA	TCG ATT ACA GCT TGC TAC ACG ATT CAG ACT TAG GAA TGT TCG T	5'-TET	43
Protector 17	DNA	*AGT TG*C CAG CGT CAA TA	3'-BHQ1	17
Protector 18	DNA	*AGT TG*C CAG CGT CAA TAT	3'-BHQ1	18
Protector 19	DNA	*AGT TG*C CAG CGT CAA TAT G	3'-BHQ1	19
Capture 23	DNA	*GGG AGT TG*C CAG CGT CAA TAT GC	N/A	23
Capture 24	DNA	*GGG AGT TG*C CAG CGT CAA TAT GCT	N/A	24
Capture 25	DNA	*GGG AGT TG*C CAG CGT CAA TAT GCT G	N/A	25
Target	RNA	**CCA GCG UCA AUA UGC UGU UU**	N/A	20
M1	RNA	**CCA GCG UCA** C**UA UGC UGU UU**	N/A	20
M2	RNA	**CCA GCG UC**C C**UA UGC UGU UU**	N/A	20
NC	RNA	UUG UAC UAC ACA AAA GUA CU	N/A	20
Extended dengue RNA sequence	RNA	UCU GGU CUC UCU C**CC AGC GUC AAU AUG CUG UUU** AUU GU	N/A	38

DNA stock solutions. The purchased oligonucleotides were dissolved in 10 mM pH 8.0 Tris-HCl buffer with 10 mM Mg^{2+} (referred to as TH-Mg buffer) and stored at –4 °C. This mentioned was used as the reaction buffer throughout.

Annealing. The annealing processes were performed using a dry bath incubator (Boekel Scientific, Feasterville, PA.). The solution of mixed DNAs was heated to 95 °C for 15 min and allowed to cool to room temperature over a period over 2 h. The annealed DNA complex solution was stored at 4 °C.

Optimization of magnesium concentration for synthesizing the DNA tetrahedron. Two hundred nM DNA 1, 2, 3, 4 and Protector DNA was added to 10 mM Tris-HCl buffer, pH 8.0, with different concentrations of magnesium chloride and preceded the annealing process, followed by further characterization.

Characterization of the DNA tetrahedron. The DNA tetrahedron was synthesized with DNA 1, 2, 3, 4 and Protector DNA by an annealing process. Native polyacrylamide gel electrophoresis (5% PAGE) was used to characterize the formation of the DNA tetrahedron. A DNA sequencing system (Model 4200, Fotodyne, Hartland, WI) was used to supply the constant direct current. The voltage was set at 80 V while the power was less than 3 W to minimize any change in temperature. Normally, the electrophoresis was complete within 90 min.

Characterization of toehold-mediated displacement reaction using a fluorescence spectrometer. To 100 nM DNA tetrahedron was added 10 nM target RNA and 100 μM Capture DNA. TH-Mg buffer was used to adjust the volume to 100 μL. The reaction was maintained at room temperature for 3 h in the dark and the fluorescence was measured.

Optimization of the concentration of DNA tetrahedron in the reaction. To different concentration of DNA tetrahedron, 10 nM target RNA and 100 μM capture DNA was added. TH-Mg buffer was used to set the volume to 100 μL. The reaction was kept at room temperature for 3 h in dark place and the fluorescence was measured. Solution without target RNA or capture DNA or both were also measured as a control.

Optimization of the pH value in the reaction. To 100 nM DNA tetrahedron, were added 10 nM of target RNA and 100 μM Capture DNA. Tris-HCl (10 mM) and 10 mM Mg^{2+} buffer with different pH values were used to adjust the volume to 100 μL. The reaction was maintained at room temperature for 3 h in the dark and the fluorescence was measured. An analogous solution lacking target RNA was used as a control.

Calculation of FRET efficiency. For the FRET model, the dipole-dipole interaction followed the power law distance dependence and the FRET distance R_0 (50% quenching distance) could be described as:[1]

$$R_0 = 9.78 \times 10^3 (\kappa^2 n^{-4} Q_{dye} J)^{1/6} \quad (1)$$

in which, κ^2 is the dipole orientation factor (defined as 2/3), n is the refractive index of the medium (1.333 for water), Q_{dye} is the quantum yield of TET and J is the overlap integral between the emission of TET and the absorption of BHQ-1. The theoretical quenching efficiency of FRET (η_{FRET}) follows the 6th-power law and is described as:[2]

$$\eta_{FRET} = \frac{1}{1 + (\frac{r}{R_0})^6} \quad (2)$$

where the *r* is the actual distance between TET and BHQ-1. The calculated R_0 was 4.41 nm. The value of *r* could be longer than the 8 bases (3.4 nm) considering the twist angle and the diameter of DNA helix structure.[3-5] In this circumstance, *r* was 3.95 nm and the calculated η_{FRET} was 0.659, which agreed with the experimental result.

Experimental setup for the fluorescence lifetime measurement. Fluorescence lifetime was measured using the time-correlated single-photon counting (TCSPC) technique. The excitation source was a fiber supercontinuum laser based on a passive modelocked fiber laser and a high-nonlinearity photonic crystal fiber supercontinuum generator (Fianium SC450-PP). The laser provides 6-ps pulses at a repetition rate variable between 0.1 and 40 MHz. The laser output passed through an Acousto-Optical Tunable Filter (Fianium AOTF) to obtain excitation pulses at the desired wavelength of 500 nm. Fluorescence emission was collected at a 90° geometry setting and detected using a double-grating monochromator (Jobin-Yvon, Gemini-180) and a microchannel plate photomultiplier tube (Hamamatsu R3809U-50). The polarization of the emission was set at 54.7° relative to that of the excitation. Data acquisition was done using a single photon counting card (Becker-Hickl, SPC-830). The typical IRF had a FWHM of 40 ps, measured from the scattering of sample at the excitation wavelength. The excitation power was kept at the repetition rate of 20 MHz. The data was fitted with a sum of exponential decay using the home-written program ASUFIT.

Calculation of total quenching efficiency. The quenching efficiency of lifetime was contributed by both radiative, non-radiative decay and FRET;[6] it is referred to as the total quenching efficiency (η_{total}) and can be described as:

$$\eta_{total} = 1 - \frac{\tau_N}{\tau_0} \tag{1}$$

where the τ_N is the lifetime of P-TH and τ_0 is the lifetime of TET-labelled DNA-4.

Calculation of relative recover efficiency. The relative recover efficiency (η) by using the average photon counts within 5 seconds was described by following equation,

$$\eta = \frac{N_{sample} - N_{background}}{N_{background}} \tag{2}$$

where the N_{sample} is the average photon counts of sample and the $N_{background}$ is the average photon counts of background.

Experimental setup for detection using fluorescence microscopy. Fluorescence detection was performed on a Nikon inverted TE2000-U microscope (Nikon Instruments Inc., Melville, NY), which can also be used for single molecule measurements. Krypton/argon laser (Melles Griot 35-KAP-431-208, IDEX Health & Science LLC., Carlsbad, CA) was used as the excitation source for all experiments. The laser beam was reflected by a double dichroic mirror (514 nm / 647 nm, Chroma Tech. Co., Bellows Falls, VT) and focused by a water immersion 60x /1.20 Plan-Apo objective lens (Nikon Instruments Inc., Melville, NY) to excite the samples on the cover glasses (Fisher Scientific International, Inc., Asheville, NC). Emitted photons were collected using the same objective lens. The collected photons were then focused through a 100 micron confocal pinhole and filtered through a 525 nm long-pass emission filter. A single photon counting APD (avalanche photodiode) (τ-SPAD, PicoQuant, Germany) detected the signal which was subsequently processed using a 6602 counter/timer module (National Instruments, Austin, TX).

The power of the laser was set at 0.1 mW to minimize photobleaching of the organic fluorescent dyes, and the signal was integrated for 5 s.

Calculation of the magnitude (*M*) of amplification. The fluorescent intensity of the free DNA 4 is I_0, the fluorescent intensity of quenched p-TH is I_q (background signal), the fluorescent intensity of partial recovered tetrahedron is I, and the order of amplification could be described as:

$$M = \frac{I - I_q}{I_0 - I_q} \times \frac{C_{DNA4}}{C_{target}} \tag{3}$$

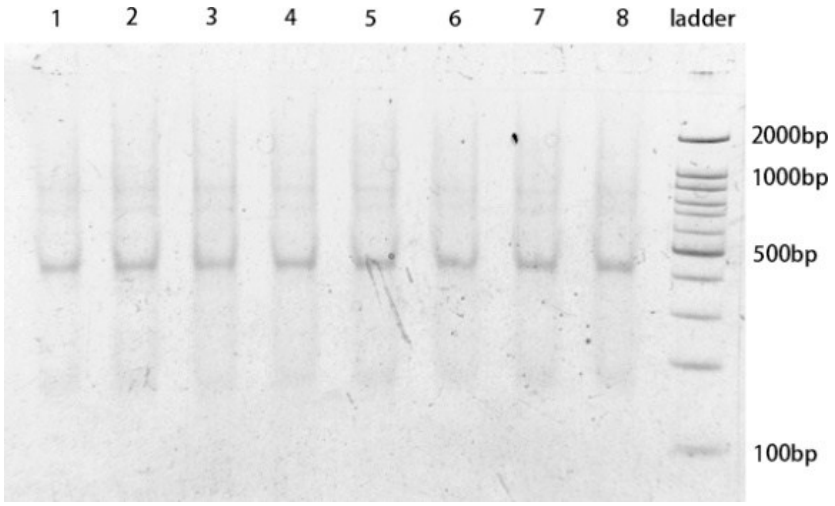


Fig. S1. PAGE analysis for the reproducibility of the synthesis P-TH. Lanes 1 to 8 were produced using different batches of P-TH. The concentration of P-TH was 100 nM.

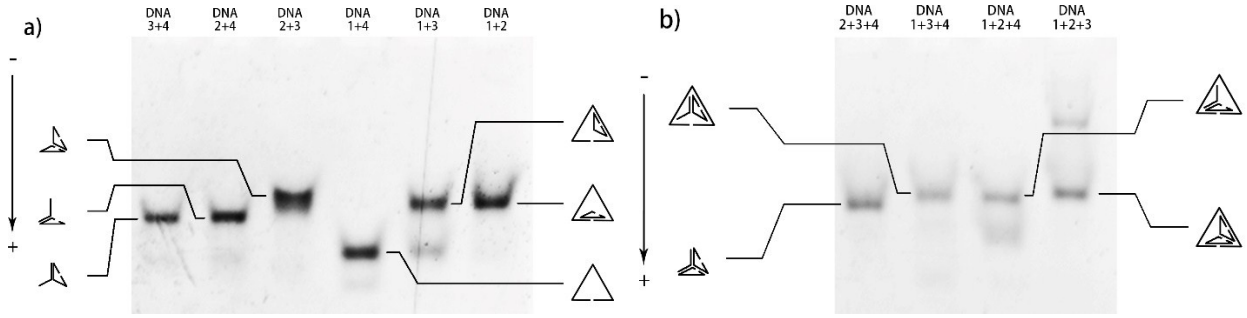


Fig. S2. PAGE analysis for the verification of sequence design. a) Native PAGE for pairwise DNA hybridization; b) Native PAGE for triple-wise DNA hybridization.

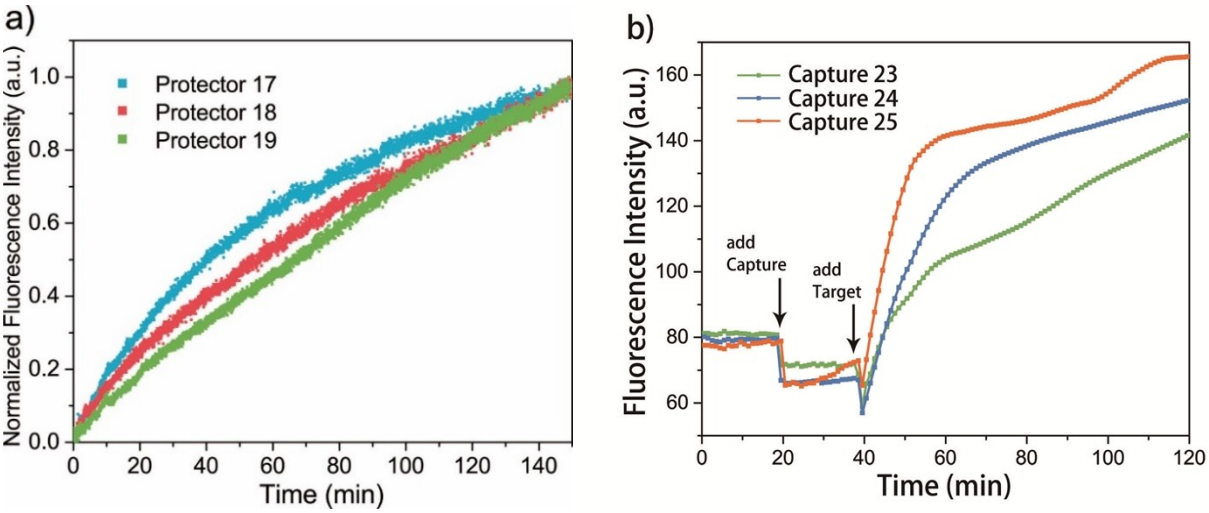


Fig. S3. The dynamic fluorescence intensity related to reaction time by changing the length of Protector DNA (a) or Capture DNA (b). The concentration of P-TH was 100 nM and the concentration of target RNA and Capture DNA were 5 nM and 100 nM, respectively. The time interval of signal collection for Protector DNA was 1 sec, the time interval of signal collection for Capture DNA was 1 min for the addition of the Capture DNA and target RNA.

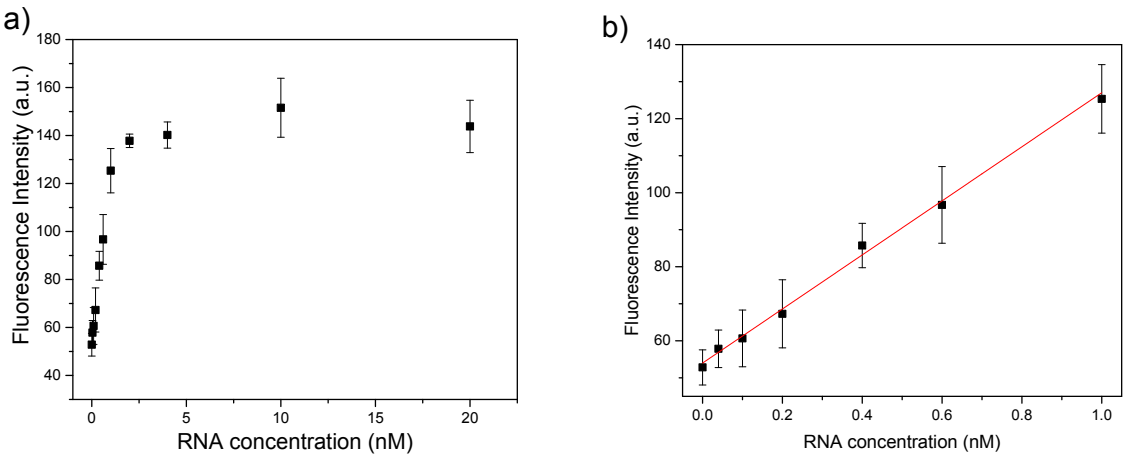


Fig. S4. Variance of fluorescence intensity as a function of the concentration of target RNA (a) in the range 40 pM to 20 nM and (b) the linear fitting of the fluorescence intensity as a function of the concentration of target RNA in the range 40 pM to 1 nM. The concentration of P-TH was 100 nM and the concentration of Capture DNA was 100 nM.

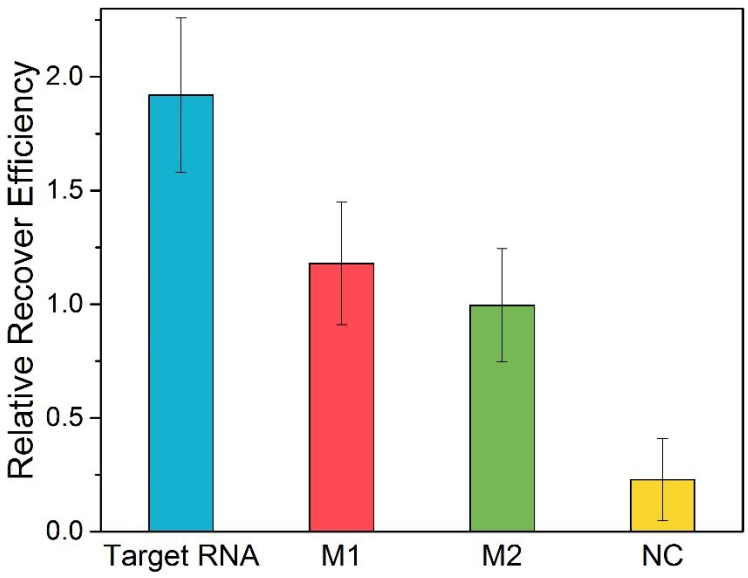


Fig. S5. The relative fluorescence recovery efficiencies following the addition of target RNA and mismatched RNAs. M1: single-base mismatched sequence, M2: double-base mismatched sequence, NC: negative control (not a matched sequence). The concentration of each RNA was 10 aM.

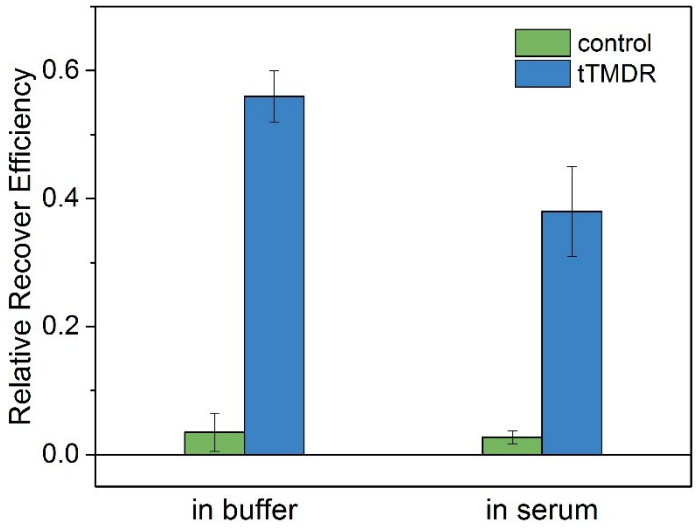
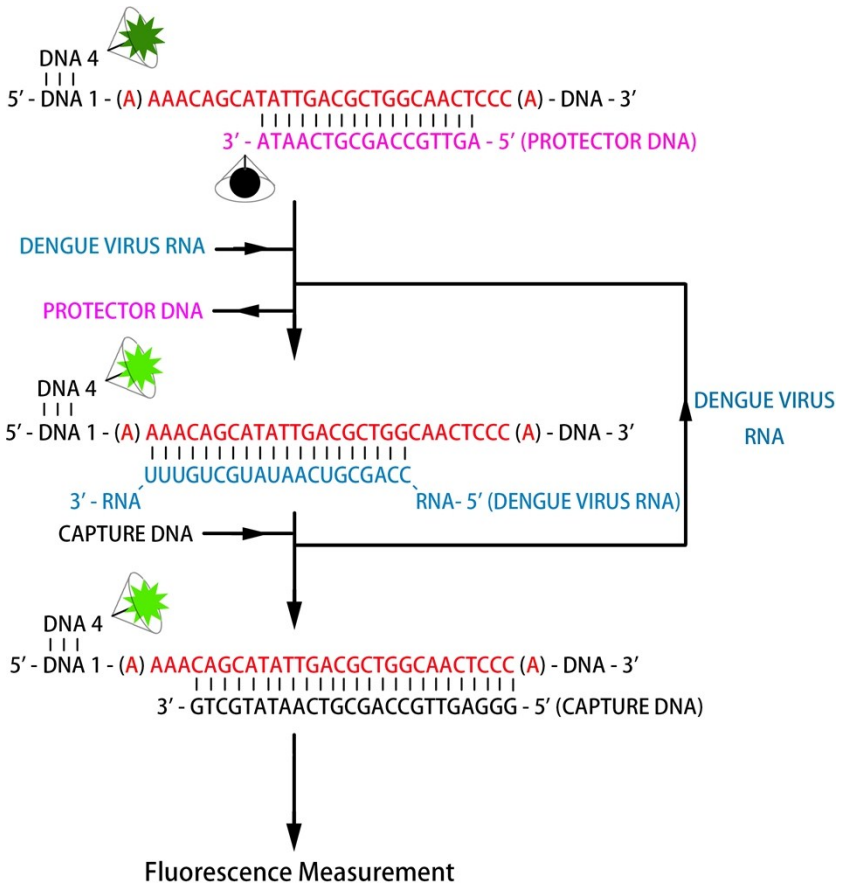


Fig. S6. The relative recovery efficiency of tTMDR in human serum with or without dengue virus RNA. The concentration of P-TH was 100 nM, the concentrations of synthetic dengue viral RNA and Capture DNA were 10 aM and 100 nM, respectively.



Scheme S1. The tTMDR strategy and the sequences of oligonucleotides used. The emission of a fluorescence donor attached to DNA 4 (green burst) is quenched by its physical proximity to a quencher (black circle) at the 3′-end of the Protector DNA. The displacement of the Protector DNA by the viral RNA produces increased fluorescence. A second displacement by the Capture DNA releases the viral RNA for additional cycles.

References

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