Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

A netlike rolling circle nucleic acid amplification technique

Xiaoli Zhu, [a] *Chang Feng,* [a] *Bin Zhang,* [a] *Hui Tong,* [a] *Tao Gao,* [b] *Genxi Li,* *[a, b]

[a] Laboratory of Biosensing Technology, School of Life Sciences, Shanghai University, Shanghai 200444, P R China

[b]State Key Laboratory of Pharmaceutical Biotechnology, Department of Biochemistry, Nanjing University, Nanjing 210093, P R China. E-mail: genxili@nju.edu.cn

.

I. Materials and Methods

All oligonucleotides used in this research (Table S1) were synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China) with HPLC purification. Deoxynucleotide solution mixture (dNTPs) and 6 × loading buffer were also obtained from the TaKaRa Biotechnology. T4 DNA Ligase, Exonuclease I, Exonuclease Ⅲ, Nb. BsrDI nicking enzyme, Bst DNA polymerase large fragment, and their corresponding buffer were purchased from New England Biolabs Inc. SanPrep Column DNA Gel Extraction Kit was purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). SYBR Green I was from Solarbio Technology Co., Ltd. Low-profile 0.2 ml 8-tube strips for PCR were obtained from Bio-Rad Laboratories, Inc. 96-well plates (Costar) were obtained from Corning Inc.

Table S1. Sequences of Oligonucleotides

Oligonucleotides	Sequence (5'-3')
Circular Probe	CAC GCG ATC CGC ATG TGG AAA ATC TCT AGC AGT CCC ACC CTC CAA CCA CCA AGG CAA TGT ACA CGA ATT CGC CGA ACG
Padlock Probe	TCT CTA GCA GTC CCA CCC TCC AAC CAC CAA GGC AAT GTA CAC GAA TTC GCC GAA CGC ACG CGA TCC GCA TGT GGA AAA
Target HIV-1 DNA (T)	ACT GCT AGA GAT TTT CCA CAT
1-base mutation of T (mT-1)	ACT GCT AGA ***C***AT TTT CCA CAT
3-bases mutation of T (mT-3)	ACT G***G***T AGA ***C***AT TT***A*** CCA CAT
5-bases mutation of T (mT-5)	AC***A*** GCT ***T***GA ***C***AT ***A***TT C***G***A CAT

unmatched variant of T (uT)	AGT CTA GGA TTC GGC GTG GGT TAA
Primer 1 (P-1)	GCG GAT CGC GTG CGT TCG GC
1-base mutation of P-1 (mP-1)	GCG GAT CGC ***C***TG CGT TCG GC
3-bases mutation of P-1 (mP-3)	GCG GA***A*** CGC ***C***TG CGT T***G***G GC
5-bases mutation of P-1 (mP-5)	GC***C*** GA***A*** CGC ***C***TG C***C***T T***G***G GC
unmatched variant of P-1 (uP)	GTA GTC GTG ATG AAC GTA TG
Primer 2 (P-2)	ACC AAG GCA ATG TAC ACG AAT TC

Isothermal amplification reactions were performed on a ThermoStat plus (Eppendorf China Ltd.). Gel electrophoresis was conducted using a Wide Mini-Sub Cell GT Cell and a Gel Doc XR Imaging System (Bio-Rad, US). Fluorescence intensity was recorded at a constant temperature of 65 °C with an interval of 1 min using a CFX96TMTouch Real-Time PCR Detection System (Bio-Rad, US). The products of NRCA, HRCA, and LRCA were also observed directly by an ex situ Agilent 5500 atomic force microscope system (Santa Clara, CA). Fluorescence intensity was measured using a SpectraMax M3 Multi-Mode Microplate Reader with SoftMax Pro software (Molecular Devices,Sunnyvale, CA, USA).

Circularization of padlock probe. The padlock probe was allowed to be ligated to form circular probe in the presence of the target HIV-1 DNA. The reaction was carried out in a 20 μL aqueous solution containing 1 × polymerase buffer, 1 mM ATP, 100 U of T4 DNA ligase, 100 nM padlock probe, and different concentrations of target DNA. The mixture was incubated at 16 ℃ for 30 min to allow the proceeding of the ligation, and then 65 ℃ for 20 min to inactivate the enzyme. Mutant variants of the target (mT-1, -3, -5, and uT, sequences shown in Table S1) were also adopted

instead of the target DNA to work as controls. After ligation, a further exonuclease treatment is either conducted or not. In the exonuclease treatment, 10 U Exonuclease I and 50 U Exonuclease III were added into the ligation solution and incubated for 30 min at 37 ℃, followed by inactivation of the enzymes at 80 ℃ for 20 min.

NRCA reaction. The NRCA reaction was conducted in a 30 μL reaction mixture containing 1 × polymerase buffer, 0.1 fM ~ 1 μM primer 1 (fixed at 1 μM while padlock probe was adopted), 1 μM Primer 2, 100 nM circular probe or 10 μL of the products from the circularization of padlock probe, 400 μM dNTPs, 10 U Nb. BsrDI nicking enzyme, and 8 U Bst. DNA polymerase. The reaction was allowed to proceed at 65 ℃ for 60 min and terminate by deactivating the enzymes at 95 ℃ for 10 min. Mutant variants of the primer 1 (mP-1, -3, -5, and uP, sequences shown in Table S1) were employed instead of the primer 1 to be involved into the amplification reaction as controls. In the case of HRCA, Nb. BsrDI was removed from the reaction system, while other conditions were kept unchanged. As for LRCA, Primer 2 and Nb. BsrDI were both removed.

Gel electrophoresis analysis. Agarose gel electrophoresis was performed for the characterization of the products of NRCA. 5 μL of the products of NRCA, or HRCA, or LRCA together with 1 μL 6 × loading buffer was loaded onto a 2% non-denaturing agarose gel. The electrophoresis experiments were carried out in 1 × Tris-acetate-EDTA (TAE) at 80 V for 30 min. Subsequently, the gel was stained with SYBR

Green I for 30 min. The imaging of the gel was performed using a Gel Doc XR Imaging System.

Polyacrylamide gel electrophoresis was also performed for the characterization of the circularization of padlock probe. 10 μL of a sample together with 2 μL 6 × loading buffer was loaded onto a 10% non-denaturing polyacrylamide gel. The electrophoresis experiments were carried out in 1 × Tris- boric acid-EDTA (TBE) at 120 V for 90 min. Subsequently, the gel was stained with SYBR Green I for 30 min. The imaging of the gel was performed using the Gel Doc XR Imaging System.

Visualization of NRCA products. The products of NRCA, HRCA, and LRCA were firstly separated using gel electrophoresis to exclude the interference of enzymes, dNTPs, and etc. DNA fragments with a length of 2000~3000 bp were extracted from the agarose gel by the SanPrep Column DNA Gel Extraction Kit. Subsequently, the DNA fragments dissolved in 10 mM $MgCl_2$ were spread onto a fresh mica slice (Yunfeng Co. Ltd., China) and allowed to incubate for 20 min under room temperature. The mica slice was then rinsed with double distilled water and blown completely dried with nitrogen gas. Morphological analysis of the RCA products on the mica slice was achieved by using an ex situ Agilent 5500 AFM system. Samples were imaged at a scan rate of 0.5-1 Hz in a tapping mode. AFM tips with resonant frequency in a range 160-260 kHz were used. Images were acquired at a resolution of 512×512 pixels.

Fluorescent detection. Real-time fluorescent signals (SYBR Green I, $\lambda_{excitation}$: 470 nm, $\lambda_{emission}$: 520 nm) of the NRCA system were recorded at 65 ℃ with an interval of 1 min by using a CFX96^{TM} Touch Real-Time PCR Detection System. NRCA reaction was conducted as described above with the only difference of the addition of 1 × SYBR Green I. In comparison, the Real-time fluorescent signals of HRCA, and LRCA were also collected.

For the detection of the target HIV-1 DNA, Fluorescence spectrum analysis was adopted. 30 μL of NRCA products and 1 μL of 20 × SYBR Green I were added into 70 μL of double distilled water, followed by incubation at room temperature for 10 min. Fluorescence spectra were obtained using a SpectraMax M3 Multi-Mode Microplate Reader. The excitation wavelength was 470 nm; and the spectra were measured in the range from 500 nm to 650 nm. The fluorescence intensity was recorded at the emission wavelength of 520 nm.

II. Supporting Figures and Discussions

Optimization of experimental conditions. Because there are two enzymes (i.e. Bst DNA polymerase and Nb. BsrDI nicking enzyme) involved in the system, a favorable integrated buffer solution, in which both enzymes may work well, should be firstly tested. We have thus studied the optimal buffer by mixing the commercial Bst buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8) and Nb. BsrDI buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.9) in different ratios. The two enzymes, as well as the two primers (primer 1 and 2), circular probe, and dNTPs, are incubated in the buffer to allow the NRCA to proceed (sequences of all the oligonucleotides shown in Table S1). As can be seen from the lane 8-13 of Figure S1, in the cases of all the ratios from Bst buffer : Nb. BsrDI buffer = 5 : 0 to 0 : 5, smear ladder-like bands typical for RCA products are observed.[1] However, to our great surprise, if only dNTPs and the two enzymes are present, ladder-like bands are also observed (lane 3-7 in Figure S1). After a literature survey, we know that similar phenomenon has also been discovered by Frank-Kamenetskii et. al., so template/primer-independent DNA synthesis may occur in the presence of a polymerase together with a restriction endonuclease.[2] A highly repetitive palindromic sequence may be produced under such circumstances. Though the phenomenon is of great interest and the mechanism how the DNA synthesis initiates without any template and primer still remains unclear, the template/primer-independent DNA synthesis is undesirable here for our development of NRCA. Fortunately, the template/primer-independent DNA synthesis shows a buffer dependent manner. In the case of Bst buffer : Nb. BsrDI buffer = 5 : 0 (lane 2 in Figure S1), no ladder-like band is observed. So, we employ this ratio, i.e., the Bst buffer only as the optimal buffer condition for our following experiments. In the meantime, we should mention that no further discussion on the template/primer-independent DNA synthesis is given anymore in the following text, since that is not the key point of this work.

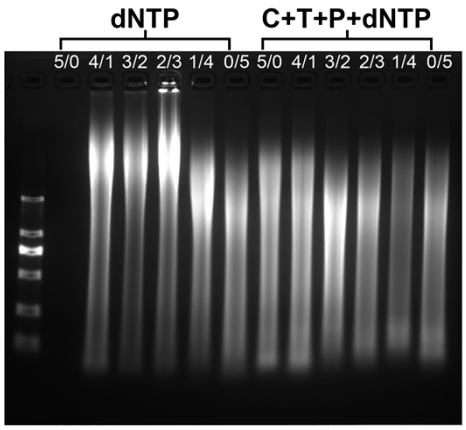

Figure S1. Agarose gel electrophoretic patterns of the products of NRCA. The NRCA has been conducted by using different buffer solutions: a mixture of Bst buffer and Nb. BsrDI buffer with different ratios from 5 / 0 to 0 / 5. Lane 1: marker; Lane 2-7: only dNTPs and two enzymes (Bst. DNA polymerase and Nb. BsrDI) are present; Lane 8-13: all the materials including circular probe, two primers, dNTPs, and the two enzymes are present.

We next study the NRCA reaction time, which has been depicted in Figure S2. Experimental results show that the size of the NRCA products becomes larger over time, representing the ladder-like bands move upward to the well. After a reaction time of 200 min, the produced DNA fragments become so large that most of them stay in the well and cannot enter into the gel. In order to facilitate the observation of the NRCA products by gel electrophoresis, we choose 60 min as the optimal reaction time. At this time, distinct uniform ladder-like bands can be observed. As for the reaction temperature otherwise, because the two enzymes share the same optimal temperature at 65 °C, we conduct the NRCA reaction under this temperature without further testing.

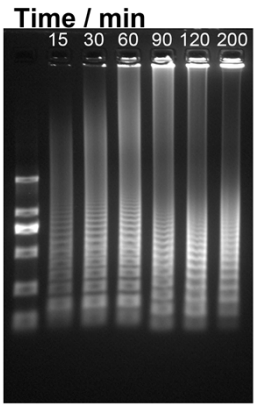

Figure S2. Agarose gel electrophoretic patterns of the products of NRCA. The NRCA reaction has proceeded for 15, 30, 60, 90, 120, and 200 min, respectively.

Amplification efficiency of NRCA. Here we study the amplification efficiency of NRCA by comparing it with LRCA and HRCA. Figure S3 shows the agarose gel electrophoretic patterns of the products of NRCA and HRCA. Different concentrations of the first primer DNA (primer 1) from 10 nM to 1 μM are adopted. Apparently, except the lanes with 1 μM of the primer 1, the bands of NRCA products are much brighter than that of HRCA in all other lanes. Especially when the concentration of the primer 1 is set at 10 nM, clear ladder-like band is observed in NRCA whereas no bands exist in HRCA. Since the reaction time for NRCA and HRCA is the same (60 min), the result suggests that NRCA has higher amplification efficiency in contrast to HRCA. As for the exception at 1 μM of the primer 1, there is no remarkable difference because of the saturation of both amplified products.

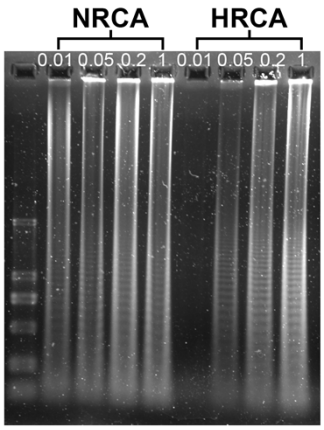

Figure S3. Agarose gel electrophoretic patterns of the products of NRCA. Comparison of NRCA (Lane 2-5) with HRCA (Lane 6-9). Different concentrations of the primer 1 from 10 nM to 1 μM was adopted.

Application in the detection of a HIV-1 DNA. In experiments, polyacrylamide gel electrophoresis is first employed to characterize the circularization of the padlock probe. As is shown in Figure S4, the target DNA as well as some mutant variants is adopted to hybridize with the padlock probe. After the ligation by a T4 DNA ligase, a clear band for the hybridization of the target DNA with the circularized probe is observed. After further treatment by exonuclease I and III, circular probe only is retained (Figure S5). However, it is noticed that similar bands are also observed in the case of 1-base (mT-1) and 3-bases (mT-3) mutations of the target DNA.

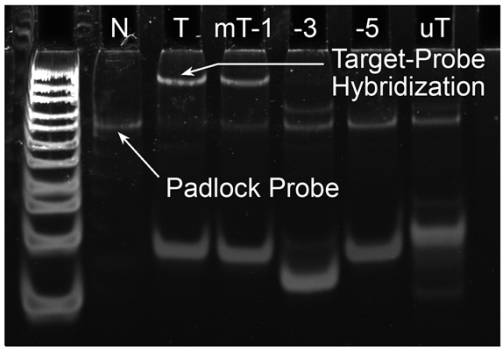

Figure S4. Ligation of the padlock probe to form circular probe. Polyacrylamide gel electrophoretic patterns of the hybridization of the target DNA as well as some mutant variants with the padlock probe in the presence of ligase. The symbol ″N″, ″T″, ″mT-1″, ″-3″, ″-5″, and ″uT″ stand for no target DNA, target DNA, 1, 3, 5-base(s) mutant and unmatched variants,

respectively.

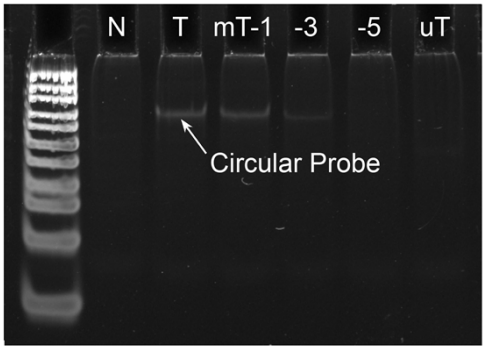

Figure S5. The samples in S4 were further treated by exonuclease I and III.

References

1 (a) D. Liu, S. L. Daubendiek, M. A. Zillman, K. Ryan and E. T. Kool, *J. Am. Chem. Soc.*, 1996, **118**, 1587-1594. (b) H. Kuhn, V. V. Demidov and M. D. Frank-Kamenetskii, Nucleic Acids Res., 2002, 30, 574-580.

2 X. Liang, K. Jensen and M. D. Frank-Kamenetskii, Biochemistry, 2004, 43, 13459-13466.