Electronic Supplementary Material (ESI) for Analyst.

DNA Nanotechnology for Nucleic Acid Analysis: Sensing of Nucleic Acids with DNA Junction-Probes

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EXPERIMENTAL SECTION

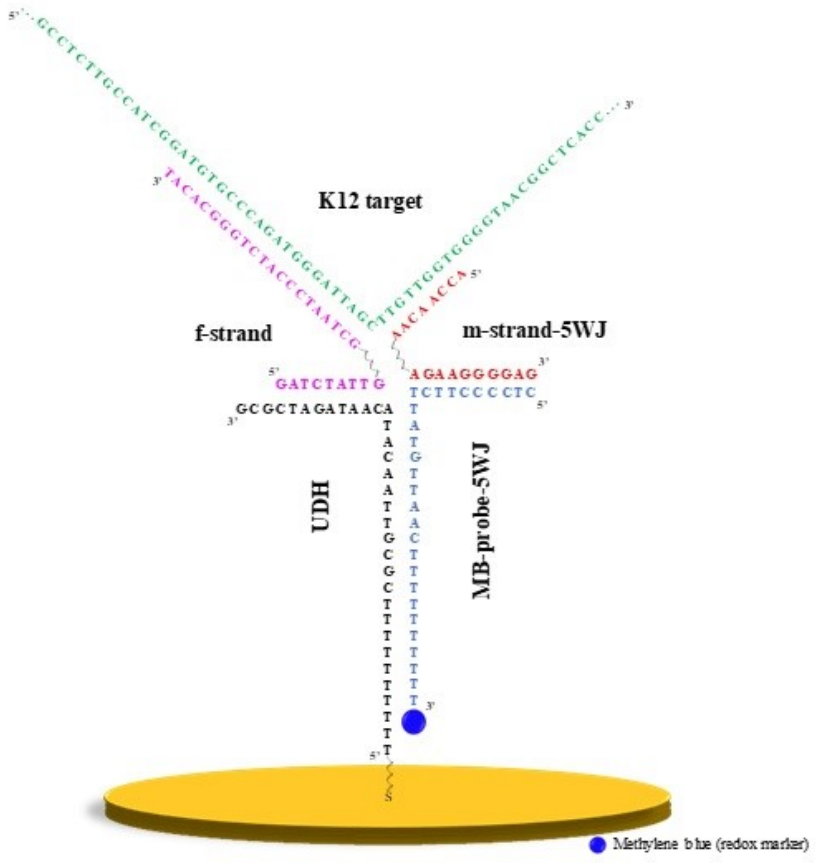
Materials

Oligonucleotides used for the sensor development were purchased from Integrated DNA Technologies (Coralville, USA). The sequences modified with a methylene blue (MB) redox marker (m-strand-X and MB probes) were purchased from Biosearch Technologies, Inc. (Petaluma, USA). All the oligonucleotide sequences used are shown in Table S1. Trizma hydrochloride (Tris-HCl), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 6-mercapto-1-hexanol (MCH) and $MgCl_2$ were purchased from Sigma Aldrich (St. Louis, USA). NaCl, NaOH and H_2SO_4 were obtained from Fisher Scientific (Pittsburgh, USA). Gold disc electrodes (GDEs) were purchased from CH Instruments (Austin, USA). Alumina slurry (1.0 µm, 0.3 µm and 0.05 µm) was obtained from Buehler (Lake Bluff, USA). All aqueous solutions were prepared with deionized water (18.2 MΩ cm) using a Siemens PURELAB Ultra system (Lowell, USA). Immobilization buffer (IB) was prepared with 50 mM Tris-HCl and 250 mM NaCl, adjusted to a pH of 7.4 using 1.0 M NaOH. The hybridization buffer (HB) was prepared with 50 mM Tris-HCl, 100 mM NaCl, and 50 mM $MgCl_2$ adjusted to pH 7.4 using 1.0 M NaOH. Monarch Total RNA Miniprep kit, 10X RNA polymerase reaction buffer, T7 RNA polymerase (50,000 U/mL), NTP mix (25mM each), 10x DTT (0.1M), and $MgCl_2$ (50 mM), were purchased from New England Biolabs (Ipswich, MA). Ampicillin, 10mg/mL bovine serum albumin (BSA), One Shot™ TOP10 chemically competent *E. coli,* Invitrogen PureLink Quick Plasmid Miniprep kit, Fast Digest PstI (10 U/µL) and 10X Fast Digest PstI buffer were purchased from Thermo Scientific (Waltham, MA). GelRed® was purchased from Biotium (Fremont, CA). RiboRuler High Range, RNA-grade glycogen, 125:25:1 ratio phenol:chloroform:isoamyl alcohol mixture, and LB media were purchased from Fisher Scientific (Pittsburg, PA). The 24:1 chloroform:isoamyl alcohol solution was purchased from Acros Organics. Non-DEPC treated RNase-free water and 3M RNase-free sodium acetate, pH 5.2 were purchased from Boston Bioproducts Inc. (Ashland, MA).

Table S1. Oligonucleotide sequences* used in the different sensor designs.

Strand	Sequence (5'-3')
UDH	/ThiolMC6-D/TTTTTTTTTTCGCGTTAACATACAATAGATCGCG
K12-Target	GCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTTGTTGGTGGGGTAACGGCTCACC
f-strand	GATCTATTG/iSp6/GCTAATCCCATCTGGGCACAT
m-strand-X	ACCAACAA/iSp9/TATGTTAACTTTTTTTTTT-MB
m-strand-5S-X	ACCAACAA/iSp9/TATGTTAACTTTCTCAACAATGTACCG
MB probe-5S-X	MB-CGGTACATTGTTGAG
m-strand-5WJ	ACCAACAA/iSp9/AGAAGGGGAG
MB probe-5WJ	CTCCCCTTCT/iSp9/TATGTTAACTTTTTTTTT-MB
O157-Target	GCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACC

*ThiolMC6-D: 5' Thiol Modifier C6 S-S; MB: Methylene Blue; iSp9: Triethylene glycol linker. Fragments of the probes/targets complementary to each other are color coded. Point-mutations in the O157-Target are underlined



Scheme S1. Predicted 5WJ complex formed on the surface of a gold electrode in the presence of the cognate K12 target.

Preparation of transcript RNA and Total RNA

Total RNA isolation protocol:

K12, MG1655 *E. coli* (accession number U00096.3, GenBank) were grown to an OD_{600} of 1.0 in LB broth, then centrifuged at 16,000 x g, 4 ºC for 10 minutes. The supernatant was decanted, with cells resuspended in 500 µL of RNase-free water and vortexed until homogenous. Cells were then treated with 1 mg/mL lysozyme for 5 min at room temperature. Treated cells were incubated with 1 mL of Lysis buffer from a New England Biolabs Monarch Total RNA Miniprep kit, vortexed, and centrifuged at 16,000 x g, 4ºC for 2 min. Supernatant was transferred into microcentrifuge tubes and total RNA was extracted according to protocols outlined in the NEB Monarch Total RNA miniprep kit. Total RNA concentrations were determined by 260 nm absorbance using a NanoDrop One^{c} absorbance spectrometer (Thermo Scientific) and confirmed with visualization in a 1% agarose gel containing GelRed nucleic acid staining dye. A GelDoc XR+ imager with ImageLab® software (BioRad, Hercules, CA) was used for visualization and quantification was done using RiboRuler High Range as an RNA reference of known concentration.

Plasmid growth and preparation: One Shot™ TOP10 chemically competent *E. coli* were transfected with pEC16SM plasmid, containing the sequence for transcription of K12, MG1655 *E. coli* according to vendor protocols, and plated on LB-ampicillin media (100 µg/µL). Transformed cells were passaged in liquid LB-ampicillin media and grown to an OD_{600} of 1.0. Plasmids were isolated using an Invitrogen PureLink Quick Plasmid Miniprep kit according to vendor protocols. Isolated plasmids were linearized in 40 µL aliquots containing 4 µL of 10X Fast Digest buffer, 2 µL Fast Digest PstI, and 11 µL Non-DEPC treated RNase-free water, and 23 µL of plasmid isolate. Samples were

digested by incubation at 37 ºC for 30 min and purified by ethanol precipitation (see "ethanol precipitation"). Linearized plasmid concentrations were determined using the same protocols outlined in "Total RNA isolation protocol".

In Vitro Transcription: *In vitro* Transcription of 16S rRNA from the linearized plasmids was performed in 50 µL reaction aliquots containing 5 µL 10X RNA polymerase reaction buffer, 2 µL of NTP mix, 0.5 µL BSA, 14 µL $MgCl_2$, 5 µL T7 RNA polymerase, 1 µg of linear plasmid, and Non-DEPC treated RNase-free water. For the transcription reaction, samples were incubated at 37 ºC for 3 hours. RNA was extracted using an acidic phenol chloroform extraction (see "phenol chloroform extraction") and ethanol precipitated (see "ethanol precipitation"). Purified RNA concentration was determined using absorbance spectrometry and gel quantification as previously described.

Phenol Chloroform extraction: One volume of sample is incubated with 1 volume of DNase/RNase-free acidic phenol:chloroform:isoamyl alcohol mixture, and mixed thoroughly. Samples were centrifuged for 5 minutes at 16,000 x g at room temperature, and the aqueous layer extracted. The aqueous layer was back titrated with one volume of 24:1 chloroform:isoamyl alcohol, then mixed, centrifuged, and extracted as previously described.

Ethanol precipitation: Samples were prepared using $1/10^{th}$ volume of 3M sodium acetate, 3X volume cold 100% ethanol, and glycogen (0.1 mg/mL final concentration). Samples were chilled at −70 ºC for 1 hour, and centrifuged for 15 min at 21,100 x g, 4 ºC for 15 min, followed by decanting of the supernatant. The isolated pellet was washed with 500 µL 70% ethanol, and centrifuged twice, followed by air drying the pellet and resuspension in 50 µL of Non-DEPC treated RNase-free water.

RESULTS

Optimization of the adaptor strand concentrations for the 5S-X and 5WJ sensors

The concentration of the adaptor strands (m-strand-X and f-strand) were already well established for the X sensor and have been described in our previous works[1-4]. However, with the addition of a new strand to form the 5S-X and 5WJ structures, it was necessary to optimize the strand concentrations in order to obtain higher signal-to-background (S/B) response for these sensors. The data from this study is shown in Tables S2 and S3.

The 5S-X sensor was evaluated using 0.10 µM or 0.25 µM of m-strand-5S-X; 0.10 µM, 0.25 µM or 0.50 µM of MB-probe-5S-X; and the concentration of the f-strand remained fixed at 0.50 µM. The sensor's response is shown in Table S2. Using 0.10 µM of MB-probe-5S-X, the lowest current density intensity was observed in the presence of the target, which was already expected, since there was a reduction in the concentration of the redox marker (MB) on the electrode's surface, consequently reducing electronic transfer. On the other hand, the highest concentration of MB-probe-5S-X (0.50 µM) resulted in the highest electrochemical signal in the presence of the target (23 ± 1 µA/cm^2). However, the signal obtained for the blank was also high (4.6 ± 0.9 µA/cm^2), which results in the S/B of 5. Thus, the best response was obtained for 0.10 µM of m-strand-5S-X and 0.25 µM of MB-probe-5S-X, which exhibited the S/B of ~21. Therefore, these concentrations were used in further experiments.

Table S2: Response of the 5S-X sensor at different concentrations of m-strand-5S-X and MB-probe-5S-X in the absence or presence of 50 nM synthetic DNA K12-target.

m-strand-5S-X (µM)	f-strand (µM)	MB-probe-5S-X (µM)	Target (nM)	j_p (µA/cm^2)
0.25	0.50	0.50	0	4.6 ± 0.9
			50	23 ± 1
0.25	0.50	0.25	0	0.8 ± 0.3
			50	9.0 ± 0.7
0.25	0.50	0.10	0	0.4 ± 0.2
			50	3.5 ± 0.9
0.10	**0.50**	**0.25**	**0**	**0.8 ± 0.2**
			50	**16.4 ± 0.5**

For the 5WJ sensor, the concentrations tested were 0.10 µM or 0.25 µM for m-strand-5WJ and for MB-probe-5WJ, while the f-strand concentration was fixed at 0.50 µM. As can be observed in Table S3, using 0.25 µM of m-strand-5WJ and MB-probe-5WJ, the highest intensity of the current density in the presence of the target was obtained (15.2 ± 0.9 µA/cm^2), but the blank response was also the highest (1.9 ± 0.3 µA/cm^2), which resulted in a low S/B response (8-fold). The use of the 0.10 µM of MB-probe-5WJ demonstrated the lowest response (4.0 ± 0.4 µA/cm^2). Thus, the best S/B (~13-fold) was obtained with 0.10 µM of m-strand-5WJ and 0.25 µM of MB-probe-5WJ. For this reason, these concentrations were used in further experiments.

Table S3: Response of the 5WJ sensor at different concentrations of m-strand-5WJ and MB-probe-5WJ in the absence and presence of 50 nM synthetic DNA K12-target.

m-strand-5WJ (μM)	f-strand (μM)	MB-probe-5WJ (μM)	Target (nM)	j_p (μA/cm²)
0.25	0.50	0.25	0	1.9 ± 0.3
			50	15.2 ± 0.9
0.25	0.50	0.10	0	0.51 ± 0.05
			50	4.0 ± 0.4
0.10	**0.50**	**0.25**	**0**	**0.6 ± 0.2**
			50	**7.6 ± 0.6**

Analysis of the 5WJ structure formation and reusability of the 5WJ E-sensor

The samples containing different combinations of the five strands constituting the 5WJ E-sensor in the absence or presence of the cognate K12-Target were analyzed using polyacrylamide gel (PAAG) electrophoresis under native conditions (Figure S1A). A band migrating at ~100-bp dsDNA marker was observed only when all five sensor strands and the target were present. The size of the associate in the band (indicated by a green arrow in panel A) is in agreement with the size of the 5WJ structure consisting 189 nts total (~95 bp) plus three triethylenglycol linkers and a MB tag. To prove the presence of the expected oligonucleotides in the slow-migrating association, the correspondent band was excised and eluted from the native gel and loaded on a denaturing PAAG. Analysis of the complex's composition by denaturing PAAG electrophoresis is shown in Figure S1B. Taking into account similar mobilities of f-strand-5WJ and MB-strand-5WJ (Figure S1B, lanes 2 and 4, respectively), as well as higher intensity of the 3rd top band in lane 6 as compared with the 2nd top band containing a one-nucleotide longer UDH, it can be concluded that the slow-migrating complex formed in the presence of all five probe's stgrands and the target is indeed the 5WJ association.

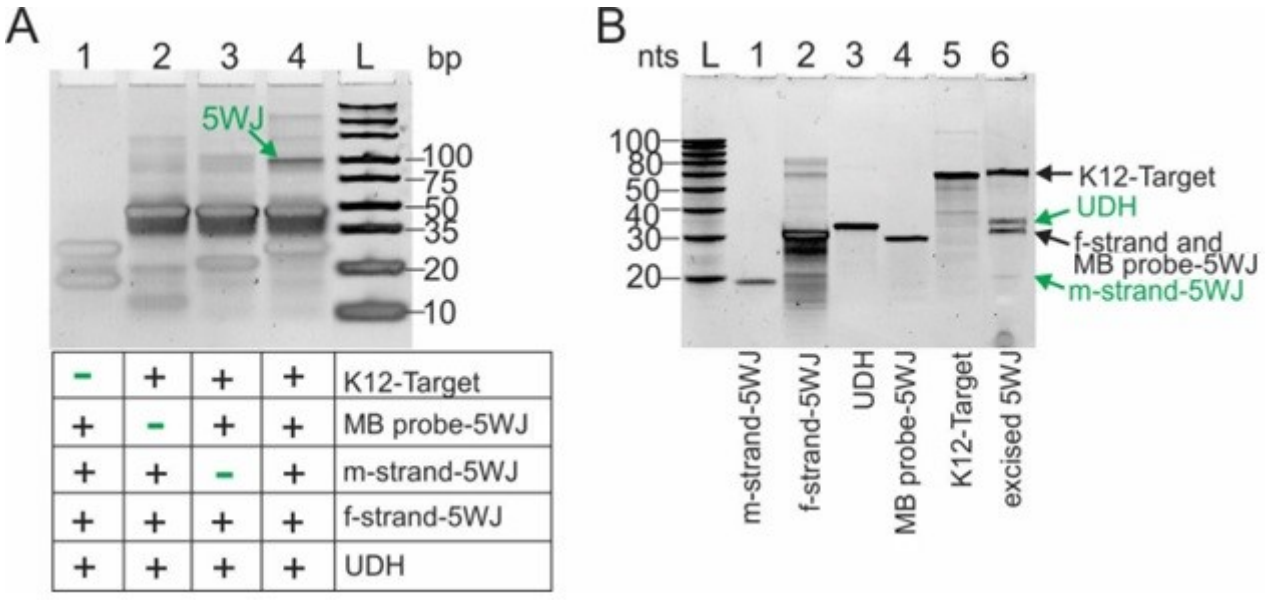


Figure S1. Analysis of the 5WJ structure by gel electrophoresis. A) Samples containing the indicated strand composition (each at 1 μM) were incubated at 22°C for 30 min and then analyzed by 8% PAAG electrophoresis under native conditions at 4°C. The gel was supplemented with 10 mM $MgCl_2$ to minimize strand dissociation during analysis. The gel was stained with GelRed, and the indicated band containing the full 5WJ complex was excised and isolated using the "crush-and-soak" method, followed by ethanol precipitation. L – low-range dsDNA ladder; the marker sizes are indicated next to the correspondent bands. B) The 5WJ complex isolated from the native gel shown in panel A (lane 6) was analyzed using 15% denaturing PAGE (7M urea) at 50°C. Individual strands (each at 1 μM) constituting the 5WJ complex were used as controls (lanes 1-5), as indicated on the bottom. L – ssDNA ladder; the marker sizes are indicated next to the correspondent bands. The bands corresponding to each strand of the 5WJ complex are indicated with arrows. Note that f-strand-5WJ and MB probe-5WJ have similar length and thus co-migrate in the gel.

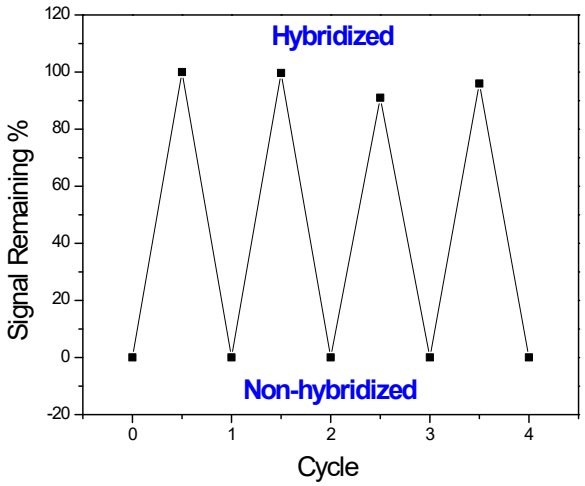


Figure S2. Regeneration of the 5WJ sensor for four consecutive cycles.

Analysis of the hybridization time for 5WJ sensor using total RNA

The 5WJ sensor was applied for the analysis of a sample of total RNA from K12 *E. coli* strain using the conventional hybridization time of 90 min. However, the observed response did not exceed the signal of the blank. As the current for this sensor density increased linearly with the hybridization time in the presence of K12-target and did not plateau even after 120 min (Figure 1B), the response of the 5WJ sensor was analyzed after prolonged hybridization (3 and 6 h), as shown in Figure S3. For both synthetic K12-target and total RNA, 3-h hybridization helped to significantly improve the intensity of current density intensity, with an S/B of ~5 triggered by total bacterial RNA. However, when the hybridization time was extended to 6 h, a decrease in the signal was observed for all samples, which could be explained by screening off the electrode from MB Red/Ox label by RNA present in the sample, which can non-specifically bind gold electrode surface[5, 6]. Therefore, 3 h of hybridization time was chosen for the analysis of total RNA in further experiments.

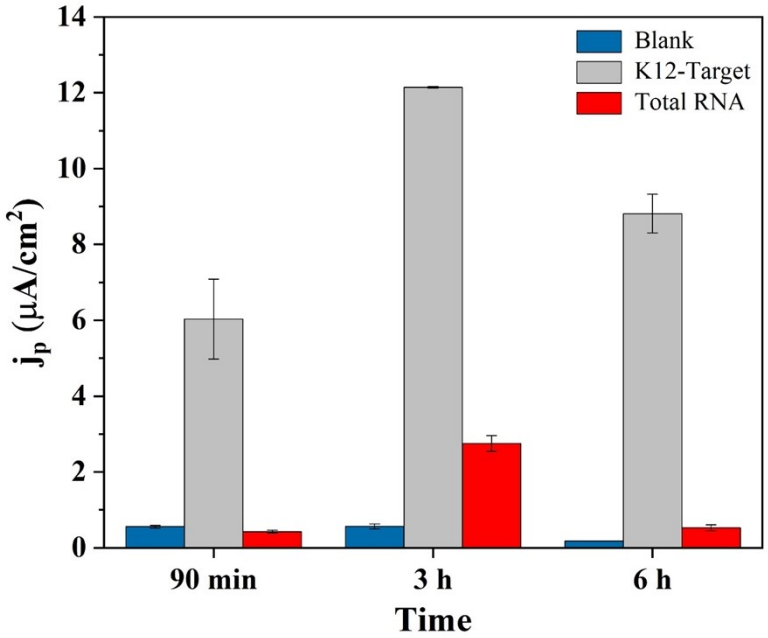


Figure S3. Response of the 5WJ sensor in the absence of the target (blank – blue bar), or in the presence of either synthetic K12-target (grey bar) or *E. coli* total RNA (red bar) at different hybridization times. All hybridization solutions were heated at 90 ºC for 2 min before adding to the UDH-modified gold electrode; total RNA concentration was 85.5 ng/μL, which corresponds to approximately 50 nM of 16S rRNA.

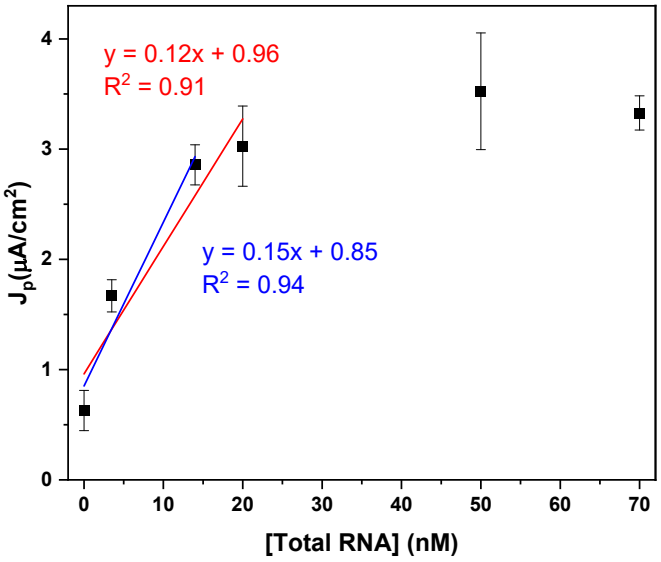


Figure S4. Response of the 5WJ sensor in the presence of E. coli total RNA at different concentrations (0, 3.5, 14, 20, 50 and 70nM). All hybridization solutions were heated at 90 ºC for 2 min before adding to the UDH-modified gold electrode.

Long analytes reduce S/B of X sensor

Prior this study we characterized the X E-sensor (Scheme 1B, main text) using Zika virus-related analytes of different lengths (ZIKV-24, ZIKV-72, ZIKV-84, ZIKV-141, Table S3) and corresponding adapter strands f-ZIKV and m-ZIKV (Table S3). The sensor's response was length-dependent, with the lowest S/B exhibited in the presence of the longest ZIKV-141 (Figure S4). The S/B obtained for ZIKV-24, ZIKV-72, ZIKV-84, ZIKV-141 was 16.7, 15.1, 15.3, and 12.1, respectively.

Table S3. Oligonucleotides used for characterization of X electrochemical sensor.

Strand	Sequence (5'-3')
USL-10T probe	S-S-$(CH_2)_6$-TTTTTTTTTTCGCGTTAACATACAATAGATCGCG
f-ZIKV	GATCTATTGGCTCTATCTTCG
m-ZIKV	CTCGACTTTCTATGTTAACTTTTTTTTTT-MB
ZIKV-24	CGAAGATAGAGCGAAAGTCGAGGT
ZIKV-72	GGGAGAAGGGCATAGCGGGATGATTGGATATGAAACTGACGAAGATAGAGCGAAAGTCGAGGTTACGCC TAA
ZIKV-84	GGGAGAAGGGCATAGCGGGATGATTGGATATGAAACTGACGAAGATAGAGCGAAAGTCGAGGTTACGCC TAATTCACCAAGAGC
ZIKV-141	GGGAGAAGGGCATAGCGGGATGATTGGATATGAAACTGACGAAGATAGAGCGAAAGTCGAGGTTACGCC TAATTCACCAAGAGCGGAAGCAACCTTGGGAGGCTTTGGAAGCTTAGGACTTGACTGTGAACCAAGGACAG G

*ThiolMC6-D: 5' Thiol Modifier C6 S-S; MB: Methylene Blue; Fragments of the probes/targets complementary to each other are color coded. Polymorphisms are underlined.

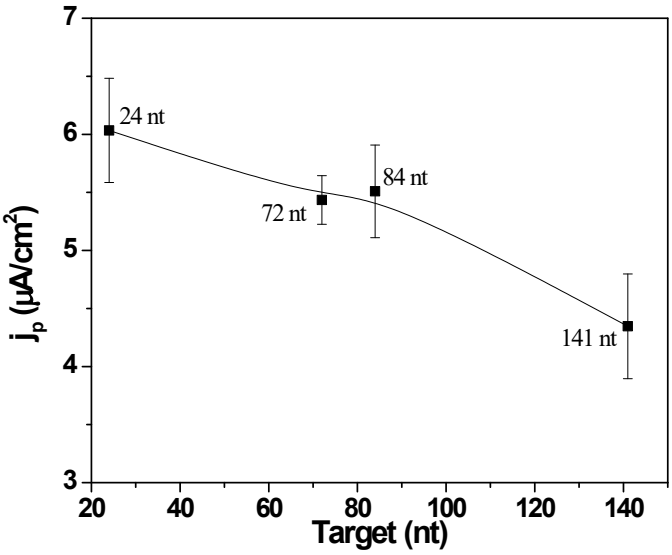


Figure S5. Response of the X sensor for Zika virus using 50 nM of synthetic DNA ZIKV-target with 24, 72, 84, and 141 nt using 10 minutes of hybridization time at room temperature.

FULL SEQUENCE* OF 16S rRNA OF K12 *E. coli*

5'– AAA UUG AAG AGU UUG AUC AUG GCU CAG AUU GAA CGC UGG CGG CAG GCC UAA CAC AUG CAA GUC GAA CGG UAA CAG GAA GCA GCU UGC UGC UUC GCU GAC GAG UGG CGG ACG GGU GAG UAA UGU CUG GGA AGC UGC CUG AUG GAG GGG GAU AAC UAC UGG AAA CGG UAG CUA AUA CCG CAU AAU GUC GCA AGA CCA AAG AGG GGG ACC UUC GG**G CCU CUU GCC AUC GGA UGU GCC CAG AUG GGA UUA GCU UGU UGG UGG GGU AAC GGC UCA CC**A AGG CGA CGA UCC CUA GCU GGU CUG AGA GGA UGA CCA GCC ACA CUG GAA CUG AGA CAC GGU CCA GAC UCC UAC GGG AGG CAG CAG UGG GGA AUA UUG CAC AAU GGG CGC AAG CCU GAU GCA GCC AUG CCG CGU GUA UGA AGA AGG CCU UCG GGU UGU AAA GUA CUU UCA GCG GGG AGG AAG GGA GUA AAG UUA AUA CCU UUG CUC AUU GAC GUU ACC CGC AGA AGA AGC ACC GGC UAA CUC CGU GCC AGC AGC CGC GGU AAU ACG GAG GGU GCA AGC GUU AAU CGG AAU UAC UGG GCG UAA AGC GCA CGC AGG CGG UUU GUU AAG UCA GAU GUG AAA UCC CCG GGC UCA ACC UGG GAA CUG CAU CUG AUA CUG GCA AGC UUG AGU CUC GUA GAG GGG GGU AGA AUU CCA GGU GUA GCG GUG AAA UGC GUA GAG AUC UGG AGG AAU ACC GGU GGC GAA GGC GGC CCC CUG GAC GAA GAC UGA CGC UCA GGU GCG AAA GCG UGG GGA GCA AAC AGG AUU AGA UAC CCU GGU AGU CCA CGC CGU AAA CGA UGU CGA CUU GGA GGU UGU GCC CUU GAG GCG UGG CUU CCG GAG CUA ACG CGU UAA GUC GAC CGC CUG GGG AGU ACG GCC GCA AGG UUA AAA CUC AAA UGA AUU GAC GGG GGC CCG CAC AAG CGG UGG AGC AUG UGG UUU AAU UCG AUG CAA CGC GAA GAA CCU UAC CUG GUC UUG ACA UCC ACG GAA GUU UUC AGA GAU GAG AAU GUG CCU UCG GGA ACC GUG AGA CAG GUG CUG CAU GGC UGU CGU CAG CUC GUG UUG UGA AAU GUU GGG UUA AGU CCC GCA ACG AGC GCA ACC CUU AUC CUU UGU UGC CAG CGG UCC GGC CGG GAA CUC AAA GGA GAC UGC CAG UGA UAA ACU GGA GGA AGG UGG GGA UGA CGU CAA GUC AUC AUG GCC CUU ACG ACC AGG GCU ACA CAC GUG CUA CAA UGG CGC AUA CAA AGA GAA GCG ACC UCG CGA GAG CAA GCG GAC CUC AUA AAG UGC GUC GUA GUC CGG AUU GGA GUC UGC AAC UCG ACU CCA UGA AGU CGG AAU CGC UAG UAA UCG UGG AUC AGA AUG CCA CGG UGA AUA CGU UCC CGG GCC UUG UAC ACA CCG CCC GUC ACA CCA UGG GAG UGG GUU GCA AAA GAA GUA GGU AGC UUA ACC UUC GGG AGG GCG CUU ACC ACU UUG UGA UUC AUG ACU GGG GUG AAG UCG UAA CAA GGU AAC CGU AGG GGA ACC UGC GGU UGG AUC ACC UCC UUA – 3`

*Bold: fragment used as a synthetic DNA K12-target; highlighted in green: a fragment complementary to f-strand; highlighted in yellow: a fragment complementary to m-strand (for all the sensor designs).

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