Electronic Supplementary Material (ESI) for ChemComm.
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The Single Strand Template Shortening Strategy Improves the Sensitivity and Specificity of Solid-State Nanopore Detection

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Methods

1. Chemicals and materials.

The reagents, LiCl and formamide, were acquired from Sigma-Aldrich (St. Louis, MO, USA). The ZIKV Trigger 27B plasmid was procured through Addgene (USA, #141856). $MgSO_4$, 1×TE, and Acryl/Bis 40% Solution were sourced from Sangon Biotech (Shanghai, China). Unless specified otherwise, all primers and oligonucleotides utilized in this study were obtained from Sangon Biotech (Shanghai, China) and subjected to dPAGE purification. Primers incorporating Spacer18 and oligonucleotides with a fluorophore were purified using HPLC. Oligonucleotide sequences are concisely presented in Table S1.

2. Fabrication of nanopores.

Conical glass nanopores were fashioned from quartz glass capillaries (outer diameter: 1 mm, inner diameter: 0.7 mm, QF100-70-10; Sutter Instrument Co.). All glass capillaries employed in the experiments underwent thorough cleaning by immersion in freshly prepared piranha solution (3:1 98% H_2SO_4/30% H_2O_2) for approximately 1 hour to eliminate organic impurities. (Caution: piranha solution is a potent oxidizing agent and reacts vigorously with organic compounds. It should be handled with utmost care.) Subsequently, the capillaries were meticulously rinsed with deionized water and subjected to vacuum drying at 70°C before utilization. The glass nanopores were then created using a CO_2-laser-actuated pipette puller (model P-2000, Sutter Instrument Co.) following a single-line program encompassing the parameters: HEAT = 760, FIL = 4, VEL = 31, DEL = 120, PUL = 175.

3. Data collection and analysis

The nanopores were integrated into custom-designed horizontal glass cells for DNA translocation experiments. The cell served as the cis reservoir, while the inner cavity of the glass capillary nanopore functioned as the trans reservoir. Two chlorinated silver electrodes were positioned in each reservoir. A potential was applied to the electrode inside the nanopore. The DNA sample was introduced into the cis reservoir (located outside the nanopore tip), designated as the electrical ground. Ion currents were recorded using a current amplifier, Axopatch 200B (Molecular Devices), equipped with a low-pass Bessel filter set at 5 kHz and digitized using a DigiData 1550B digitizer (Molecular Devices) at a sample rate of 250 kHz. All experiments were conducted at a temperature of 25°C. The current signals were processed using Clampfit 11.2 software (Molecular devices).

4. Experiments

4.1 Nanopore reproducibility testing

We employed electrochemical measurements to estimate the diameter of the nanopores using the classical Equation. The current data within the range of -100 mV to 100 mV was selected due to the proximity of the linear and rectification ratio to 0 in this interval. The equation utilized for this estimation is expressed as: $a = \frac{1}{\Pi \mathrm{kR} tan\theta/2}$

R represents the measured pipette resistance

k denotes the specific resistance of the electrolyte used (k=1.2S/m in 0.1 M KCl)

θ signifies the cone angle (θ =12°),

a stand for the radius of the nanopore at the tip of the nanopipette.

4.2 Synthesis of circular DNA

A mixture composed of 10 μL of 5' end phosphorylated blocking probe, 15 μL of ligation sequence, 10 μL of 10× T4 buffer, and 63 μL of ddH_2O was prepared and annealed at 95°C for 5 minutes. The annealed mixture was then gradually cooled to 25°C at a rate of 0.1°C/s. Following cooling, 2 μL of T4 DNA Ligase was added to initiate the ligation reaction, which was incubated at 25°C for 2.5 hours. The resulting product was purified and ultimately diluted to a concentration of 3.5 μM. Additionally, 2 μL of T4 DNA Ligase was introduced to a similar cooled mixture for a ligation reaction, and this second reaction was also incubated at 25°C for 2.5 hours. The incubated product was subsequently purified and further diluted to generate a 3.5 μM ring stock solution for future use.

4.3 Rolling circle amplification (RCA)

2 μL of 3.5 μM ring stock solution, 1 μL of 10 mM primer, 1.25 μL of 10 mM dNTP, 5 μL of 10×phi29 buffer (50 mM Tris-HCl, 10 mM MgCl2, 10 mM $(NH_4)_2SO_4$, 4 mM DTT, PH=7.5), and 38.25 μL of ddH_2O should be mixed. Then, anneal the mixture for 5 minutes at 95 ℃. After annealing at 95°C for 5 minutes, the mixture was cooled to 25 °C at a rate of 0.1 °C/s for use. Add 1 μL of phi29ase, 1 μL of Ppase, and 0.5 μL of 100 x BSA to the cooled mixture and perform the amplification reaction at 30°C for 1 hour.

4.4 Gel and nanopore detection

During agarose gel characterization, combine 10 μL of the amplified product with 2 μL of 6× DNA Loading Buffer. Subsequently, apply the mixture to a 2% agarose gel for electrophoresis, conducting the electrophoresis at 120 V for 45 minutes, followed by observation under UV light.

During end-point nanopore testing, take 6 μL of the resulting product, along with 16 μL of 4 M LiCl, 8 μL of 20% v/v formamide, and 10 μL of 1× TE (10 mM Tris-HCl and 1 mM EDTA, pH = 8.0). Combine these components to yield a 40 μL nanopore sample test solution. Add the appropriate volume of the sample test solution to the sample cell for nanopore detection. The nanopore sample test solution is introduced into the sample cell at

the cis end. The reaction is conducted at 25°C. The wells are purged with a voltage-driven (bias: 600 mV, direction: cis to trans) buffered electrolyte for 5 minutes between each sample test.

4.5 Versification of the AcyNTP blocking effect

The AcyNTP powder utilized in the reaction was diluted to 10 mM with water and stored in the refrigerator at -20°C. For preparation, 2 μL of a 3.5 μM ring stock solution, 1 μL of a 10 mM primer, 1.25 μL of a 10 mM dNTP, 1.25 μL of a 10 mM AcyNTP, 5 μL of 10× phi29 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT, pH = 7.5), and 37.25 μL of ddH_2O were combined to form a 47.5 μL reaction solution at 95°C. Subsequently, $(NH_4)_2SO_4$, 4 mM DTT, pH = 7.5, and 37.25 μL of ddH_2O were mixed to create an additional 47.5 μL reaction solution. The two solutions were annealed at 95°C for 5 minutes and cooled to 25°C at a rate of 0.1°C/s. Following this, 1 μL of phi29ase, 1 μL of Ppase, and 0.5 μL of BSA were added to the cooled mixture, and the reaction was carried out at 30°C for 1 hour. 10 μL aliquot of the product was mixed with 2 μL of 6× DNA Loading Buffer and subsequently applied to a 2% agarose gel for electrophoretic detection. The resulting products were electrophoresed at 120 V for 45 minutes and visualized under UV light.

4.6 Optimization of reaction conditions

We systematically explored multiple combinations of reaction time and AcyNTP mixing concentrations, employing diverse time gradients ranging from 3 minutes to 60 minutes and AcyNTP to dNTP mixing ratios spanning from 1:64 to 6:1. Throughout the optimization of reaction conditions, a dual analytical approach involving gel electrophoresis and nanopore characterization was concurrently applied to analyze the resultant products. The detailed reaction conditions are outlined in **Table S2**. Eventually, we chose a reaction time of 20 minutes and an AcyNTP mixing ratio of 2:1 as our single-stranded DNA generation system.

4.7 Detection of miRNA

2 μL of 3.5 μM ring stock solution, 1 μL of 10 μM miRNA, 1.25 μL of 10 mM dNTP, 2.5 μL of 10 mM AcyNTP, 5 μL of 10×phi29 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT, PH=7.5), and 35.75 μL of ddH_2O should be mixed. Then, anneal the mixture for 5 minutes at 95 ℃. After annealing at 95 °C for 5 minutes, the mixture was cooled to 25 °C at a rate of 0.1 °C/s for use. Add 1 μL of

phi29ase, 1 μL of Ppase, and 0.5 μL of 100 x BSA to the cooled mixture and perform the amplification reaction at 30 °C for 20 minutes.10 μL aliquot of the product was mixed with 2 μL of 6× DNA Loading Buffer and subsequently applied to a 2% agarose gel for electrophoretic detection. The resulting products were electrophoresed at 120 V for 45 minutes and visualized under UV light.

4.8 Preparation and characterization of structure tags

All pertinent sequences were appropriately diluted in 1×ST buffer (20 mM Tris-HCl and 50 mM $MgSO_4$, pH=8.0) for experimental use. The samples consisted of 10 μL 2.8 μM A26, 2.8 μM B26, 2.8 μM C26, and 2.8 μM D26, resulting in a total volume of 40 μL for the tetrahedral DNA structure reaction. Subsequently, the individual mixtures were annealed at 95°C for 10 minutes and gradually cooled to 4°C at a rate of 0.1°C/s. For further analysis, 10 μL of the tetrahedral structure products were combined with 2 μL of 6× gel loading buffer. The resulting mixture was added to 8% or 10% native PAGE and prepared with 1×TBE buffer. The native PAGEs were run at 350 V for 1.5 hours and subjected to visualization under UV light after a 20-minute immersion in SYBR Gold.

To prepare the reaction solution, 25 μL of buffer, 2 μL of FP, 2 μL of RP, and 6.5 μL of H_2O were added to the RPA kit, ensuring complete dissolution of the kit powder. After dissolution, 25 μL of MgOAc was added to the sample tube cap (note: MgOAc should not be mixed with the solution before adding the template). Subsequently, 2 μL of 10 ng/μL Zikv Trigger 27B plasmid was introduced into the sample tube. The MgOAc was then mixed with the reaction solution. The reaction was carried out at 37°C for 30 minutes, followed by a cooling step to 4°C at a rate of 0.1°C/s for preservation. The assembled product was characterized using 1% agarose gel electrophoresis. For electrophoresis, 10 μL of the double-stranded product was combined with 2 μL of 6× DNA Loading, and the mixture was subjected to electrophoresis for 30 minutes at 120 V. Post-electrophoresis, observation was conducted under UV light.

4.9 Binding of the structure tag to single strand templates and nanopore characterization

After the preparation of the polymerized products and the tetrahedral DNA structures are completed. For the first reaction, combine 25 μL of single-stranded template DNA, 10 μL of tetrahedron, 40 μL of FNA buffer, and 5 μL of 10× TNaK. Incubate the mixture at 37°C for 2 hours. Subsequently, take 16 μL of the incubation solution and add 8 μL of formamide (100%) and 16 μL of 10 M LiCl. This creates a 40 μL nanopore test

reaction solution. Add the appropriate volume of the test reaction solution to the sample cell for nanopore detection.

For the second reaction, mix 20 μL of single-stranded template DNA, 20 μL of double-stranded substrate, 40 μL of FNA buffer, and 5 μL of 10× TNaK. Incubate the mixture at 37°C for 2 hours. Similarly, combine 16 μL of the incubation solution with 8 μL of formamide (100%) and 16 μL of 10 M LiCl. This forms a 40 μL nanopore test solution. Add the appropriate volume of the test solution into the sample cell for nanopore detection.

5. Supporting Figures and Tables

When the transit time of the product through the nanopore is less than one sampling period, the resulting signal needs to be more significant to be captured and contribute to current drop data. It generates a current signal only when the product is sufficiently large to exceed the sampling period.

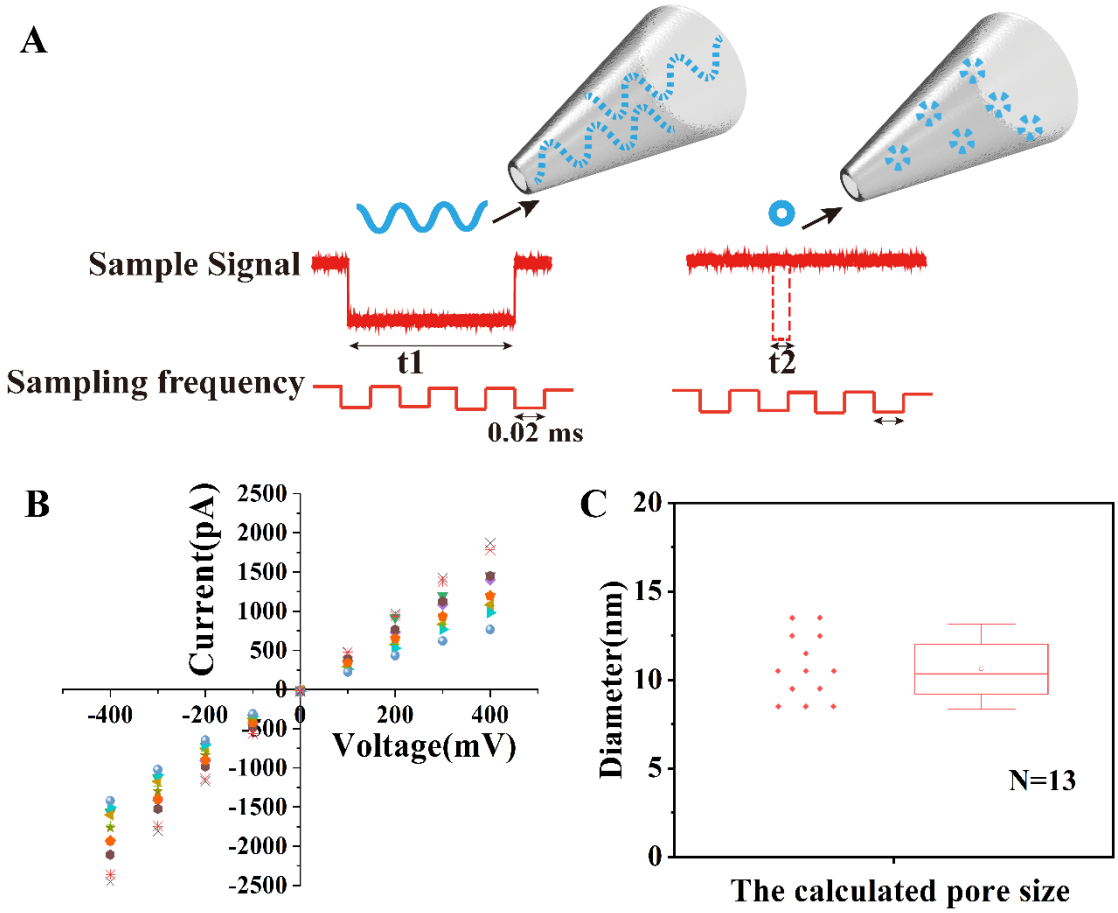

Figure S1. (A) Principal schematic of Nanopore Detection of RCA Products. (B) I-V characteristics of conical glass nanopores in 0.1 M KCl (N=14). (C) Calculated nanopore diameters are 11nm±1.5nm. Note: The reproducibility of nanopore combined with the calculation method as 4.1 described.

The altered sugar ring in AcyNTP is responsible for its blocking effect. Due to the absence of a complete pentose structure, it cannot form a phosphodiester bond with the next nucleotide after it binds to the DNA strand, thus interrupting the process of DNA synthesis.

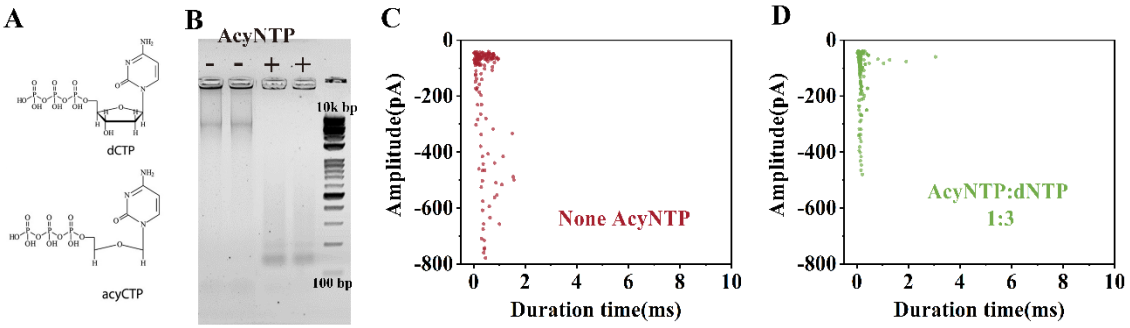

Figure S2. (A) Structural formulas of AcyCTP and dCTP. (B) The blank sample without AcyNTP and mixed with AcyNTP are characterized by 2% gel electrophoresis. (C) Scatter plot of perforations in a blank sample without AcyNTP. (D) Scatter plot of perforation of sample mixed with AcyNTP. Note: The scatter plots are obtained from recording with the same pore for 10 minutes at 600 mV.

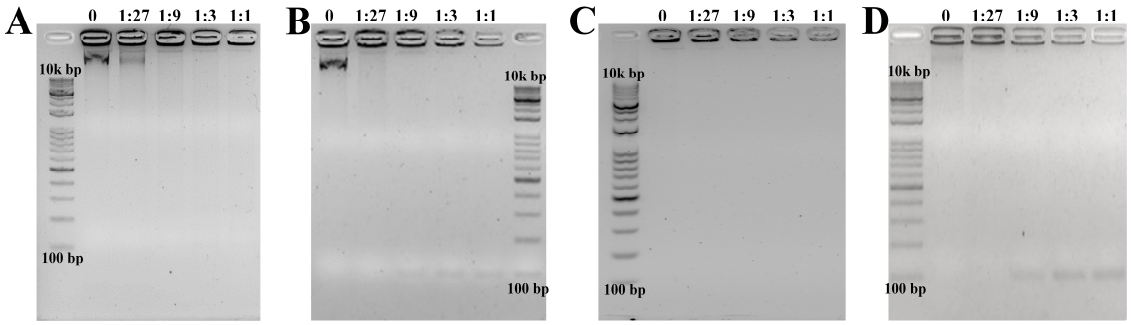

Figure S3. Different AcyNTP mixing ratios at different reaction times are characterized by 2% gel electrophoresis. (A) 60 min. (B) 30 min. (C) 20 min. (D) 10 min.

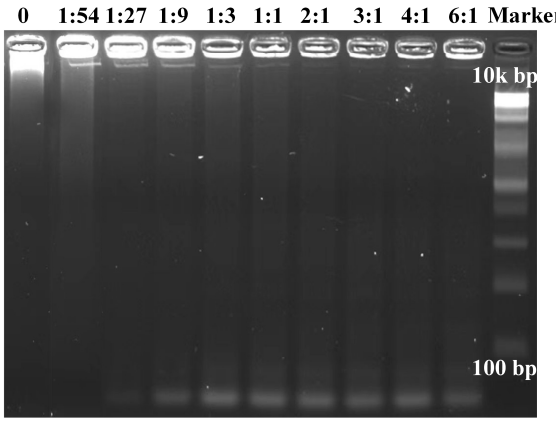

Figure S4. Different AcyNTP mixing ratios at 60 minutes are characterized by 2% gel electrophoresis.

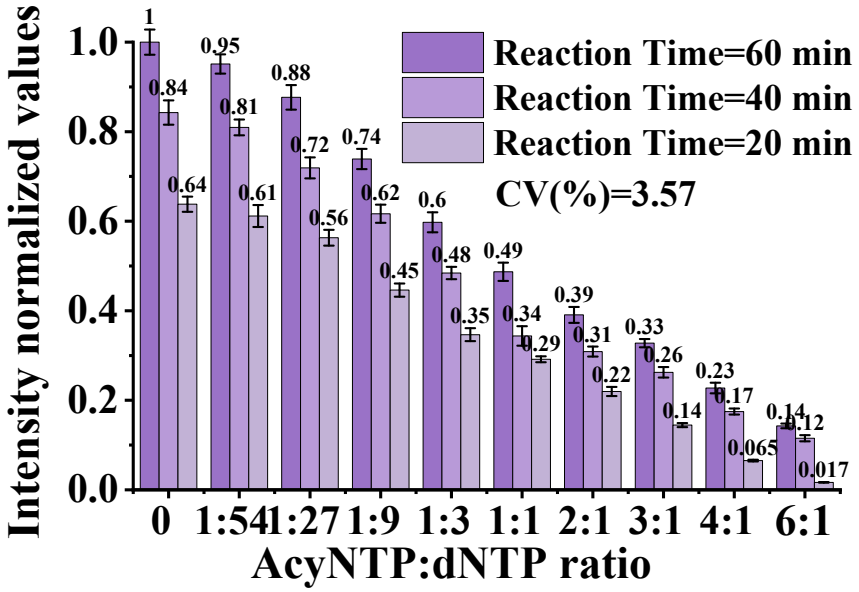

Figure S5. Statistical charts standardizing product concentrations from different samples with different reaction times. Note: The intensity of the product bands generated within a 60 minute reaction for the samples without added AcyNTP was used as a benchmark, and the intensity of the bands for the remaining samples was normalized.

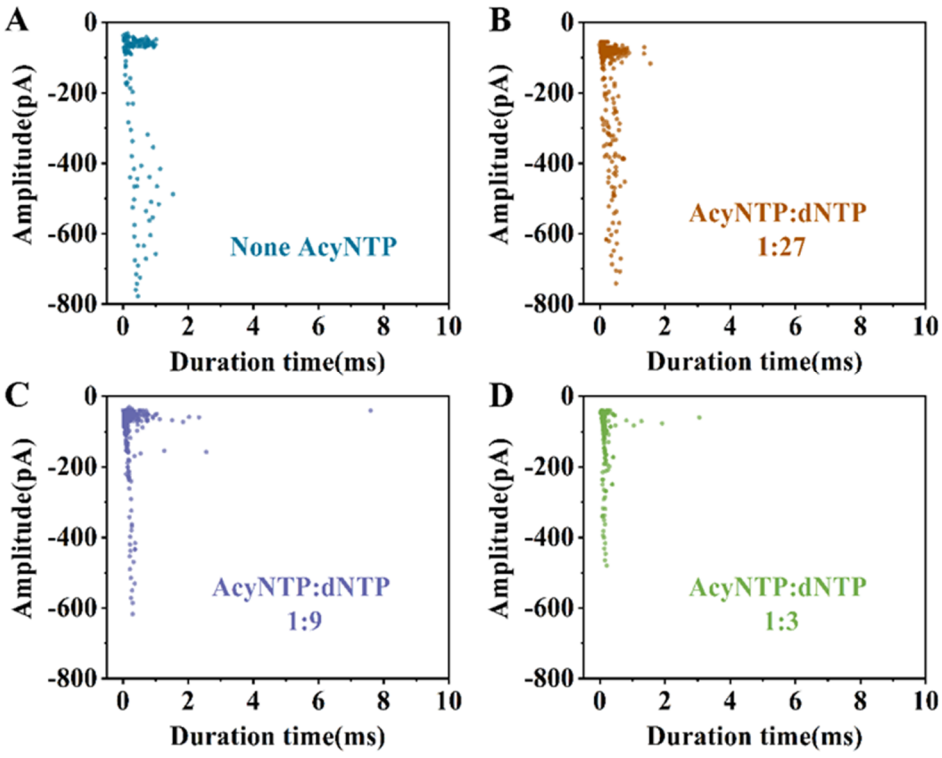

Figure S6. Nanopore scattering statistics for different AcyNTP to dNTP mixing ratios (A) 0 (B) 1:27 (C) 1:9 (D) 1:3. Note: The scatter plots are obtained from recording with the same pore for 10 minutes at 600 mV.

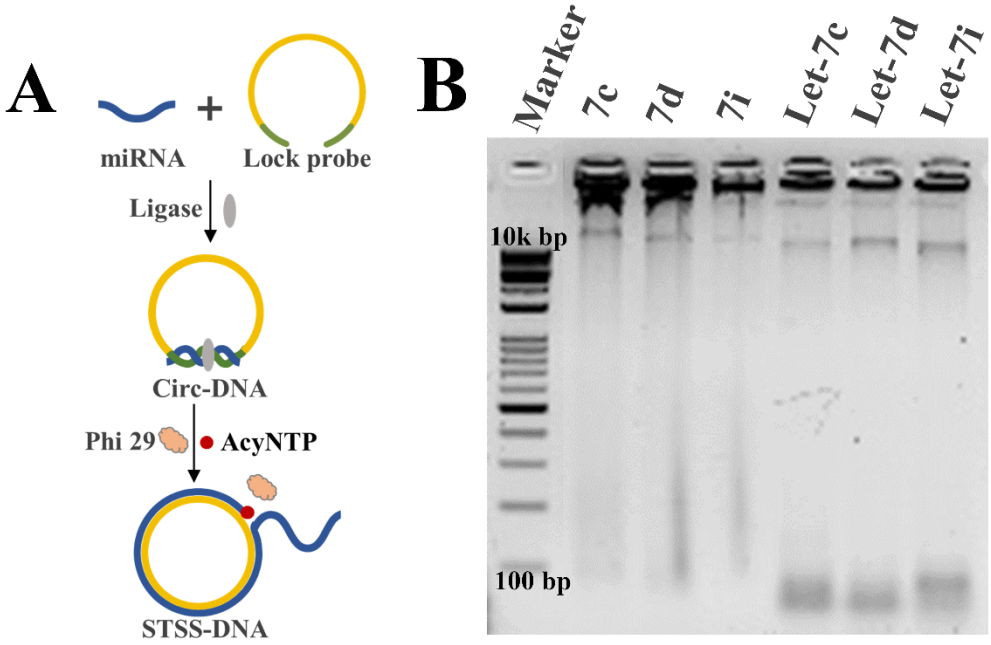

Figure S7. (A) Schematic illustration of miRNA detection employing STSS. (B) 2% gel electrophoresis of the products generated by ssDNA and miRNA. Note: 7c,7d, and 7i are ssDNAs that have the same sequence with Let-7c, Let-7d, and Let-7i.

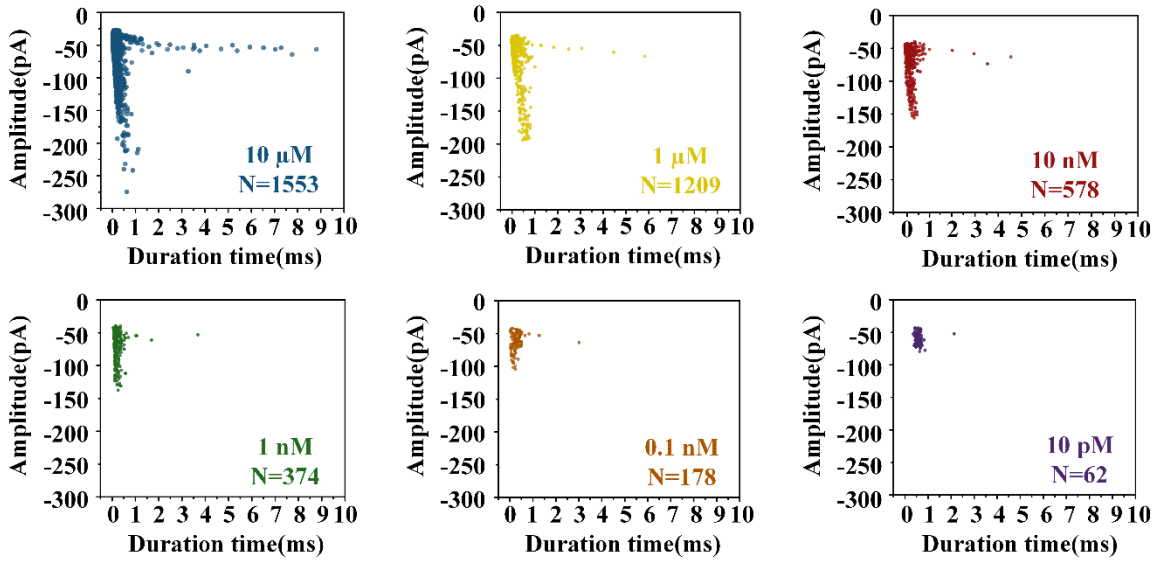

Figure S8. Nanopore scattering statistics for samples with different miRNA concentrations (A) 10 μM (B) 1 μM (C) 10 nM (D) 1 nM (E) 0.1 nM (F) 10 pM. Note: The scatter plots are obtained from recording with the same pore for 10 minutes at 600 mV.

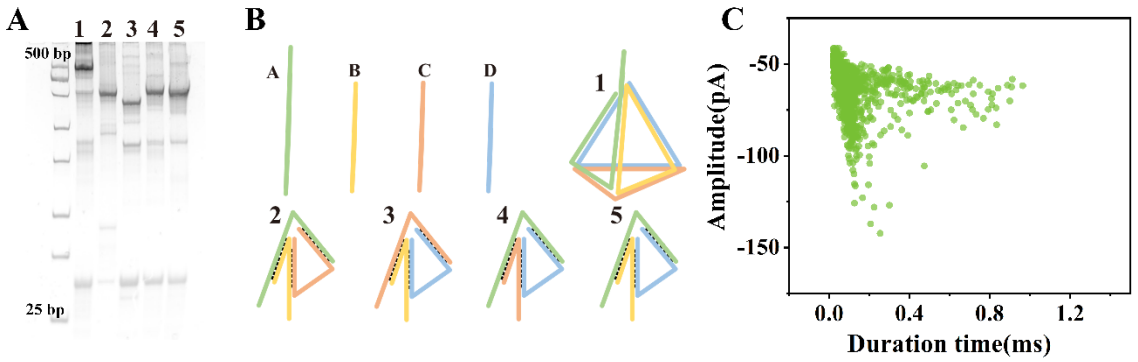

Figure S9. (A) Characterization of DNA tetrahedral self-assembly by 10% native PAGE electrophoresis lane 1: DNA tetrahedral nanostructures; lanes 2-5: hybridization products of the three DNA strands; the left lane is Marker (B)The structure of tetrahedron and three DNA strands (C) Scatterplot of tetrahedron perforated alone. Note: The scatter plots are obtained from recording for 5 minutes at 600 mV.

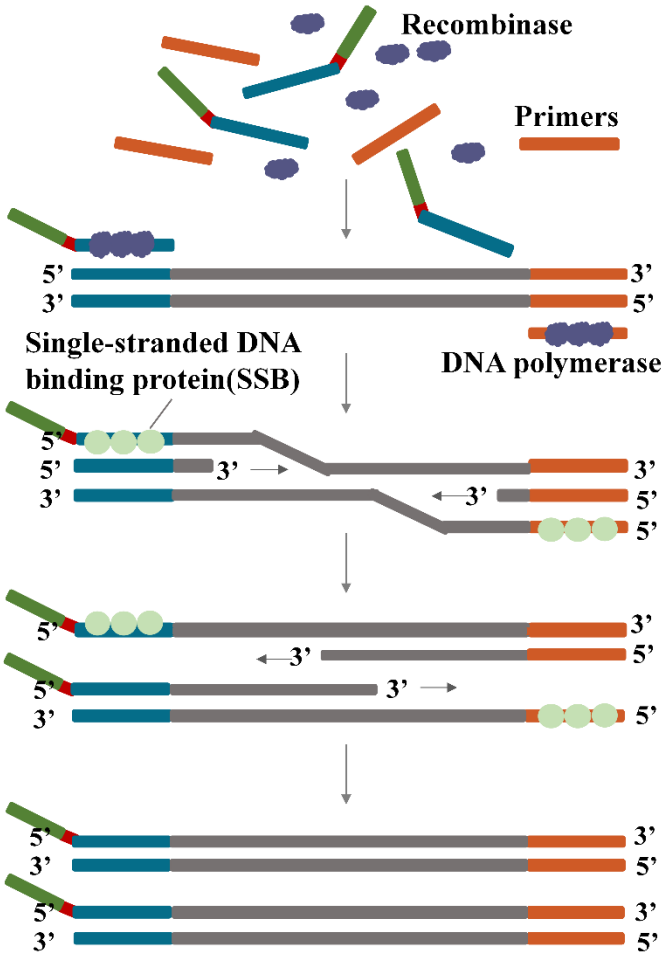

Figure S10. Principle schematic of RPA amplification

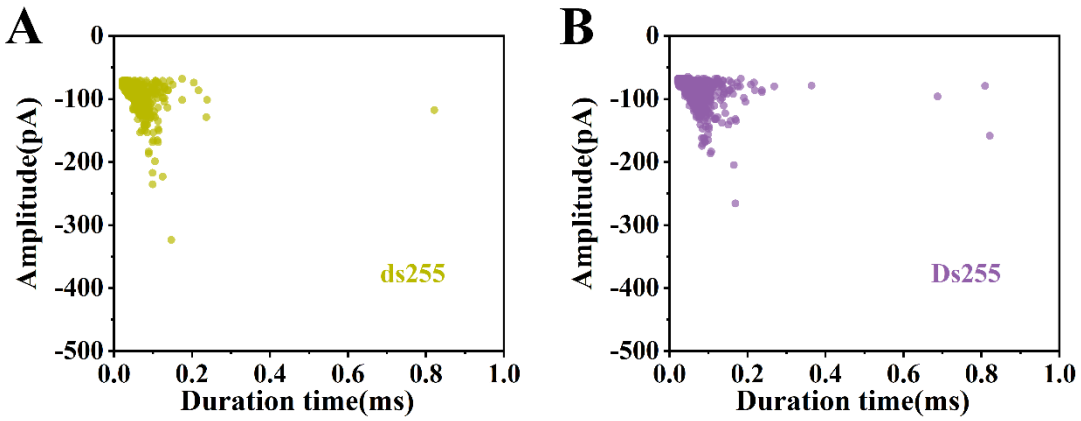

Figure S11. (A) Nanopore scattering statistics for ds255. (B) Nanopore scattering statistics for Ds255.

Note: The scatter plots are obtained from recording with the same pore for 5 minutes at 600 mV.

Table S1 Oligonucleotides used in this paper.

Name	Sequence 5'-3'	Lable and notes
	Sequence for generating ssDNA.	
RCA-Ct-5P	GGCGAAGACAGGTGCTTAGTCGAAAGATACCTGG GGGAGTATTGCGGAGGAAGGTTCAGATATC	
RCA-Lt	TGTCTTCGCCGATATCTGAACC	
RCA-primer	ATGTTGAGATATCACTTGGAAGGAGGCGT	
	Sequence for detecting miRNA.	
Let-7c	UGAGGUAGUAGGUUGUAUGGUU	
Let-7d	AGAGGUAGUAGGUUGCAUAGUU	
Let-7i	UGAGGUAGUAGUUUGUGCUGUU	
Let-7c	TGAGGTAGTAGGTTGTATGGTT	
Let-7d	AGAGGTAGTAGGTTGCATAGTT	the ssDNA sequence is same with the miRNA
Let-7i	TGAGGTAGTAGTTTGTGCTGTT	
Let-7c-5P`	CTACTACCTCAGTGCTTAGTCGAAAGATACCTGGG GGAGTATTGCGGAGGAAAACCATACAAC	
Let-7d-5P`	ATACTACCTCTGTGCTTAGTCGAAAGATACCTGGG GGAGTATTGCGGAGGAAAACTATGCAAC	
Let-7i-5P`	CTACTACCTCAGTGCTTAGTCGAAAGATACCTGGG GGAGTATTGCGGAGGAAAACAGCACAAA	
	Sequences for forming structure tag.	
FNA-A26	GCCTGGAGATACATGCACATTACGGCTTTCCCTAT TAGAAGGTCTCAGGTGCGCGTTTCGGTAAGTAGA CGGGACCAGTTCGCC	
FNA-A26`	[a|AAA|b]	The extra a

Name	Sequence 5'-3'	Lable and notes
	[AAAGATACCTGGGG|AAAAAAAAA|GCCTGGAGATACATGCACATTACGGCTTTCCCTATTAGAAGGTCTCAGGTGCGCGTTTCGGTAAGTAGACGGGACCAGTTCGCC]	sequence and RCA-Ct-5P are complimentary
FNA-B26	CGCGCACCTGAGACCTTCTAATAGGGTTTGCGACAGTCGTTCAACTAGAATGCCCTTTGGGCTGTTCCGGGTGTGGCTCGTCGG	
FNA-C26	GGCCGAGGACTCCTGCTCCGCTGCGGTTTGGCGAACTGGTCCCGTCTACTTACCGTTTCCGACGAGCCACACCCGGAACAGCCC	
FNA-D26	GCCGTAATGTGCATGTATCTCCAGGCTTTCCGCAGCGGAGCAGGAGTCCTCGGCCTTTGGGCATTCTAGTTGAACGACTGTCGC	
Linker	AAAGATACCTGGGG	
	Sequences for RPA amplification.	
255-FP	AGGCAGACATCTGTGAATCGCTTCACGACCACGCT [a|isp/isp|255-FP]	isp18 means the modification of Spacer18
255-FP`	[AAAGATACCTGGGG/iSp18//iSp18/AGGCAGACATCTGTGAATCGCTTCACGACCACGCT]	
255-RP	GATGCCGCATAGTTAAGCCAGTATACACTCCGCTA	

Table S2 The specific reaction conditions

AcyNTP: dNTP	Circ-DNA (μL)	Primer (μL)	10×phi29 buffer (μL)	dNTP (μL)	AcyNTP (μL)	ddH_2O (μL)	Polymerase (μL)
0	2	1	5	1.25	0.00	38.25	2.5
1:54					0.02	38.23	
1:27					0.05	38.20	
1:16					0.08	38.17	
1:9					0.14	38.11	
1:4					0.31	37.94	
1:3					0.42	37.83	
1:1					1.25	37.00	
2:1					0.63	37.63	
3:1					0.42	37.83	
4:1					0.31	37.94	
6:1					0.21	38.04	