Electronic Supplementary Material

Enzyme-powered Cascade Three-Dimensional DNA Machine for the Ultrasensitive Determination of Kanamycin

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Contents

Supplementary Experimental Section

***Chemicals and Materials*:** All oligonucleotides used in this study were synthesized and HPLC purified by Sangon Biotech Co., Ltd. (Shanghai, China). Sequences of the synthesized oligonucleotides are list in Supporting Information Table S1. Gold chloride ($HAuCl_4$ $3H_2O$), Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)，Tween 20, dithiothreitol (DTT)，tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl), Kanamycin sulfate were purchased from Sigma-Aldrich (St. Louis, MO). Tetracycline, Chloramphenicol, Ampicillin, Oxytetracycline, streptomycin and florfenicol were brought from Macklin Inc. (Shanghai, China). Nicking endonuclease (*Nt.AlwI*) and CutSmart buffer (NEB buffer) were ordered from New England Biolabs, Inc. (Beverly, MA, USA). Phosphate-Buffered Saline (10×, cat. no. AM9625) was purchased from Thermo Fisher Scientific, Inc. All chemical reagents were of analytical grade and used without further purification. The milk sample was acquired from local supermarket in Shanghai, China. All solutions were prepared with ultra-pure water (18.25 MΩ·cm) from a Millipore system.

***Apparatus*:** The transmission electron microscopy (TEM) images were obtained by a JEM-2100 transmission electron microscope (JEOL, Japan). Zeta potential and dynamic light scattering (DLS) were recorded on the Malvern Zetasizer Nano ZS90 (Malvern, USA). The fluorescence spectra and the real-time fluorescence dates were measured on RF-6000 spectrometer with 10 nm band-pass spectrometer slits (Shimadzu, Japan). The UV-vis absorption spectra were recorded on TU-1901 spectrometer (Persee, Beijing, China).

***Synthesis of gold nanoparticles*:** The gold nanoparticles were prepared by the classic citrate reduction method. Briefly, 100 mL of $HAuCl_4$ (1 mM) was heated under reflux. 10 mL of trisodium citrate (38.8 mM) solution was quickly added to a stirred boiling solution of $HAuCl_4$. After the color of the solution changed from pale yellow to deep red, the solution kept at boiling and stirred for another 20 min. Then, the solution was cooled down to room temperature, filtered by a 0.45 μm Millipore membrane filter and stored at 4 ℃ for future use. The concentrations of Au NP were estimated based on the absorbance of 520 nm with extinction coefficient of $2.7*10^8$ M^{-1} cm^{-1}.

Preparation of unlocked DNA machines system: To study the signal enhancement performance, three kinds of unlocked DNA machines were synthesized. The unlocked machine 1 and machine 2 were prepared as described in the main manuscript except without addition of aptamer strand. For the machine 3, the substrate strand of H1 and H2 were co-immobilized on the surface of gold nanoparticles. In brief, 5 μL of 6.8 μM walker strand, 20 μL of 25 μM H1, and 20 μL of 75 μM H2 were mixed with 45 μL of 100 μM TCEP·HCl at 25 °C for 1 h to break the disulfide bond. After that, 2 mL of 1 nM gold nanoparticles were added. The mixture was incubated at 25 °C for 16 h before aging. After aging process, the machine 3 was finally redispersed in 1×PBS buffer.

Characterization of unlocked DNA machines system: To characterize unlocked DNA machines, the real time fluorescence responsive of three DNA machine systems were recorded ($\lambda_{ex/em}$=485/519 nm) in presence of nicking enzyme *Nt.AlwI* (10 U) and the control experiments were proceed in presence of denature enzyme.

Determination of density of track molecules on the cascade DNA machines: The unlocked machine 1 and machine 2 were decorated with FAM labeled H1 and Cy5 labeled H2, respectively. The standard curves of two fluorophore labeled track molecules were obtained by plotting the linear relations between different concentrations of FAM -labeled tracks and Cy5-labeled tracks with their fluorescence intensities (Fig. S2). To estimate initial loading amount of H1 or H2 on the machines,

20 μL of 1 nM machine was incubated with 8 μL of 1 M DTT. Then, 1×PBS was added to a total volume of 400 μL. The mixture was shaken at room temperature overnight. The released H1 or H2 were separated via centrifugation and the substrate loading amount ($N_{initial}$) was calculated by the fluorescence measurements according to the corresponding standard curve of dye-labeled hairpin substrate ($\lambda_{ex/em}$=485/519 nm for FAM, $\lambda_{ex/em}$=649/670 nm for Cy5). The amount of residue track molecules ($N_{residue}$) on DNA machines were calculated by the dissociation of DTT after enzymatic cleavage.

In order to overcome the nonspecific dissociate induced by dithiothreitol, the cascade DNA machines were incubated with denature nicking enzyme at 37 °C for 1h, and the fluorescence intensity of fluorescent dyes were recorded to calculate the released nucleic acid ($N_{release}$). Thus, actual cleavage ($N_{cleavage}$) of substrate strand on each machine was calculated by the following equation:

$$N_{cleavage} = N_{initial} - (N_{release} + N_{residue})$$

The cleavage efficiency refers to the ratio of the cleaved DNA substrates by nicking enzyme to the total track molecules anchored on the gold nanoparticles surface after subtracting the nonspecific released ones.

Table S1. Sequence information for oligonucleotides used in this work

Name	Sequence (5'-3')
Hairpin 1 (H1)	HS-TTTTTTGGGTGGCGATAAGGCTAATGGGATCATAT*AGTAGTA TAGATCCACCCA-FAM (*cleavage site)
Hairpin 2 (H2)	HS-TTTTTGATGGATCTATA*CTACTCGACGCTGGGTAGTATA-FAM (*cleavage site)
Walking strand	HS-T-40-T-TCGGCTTAGCTACTATATGATCCGAACCCCCA
Kanamycin aptamer	TGGGGGTTGAGGCTAAGCCGA

The underlined portion of the Hairpin 1 and Hairpin 2 represents the recognition site of the nicking enzyme *Nt.AlwI*, and the asterisk indicates the cleavage site of the nicking enzyme.

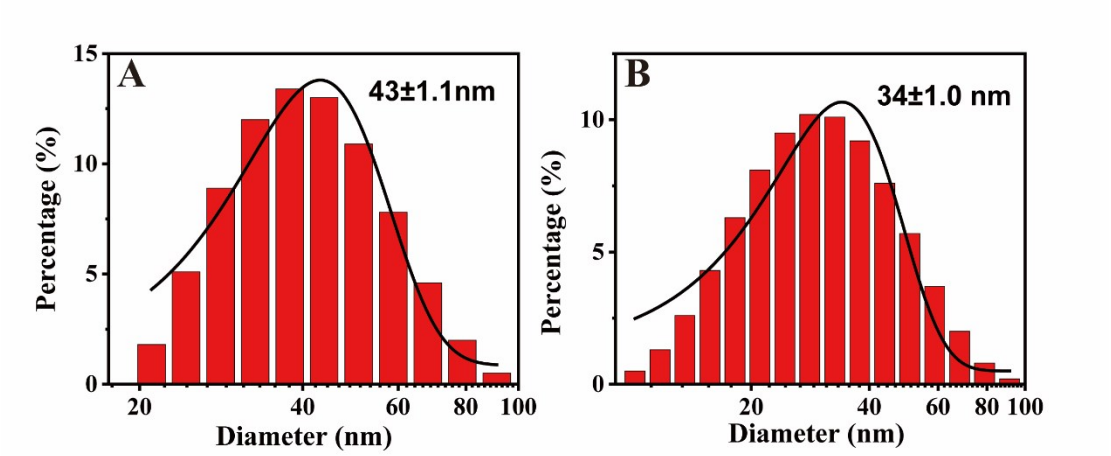

Fig. S1. Dynamic light scattering (DLS) measurements of machine 1 (A) and machine 2 (B).

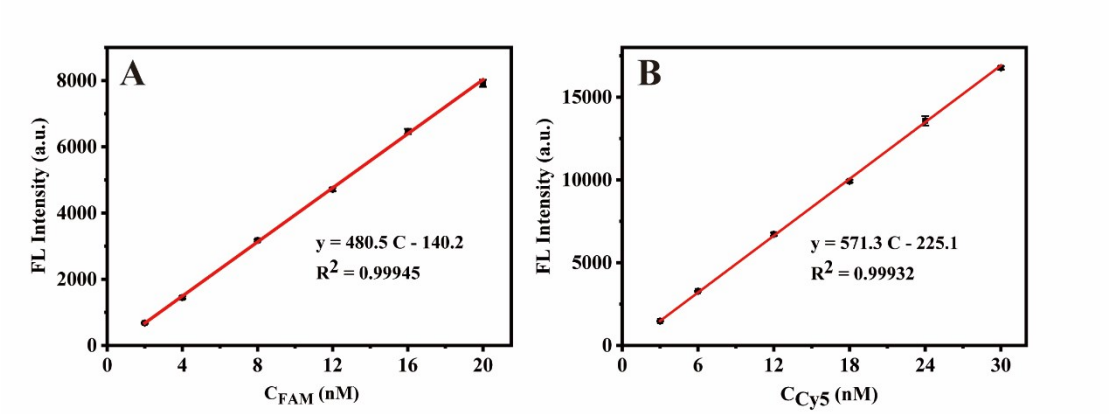

Fig. S2. Standard linear calibration curves of fluorophore FAM (A) and fluorophore Cy5 (B).

In order to demonstrate the cleavage mechanism of driven power and the occurrence of cascade reaction, the polyacrylamide gel electrophoresis analysis was used to study the products at various conditions. As can be seen in Fig. S3, in the absence of nicking enzyme, the bright bands around at 40 pb represented the intact H1 strand, which indicated no enzymatic cleavage occurred (lane 6, lane 7, and lane 8). When the kanamycin and nicking enzyme were added, an obviously band that moving fast was appeared in lane 5, which suggested the machine 1 was triggered and plenty of dye-labelled fragments produced. In contrast, in the absence of H 1, there was no digestion product appeared even in presence of H 2 (lane 2). For the cascade DNA machines, in the absence of kanamycin, there were only the bands of intact H 1 and H 2 and no digestion products was observed, which illustrating no cross-talk reaction between the machine 1 and machine 2 (lane 3 and lane 4). Upon adding kanamycin, the cascade DNA machine was constructed and the corresponding band of digestion product appeared in lane 1. Moreover, due to the FAM-labelled for both H 1 and H 2, the band of digestion product at lane 1 was brighter than that of lane 5, which also demonstrate the occurrence of cascade reaction. These results illustrated that the nicking enzyme powered cascade DNA machine had been successfully prepared and were also demonstrated the capability for the determination of kanamycin.

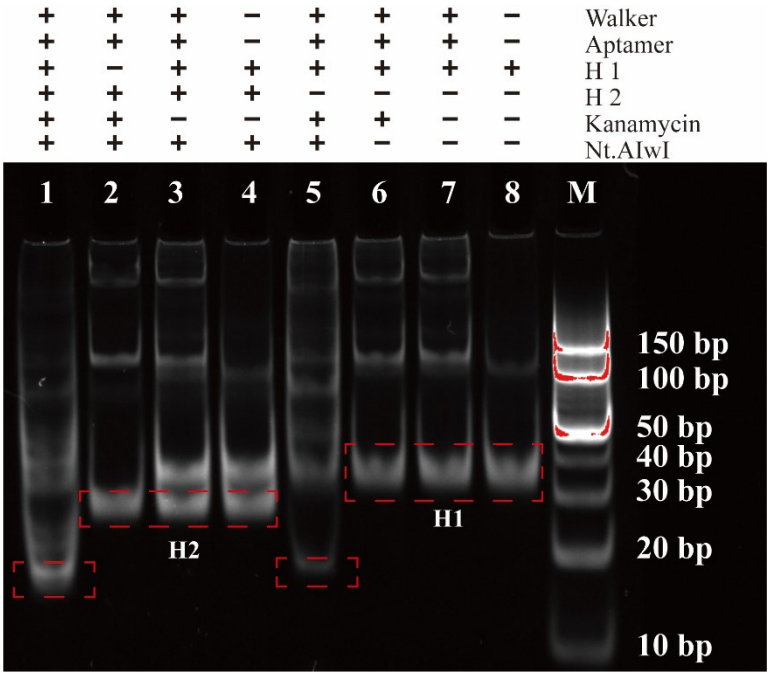

Fig. S3. Polyacrylamide gel electrophoresis analysis of the kanamycin detection under different conditions. Lane 1, a mixture of walker strand, aptamer, H1, H2, kanamycin, and nicking enzyme. Lane 2 and lane 3 were the mixture of that in lane 1 in the absence of H1 and kanamycin, respectively. Lane 4, a mixture of H1, H2 and nicking enzyme. Lane 5, a mixture of walker strand, aptamer, H1, kanamycin, and nicking enzyme. Lane 6, a mixture of walker strand, aptamer, H1, and kanamycin. Lane 7, a mixture of walker strand, aptamer, and H1. Lane 8, H1. The concentration of all nucleic acid strand, kanamycin, and *Nt.AIwI* were 1 μM, 1 μM and 10 U, respectively.

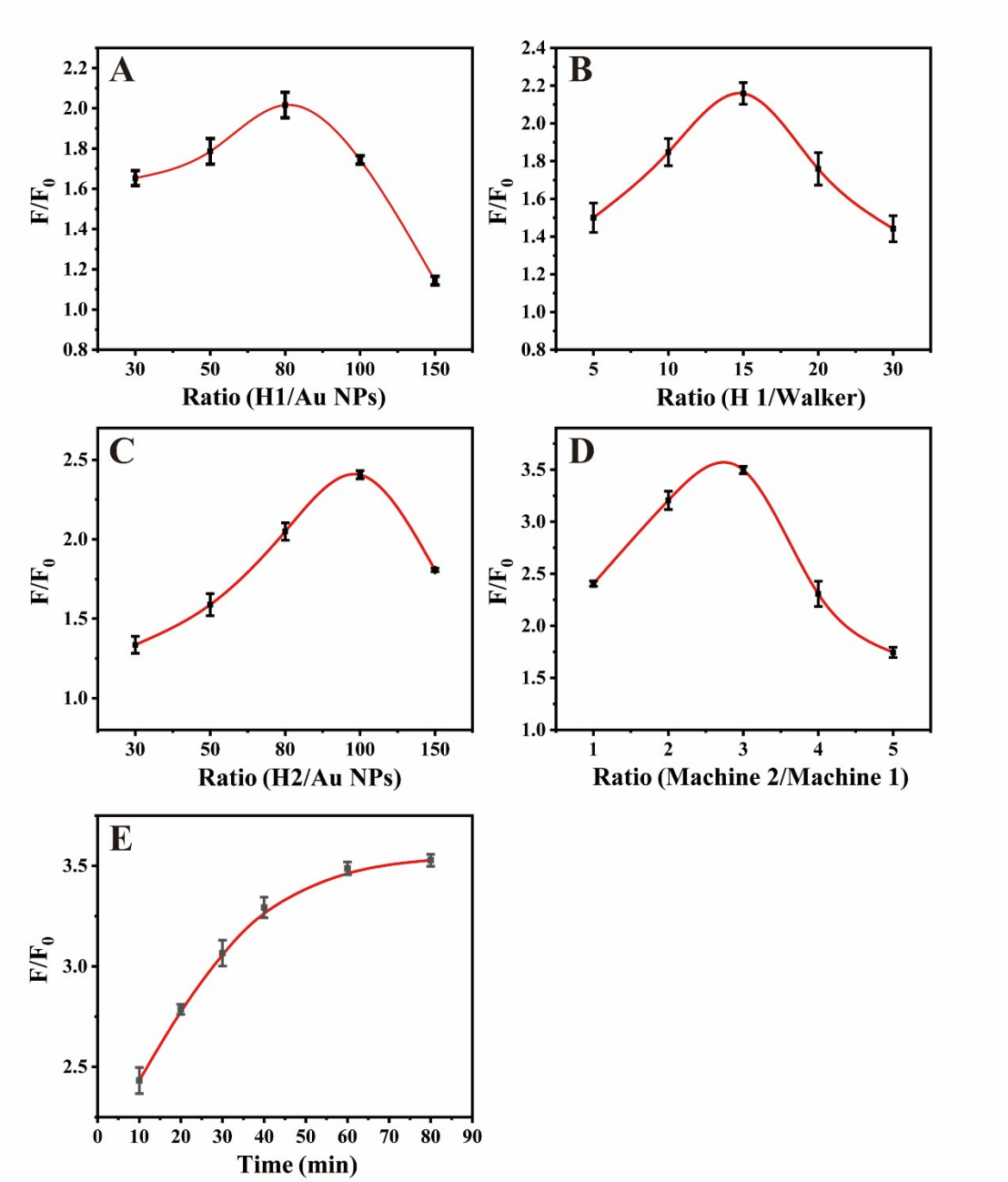

Fig. S4. The influence of molar ratio of H1 to Au NPs (A) and H1 to walking strand (B) for the machine 1. Effects of molar ratio of H2 to Au NPs (C), machine 2 to machine 1 (D), and the reaction time of cascade DNA machines for the detection of kanamycin. The concentration of kanamycin and *Nt.AIwI* were 0.5 nM and 10 U, respectively.

Table S2. Comparison of the cascade signal amplification strategies for kanamycin detection.

Methods	Signal amplification strategy	Dynamic Range	Limit of detection	Detection time (min)	Ref.
Fluorescence	Exo I-HCR	2-5000 pM	1.2 pM	280	**1**
Electrochemistry	SDA-HCR	0.05-200 pM	36 fM	370	**2**
Electrochemistry	Exo-III-DNA walker	10 fM-100 pM	7.1 fM	240	**3**
Fluorescence	DNA walker-HCR	5 pM-100 nM	1.01 pM	150	**4**
Electrochemistry	SDA- Exo-III	100 fM-1 nM	83 fM	270	**5**
Electrochemistry	SDA- Exo-III	5 fM-100 pM	1.3 fM	250	**6**
Electrochemistry	SDA-HCR	0.05 pM-50 nM	16 fM	110	**7**
Fluorescence	Cascade DNA walker	0.5-5000 pM	28 fM	60	This work

Exo: Exonuclease; *HCR*: Hybridization Chain Reaction; *SDA*: Strand Displacement Amplification

Table S3. Recovery of kanamycin in spiked milk samples.

Samples	Added (nM)	Founded (nM)	Recovery (%)	RSD (%, n=3)
1	0.2	0.208	104.40	9.61
2	2	2.124	106.20	7.12
3	20	20.008	100.04	4.88

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