Electronic Supplementary Information for Analyst

Label-free fluorometric detection of influenza viral RNA by strand displacement coupled with rolling circle amplification†

Hyobeen Lee[a], Dong-Min Kim[a], Dong-Eun Kim[a,*]

[a]*Department of Bioscience and Biotechnology, Konkuk University, Gwangjin-gu, Seoul 05029, Republic of Korea. *Tel.: +82-2-2049-6062; Fax: +82-2-3436-6062; E-mail: kimde@konkuk.ac.kr*

Supplementary experimental methods

1. Generation of ligated dumbbell padlock DNA

The two termini of dumbbell padlock DNA were pre-ligated before RCA. One micromolar of the 5'-phosphorylated linear dumbbell padlock DNA was thermally denatured at 95 °C for 3 min and cooled to 25 °C for 10 min, and then added to the reaction mixture containing 175 U of T4 DNA ligase and 1× T4 DNA ligase buffer (66 mM Tris-HCl, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP, pH 7.6), yielding a total volume of 10 μL. The mixture was incubated at 25 °C for 1 h. To eliminate un-ligated linear padlock DNA, the ligation product was mixed with 5 μL of the mixture containing 20 U of Exonuclease I, 100 U of Exonuclease III, and 1× Exonuclease I buffer (67 mM Glycine-KOH, 1 mM dithiothreitol, 6.7 mM $MgCl_2$, pH 9.5), followed by incubation at 37 °C for 2 h; the reaction was terminated by treatment at 95 °C for 10 min. The ligation and exonuclease-treated products were analyzed via urea-PAGE (10% resolving gel).

2. GQ-RCA assay with synthesized model SDA products and fluorescence analysis

Pre-ligated padlock and a model SDA product were subjected to the GQ-RCA assay. The 25-μL reaction mixture contained 100 nM of the model SDA product, 500 μM of dNTPs, 75 nM of the ligated dumbbell padlock template, 1× CutSmart buffer, 115 U ϕ29 polymerase, 15 μM of ThT, and 2 mM of KCl; the reaction mixture was incubated at 37 °C for 40 min, and then, the reaction was terminated by treatment at 65 °C for 10 min. The mixture was heat-denatured at 95 °C for 3 min and cooled to 25 °C for 10 min before adding ϕ29 polymerase. To determine the fluorescence intensity of ThT, 25 μL of the RCA products were mixed with 25 μL of distilled water and transferred to quartz cuvettes of a fluorescence spectrophotometer (Model Cary Eclipse, Agilent Technologies, Santa Clara, CA, USA), with the following instrument settings: λ_{ex} = 425 nm with a slit width of 10 nm and λ_{em} = 488 nm with

a slit width of 20 nm; PMT voltage of medium (600 V).

3. Optimization of the volume of the RT-PCR aliquot and the temperature of the PG-SDA/GQ-RCA reaction

To achieve the maximum signal-to-background ratio (S/B ratio), the volume of the RT-PCR aliquot and the temperature of the PG-SDA/GQ-RCA reaction were optimized. Aliquots of 1 µL, 2.5 µL, 5 µL, and 7.5 µL of the RT-PCR product (out of 25 µL) were subjected to PG-SDA/GQ-RCA and the fluorescence intensity was measured. The S/N ratio was determined using the formula F/F0, where F is the ThT fluorescence intensity in the presence of target H3N2 RNA and F0 is the fluorescence intensity in the absence of the target H3N2 RNA. To optimize the PG-SDA/GQ-RCA reaction temperature, the assay was performed at various temperatures (27 °C, 30 °C, 32 °C, 37 °C, and 40 °C) and the fluorescence intensity was determined to compute the maximal S/B ratio. The instrument settings for fluorescence measurement were as follows: λ_{ex} = 425 nm with a slit width of 10 nm and λ_{em} = 488 nm with a slit width of 20 nm; PMT voltage of medium (600 V).

Supplementary data (tables and figures)

Table S1. Oligonucleotides (5′ to 3′) used in the assay

Name		Sequence* (5′ → 3′)	Size (nt)
Dumbbell padlock template		p-CGAAATCTATTGCCCTATTCCCTAACCCTAACCCTAACCCTATTTCGGCAGTGTCCTTTTCAACATCAGTCTGGTAAGCTACCCTTTCACTGC	93
Model SDA product		TGAGGGCTTACCAGACTGATGTTG	24
H3N2	Forward primer	GCACAGGGAATCTAATTGCTCCTA	24
	Reverse primer (normal)	TTGCTTAACATATCTGGGACAGGC	24
	Reverse primer (tailed)	CAACATCAGTCTGGTAAGCCCTCAGCTTGCTTAACATATCTGGGACAGGC	50
H1N1	Forward primer	TTCAGACAATGGAACGTGTTACCC	24
	Reverse primer (normal)	AGAAGCTTTTTGCTCCAGCATGAG	24
	Reverse primer (tailed)	CAACATCAGTCTGGTAAGCCCTCAGCAGAAGCTTTTTGCTCCAGCATGAG	50
Influenza B	Forward primer	ACTGTTTGGGGGTTCCATTCAG	22
	Reverse primer (normal)	GTCTTCTGTTTGATCTGGGAAGCC	24
	Reverse primer (tailed)	CAACATCAGTCTGGTAAGCCCTCAGCGTCTTCTGTTTGATCTGGGAAGCC	50

* Red segment, complementary sequences of RCA primer site; blue segments, nicking site; green segment, C-rich region; underlined sequence, stem forming site; p, phosphorylation; nt, nucleotides.

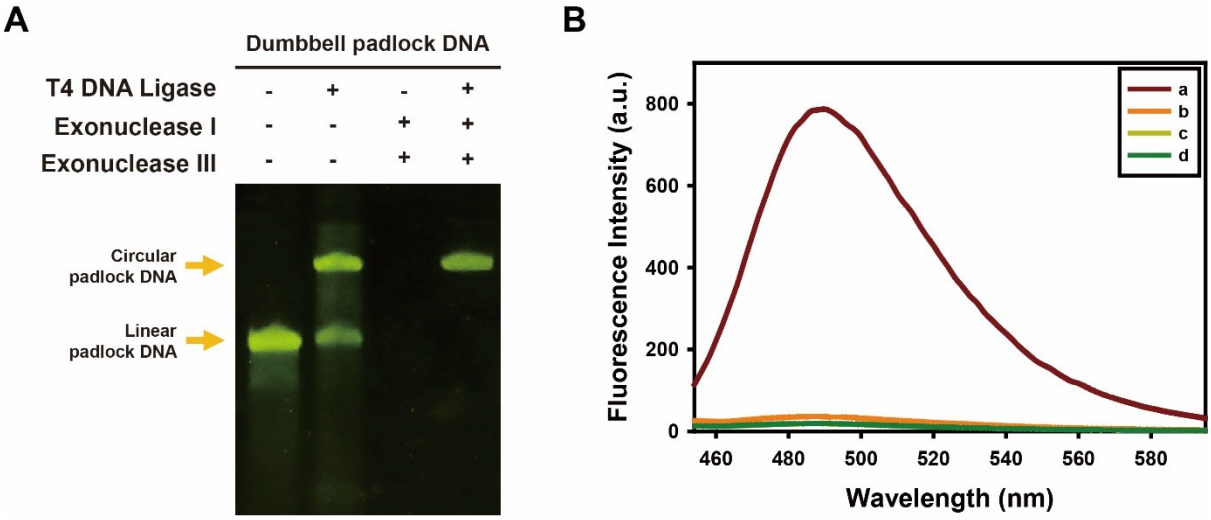

Fig. S1 Validation of dumbbell padlock ligation and GQ-RCA with synthesized model SDA products. (A) PAGE analysis of dumbbell padlock ligation. (B) Fluorescence emission spectra (λ_{ex} = 425 nm) of GQ-RCA was obtained using the ligated dumbbell padlock and synthesized model SDA products: a, without padlock and model SDA product; b, without model SDA product; c, without padlock; d, all included.

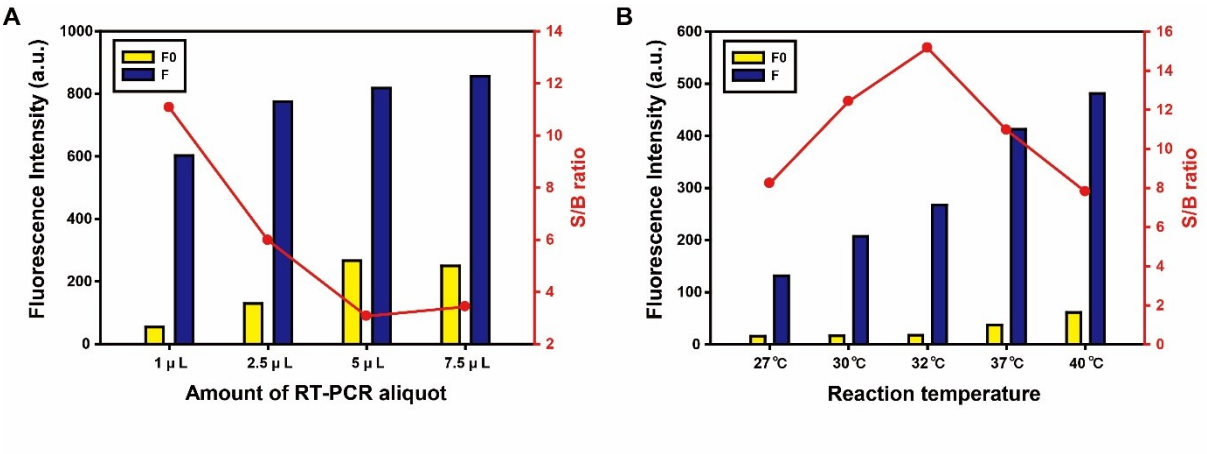

Fig. S2 Optimization of experimental conditions. (A) Fluorescence intensities and the S/N ratio of PG-SDA/GQ-RCA with different volumes of RT-PCR aliquots (1 μL, 2.5 μL, 5 μL, and 7.5 μL). (B) Fluorescence intensities and the S/N ratio of PG-SDA/GQ-RCA at different reaction temperatures (27 °C, 30 °C, 32 °C, 37 °C, and 40 °C).

Table S2. Comparison of the limit of detection (LOD) and operation time for detecting target genes between the RT-PCR-coupled PG-SDA/GQ-RCA method and other published methods

Method	Target genes	LOD	Time	Reference
RT-PCR coupled PG-SDA/GQ-RCA	Influenza A H3N2 (ssRNA)	6 pg	less than 2 h	This study
Asymmetric tailing-PCR triggered RCA	*Cronobacter* spp. (dsDNA)	4.5 × 102 cfu/mL	less than 4.5 h	1
One-step RT-PCR and SDA based signal amplification	Human Immunodeficiency virus-1 (ssRNA)	6 fM	less than 3 h	2
Hybridization cascade plus strand-displacement isothermal amplification	Dengue virus (ssRNA)	3.6 fM	less than 4 h	3
Endonuclease IV-aided signal amplification	Human Immunodeficiency virus-1 (ssRNA)	1.22 nM	less than 4 h	4
Exonucleolytic digestion-triggered exponential RCA	*Cryptococcus neoformans* (dsDNA)	0.02 fM	less than 10 h	5
Triplex DNA-assisted SDA strategy	Simian Virus 40 (dsDNA)	0.4 pM	less than 1 h	6
NASBA-DNAzyme system	Classic Swine Fever Virus (ssRNA)	10 copies/ml	less than 3 h	7

References

1. J. Liu, Z. Zhan, T. Liang, G. Xie, Z. P. Aguilar and H. Xu, *J. Dairy. Sci.*, 2020, **103**, 3055-3065.
2. F. Du, F. Streckenbach, H. Chen, X. Huang, Z. Tang and A. Marx, *Analyst*, 2013, **138**, 1544-1548.
3. W. Saisuk, C. Srisawat, S. Yoksan and T. Dharakul, *Anal. Chem.*, 2019, **91**, 3286-3293.
4. X. Q. Wang, Z. J. Huang, J. M. Chen, Z. W. Luo, Y. Xu and Y. X. Duan, *Anal. Methods,* 2019, **11**, 2190-2196.
5. X. Y. Li, Y. C. Du, Y. P. Zhang and D. M. Kong, *Sci. Rep.*, 2017, **7**, 6263.
6. Y. B. Li, R. M. Li, L. Zou, M. J. Zhang and L. S. Ling, *Microchim. Acta*, 2017, **184**, 557-562.
7. X. L. Lu, X. Y. Shi, G. G. Wu, T. T. Wu, R. Qin and Y. Wang, *Sci. Rep.*, 2017, **7**, 44211.