Electronic Supplementary Material (ESI) for ChemComm.

DNAzyme-activated CRISPR/Cas assay for sensitive and one-pot detection of lead contamination

Ruijie Deng[b], Yaxuan Bai[a], Yumei Liu[b], Yunhao Lu[b], Zhifeng Zhao[b], Yi Deng[a], and Hao Yang*[ab]

[a] *School of Chemical Engineering, Sichuan University, Chengdu 610065, China.*

[b] *College of Biomass Science and Engineering, Healthy Food Evaluation Research Center, Sichuan University, Chengdu 610065, China*

* Corresponding author

e-mail: hyang23@scu.edu.cn

Experimental Procedures

Materials and reagents

All the oligonucleotides were synthesized by Sango Biotech. (Shanghai, China) and the sequences were listed in Table S1 (in the Supporting Information). Except for oligonucleotides with modifications purified by HPLC, other oligonucleotides were purified by PAGE. Biological reagents including EnGen® Lba Cas12a (Cpf1) (10 μM), T4 polynucleotide kinase (T4 PNK, 10 U/μL), Klenow Fragment (3'-5' exo^-, 50 U/μL), phi29 DNA Polymerase (10 U/μL), T7 RNA Polymerase (20 U/μL), DNase I (20 U/μL), dNTP mix (10 mM) and rNTP mix (100 mM) were purchased from New England Biolabs (Beijing) LTD. Supplies including agarose, TAE buffer, loading buffer, and Gelred dye were sourced from Beijing DingGuo Biotech. (Beijing, China). Buffer and metal-salt reagents including $Pb(CH_3COO)_2$, Tris-HCl (pH 7.5), HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid), $MgCl_2$, NaCl, $Al(NO_3)_3$, $Cd(NO_3)_2$, $MnCl_2$, $CuSO_4$, $NiCl_2$ and $CoCl_2$ were purchased from Sigma-Aldrich (Beijing, China). HNO_3, and $HClO_4$ were obtained from equipment division of Sichuan University. Molecular Biology Grade Water was obtained from Corning Incorporated (New York, USA).

Preparation of guide RNA (gRNA)

The first step involving synthesis of double-stranded DNA templates took place at 30 °C for 30 minutes within a 20 μL reaction volume. The reaction mixture comprised 1× phi29 DNA polymerase buffer consisting of 33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate, 1 mM dithiothreitol (DTT), and 0.1% (v/v) Tween 20, with pH adjusted to 7.9 at 37 °C, along with a final concentration of 1 μM for both the promoter and L-gRNA, 4 U of phi29 DNA polymerase, and 0.5 mM of dNTPs. Following the DNA template synthesis, the transcription of Cas12a-gRNA was carried out at 37 °C for 3 hours in a 40 μL reaction volume. This reaction mixture contained 20 μL of the previously prepared DNA template solution, 1× transcription buffer, 20 U of T7 RNA polymerase, and 0.5 mM of rNTPs. Finally, to remove remaining DNA templates, 1 μL of DNase I (20 U/μL) was added to the reaction mixture, and the solution was then incubated at 37 °C for 2 hours followed by heating at 75 °C for 10 minutes.

Pb^{2+} detection procedure

First, the Pb^{2+}-specific DNAzyme cutting process was carried out in a 20 μL volume containing 2 μL 10× NEBuffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 @ 25°C), 4 μL Dz (1 μM), 4 μL Sub (1 μM) and 4 μL Pb^{2+} standard solutions with different concentrations at 25 ℃ for 30 min. The 5'-fragment of Sub bearing a 2',3'-cyclic phosphate produced by DNAzyme cutting can serve as the pre-primer for Klenow-mediated extension. The 2',3'-cyclic phosphate of pre-primer can be removed by T4 PNK, at which point tconverting the pre-primer into a mature primer. So then, the extension-bridged CRISPR reporting process was performed in a 40 μL volume containing 20 μL foregoing DNAzyme cutting reaction mixture, 2 μL 10× NEBuffer™ 2, 0.5 μL T4 PNK (10 U/μL), 4 μL Template (1 μM), 2 μL dNTP mix (10 mM), 0.5 μL Klenow Fragment (3'-5' exo^-, 50 U/μL), 1 μL Cas12a (4 μM), 4 μL gRNA (2 μM) and 4 μL reporter (4 μM) at 37 ℃ for 60 min. The fluorescence signal was measured using a microplate reader Synergy H1 (BioTek, USA), with excitation at 480 nm and emission detected between 510 and 600 nm.

Gel Electrophoresis

The nondenaturing gel electrophoresis analysis was performed using a 6 μL sample volume, composed of 1 μL of gel loading buffer and 5 μL of the nucleic acid mixture, on a 3% agarose gel prepared with 1× TAE buffer and 0.5× GelRed. Electrophoresis was carried out in 1× TAE buffer at 150 V for 30 minutes, followed by imaging using a Gel Doc XR + system (Bio-Rad, USA).

Analysis lead contamination in practical samples

The practical samples including fresh egg, drinking water, tap water and river water spiked with various Pb^{2+} amounts were prepared in advance. Before the spike-recovery test using the proposed assay, the practical samples need to undergo an acid-digestive pretreatment. First, taking 0.1 g Pb^{2+}-spiked samples were added into a digestion tube with an acid mixture containing 10 mL concentrated HNO_3 and 0.5 mL $HClO_4$, and put in a few glass beads to prevent bumping. The digestion of above mixture was conducted with the procedure: 120 °C for 1 h, 180 °C for 3 h, 200 °C for 1 h. If the digestion solution turns brown, add a small amount of nitric acid

until it fumes. Continuing digestion until the solution becomes colorless or slightly yellow. Evaporating the acid to near dryness, stop the digestion, allow it to cool. Then, the mixtures were diluted to a volume of 10 mL and adjust the pH to approximately 7.0 using NaOH solution, to be used for later analysis.

Table S1. Oligonucleotide sequences

Oligonucleotide	Sequences (5' to 3')
	DNAzyme
Dz 5-5	TCTCTGAAGTAGCGCCGCCGTATAG
Dz 6-6	ATCTCTGAAGTAGCGCCGCCGTATAGT
Dz 7-7	CATCTCTGAAGTAGCGCCGCCGTATAGTG
Dz 8-8	TCATCTCTGAAGTAGCGCCGCCGTATAGTGA
Dz 9-9	ATCATCTCTGAAGTAGCGCCGCCGTATAGTGAG
	Substrate
Sub 5	CTCACTATrAGGAAGAGATGATGTCTGT
Sub 18	TTGGTTACACTCACTATrAGGAAGAGATGATGTCTGT
	Cas12a-gRNA
gRNA-1	UAAUUUCUACUAAGUGUAGAU**AUUCGAUGCAACGCGAAGAACCUUACCUGG**
gRNA-2	UAAUUUCUACUAAGUGUAGAU**AUUCGAUUCAACGCGAAGAACUUUACCUAC**
gRNA-3	UAAUUUCUACUAAGUGUAGAU**CGGCGUGGACUACCAGGGUAUCUAAUCCUG**
gRNA-4	UAAUUUCUACUAAGUGUAGAU**AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
gRNA-5	UAAUUUCUACUAAGUGUAGAU**CGCCCAAUAAAUCCGGACAACGCUUGCCAC**
	DNA template for transcription of gRNA
T-gRNA-1	CCAGGTAAGGTTCTTCGCGTTGCATCGAATATCTACACTTAGTAGAAATTAC CCTATAGTGAGTCGTATTA
T-gRNA-2	GTAGGTAAAGTTCTTCGCGTTGAATCGAATATCTACACTTAGTAGAAATTAC CCTATAGTGAGTCGTATTA
T-gRNA-3	CAGGATTAGATACCCTGGTAGTCCACGCCGATCTACACTTAGTAGAAATTAC CCTATAGTGAGTCGTATTA
T-gRNA-4	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTATCTACACTTAGTAGAAATTAC CCTATAGTGAGTCGTATTA
T-gRNA-5	GTGGCAAGCGTTGTCCGGATTTATTGGGCGATCTACACTTAGTAGAAATTAC CCTATAGTGAGTCGTATTA
	Templates complementary to the 5'-products of cleaved Substrate
Template-1	TTTAATTCGATGCAACGCGAAGAACCTTACCTGGTATAGTGAGTGTAACCAA
Template-2	TTTAATTCGATTCAACGCGAAGAACTTTACCTACTATAGTGAGTGTAACCAA
Template-3	TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTATAGTGAGTGTAACCAA
Template-4	TTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAATATAGTGAGTGTAACCAA
Template-5	TTTACGCCCAATAAATCCGGACAACGCTTGCCACTATAGTGAGTGTAACCAA
	Others
promoter	TAATACGACTCACTATAGGG
TG reporter	**/FAM/**-GGGTTTTTTGGG-**/BHQ1/**

* The red letters (rA) represent the adenosine ribonucleotide at this position. The bold bases represent the targeting regions of the Cas12a-gRNA. The underline part represents the protospacer-adjacent motif (PAM) sequence.

Table S2. Detection performance comparison of different DNAzyme assays for Pb^{2+} detection

Strategy	Separation-free	Nanomaterial involved	Linear range	LOD	Samples tested	Ref.
DNAzyme-CRISPR tandem assays						
Extension-bridged DNAzyme-Cas12a assay	**Yes**	**None**	**0-5 nM**	**27 pM**	**Eggs and water**	**This work**
Csm6-DNAzyme assay	Yes	None	0.1-100 nM	70 pM	Eggs and water	[S1]
Cas12a-amplified DNAzyme assay	No	Magnetic beads	0.01-10 nM	53 pM	Water	[S2]
SNA-based DNAzyme-Cas12a assay	No	AuNPs	0.1 pM–1 μM	86 fM	Soil and serum	[S3]
Strand displacement-based DNAzyme-Cas12a assay	Yes	CDs@ZIF-8	0-2 nM	18 pM	Water and fish	[S4]
Colorimetric DNAzyme-Cas12a assay	No	Magnetic beads	0.8-2500 nM	540 pM	Oil, wine, and liquor	[S5]
Dual-functional DNAzyme powered Cas12a assay	No	Magnetic beads	4.82 pM-48.2μM	4.82 pM	Water	[S6]
Nanocage-confined DNAzyme-Cas12a assay	Yes	DNA nanocage	10-800 nM	1.025 nM	Milk	[S7]
DNAzyme-Cas14a assay	No	Magnetic beads	0.24-48 nM	480 pM	Water	[S8]
Other DNAzyme assays						
Label-free colorimetric nanosensor	Yes	AuNPs	0.5-5 nM	200 pM	Water	[S9]
Evanescent-wave biosensor	No	Optical fiber	0-10 μM	20 nM	Water	[S10]
Graphene electronic biosensor	Yes	Graphene	None	790 pM	Water	[S11]
Photoelectrochemical assay	Yes	TiO2/Au/CdS	0.5 pM-10 nM	0.13 pM	Water and serum	[S12]
DNAzyme-templated EXPAR assay	Yes	None	0.1-5 nM	95 pM	Water, egg and juice	[S13]

Table S3. Discrimination factor using other interfering metal ions

Metal ions	10 nM	5 nM	1 nM
Al^{3+}	11.19	7.95	3.29
Cd^{2+}	8.77	10.24	2.70
Mn^{2+}	9.44	8.19	3.22
Cu^{2+}	13.26	8.81	2.93
Ni^{2+}	9.94	8.13	3.21
Co^{2+}	15.21	9.22	2.70

Table S4. Determination of Pb^{2+} in practical samples

Practical samples	Spiked (nM)	Found (nM)	Recovery (%)	RSD (%) n=3
Fresh egg	5.00	4.72	94.43	2.42
	3.00	2.61	86.98	3.12
	1.00	0.96	95.88	7.14
Drinking water	5.00	4.86	97.13	4.23
	3.00	2.75	91.64	2.25
	1.00	0.89	89.18	14.77
Tap water	5.00	4.82	96.46	5.67
	3.00	3.06	101.94	2.44
	1.00	1.07	106.70	3.66
River water	5.00	4.86	97.13	3.65
	3.00	2.99	99.63	3.35
	1.00	1.02	102.40	6.51

Figure S1. Illustration of the secondary structure and sequences of different Cas12a-gRNA used in this work.

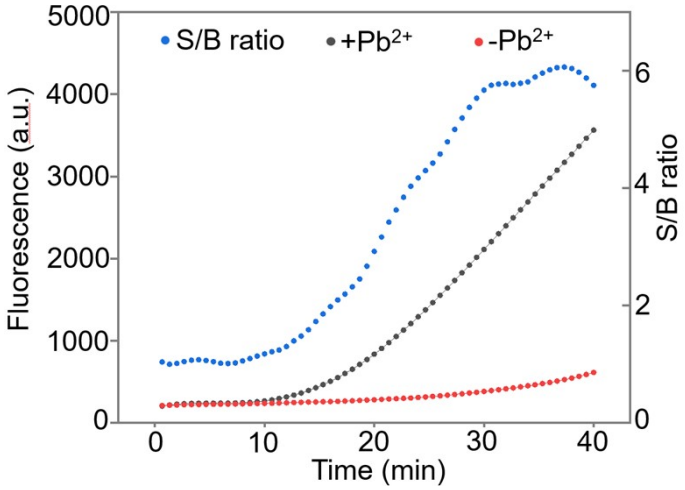

Figure S2. The kinetics of Cas12a-mediated *trans*-cleavage. The reaction was carried at 37 ℃. The excitation wavelength was 480 nm, and the emission wavelength was 520 nm.

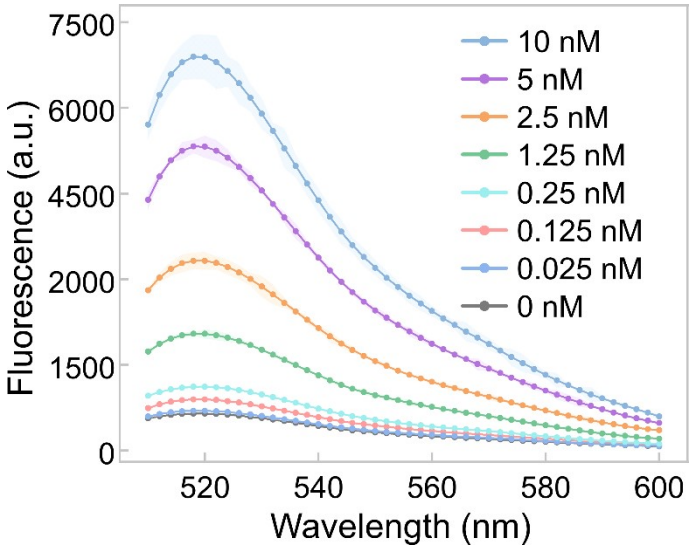

Figure S3. The fluorescence spectra of the DzCas12T assay response to different Pb^{2+} concentration (0, 0.025, 0.125, 0.25, 1.25, 2.5, 5 and 10 nM). The excitation wavelength was set to 480 nm, with the corresponding emission wavelength ranging from 510 nm to 600 nm.

References:

[S1] Yang H., Li F., Xue T., et al. Csm6-DNAzyme Tandem Assay for One-Pot and Sensitive Analysis of Lead Pollution and Bioaccumulation in Mice. *Analytical Chemistry,* 2022, 94, 48, 16953-16959.

[S2] Li J., Yang S., Zuo C., et al. Applying CRISPR-Cas12a as a Signal Amplifier to Construct Biosensors for Non-DNA Targets in Ultralow Concentrations. *ACS Sensors,* 2020, 5, 4, 970-977.

[S3] Li Y., Li H., Fang W., et al. Amplification of the Fluorescence Signal with Clustered Regularly Interspaced Short Palindromic Repeats-Cas12a Based on Au Nanoparticle-DNAzyme Probe and On-Site Detection of Pb^{2+} Via the Photonic Crystal Chip. *ACS Sensors,* 2022, 7, 5, 1572-1580.

[S4] Yang C., Du C., Yuan F., et al. CRISPR/Cas12a-Derived Ratiometric Fluorescence Sensor for High-sensitive Pb^{2+} detection Based on CDs@ZIF-8 And DNAzyme. *Biosensors and Bioelectronics*, 2024, 251, 116089.

[S5] Xu S., Wang S., Guo L., et al. Nanozyme-Catalysed CRISPR-Cas12a System for The Preamplification-Free Colorimetric Detection of Lead Ion. *Analytica Chimica Acta*, 2023, 1243, 340827.

[S6] Wen J., Deng H., He D., et al. Dual-Functional DNAzyme Powered CRISPR-Cas12a Sensor for Ultrasensitive And High-Throughput Detection of Pb^{2+} in Freshwater. *Science of The Total Environment*, 2024, 911, 168708.

[S7] Yu Y., Zhang Y., Li W., et al. DNA Nanocage Confined DNAzyme for Detection of Lead Ions Coupled with CRISPR-Cas12a System. *Chemical Engineering Journal*, 2024, 480, 148177.

[S8] Chen Y., Wu H., Qian S., et al. Applying CRISPR/Cas System as A Signal Enhancer for DNAzyme-Based Lead Ion Detection. *Analytica Chimica Acta*, 2022, 1192, 339356.

[S9] Memon A. G., Zhou X., Xing Y., et al. Label-free colorimetric nanosensor with improved sensitivity for Pb^{2+} in water by using a truncated 8–17 DNAzyme. *Frontiers of Environmental Science & Engineering*, 2019, 13, 12.

[S10] Han S., Zhou X., Tang Y., et al. Practical, highly sensitive, and regenerable evanescent-wave biosensor for detection of Hg^{2+} and Pb^{2+} in water. *Biosensors and Bioelectronics*, 2016, 80, 265-272.

[S11] Li Y., Wang C., Zhu Y., et al, Fully integrated graphene electronic biosensor for label-free detection of lead (II) ion based on G-quadruplex structure-switching. *Biosensors and Bioelectronics*, 2017, 89, 758-763.

[S12] Meng L., Liu M., Xiao K., et al. Sensitive photoelectrochemical assay of Pb^{2+} based on DNAzyme-induced disassembly of the “Z-scheme” TiO2/Au/CdS QDs system†. *Chemical Communication*, 2020, 56, 8261.

[S13] Yang H., Liu Y., Wan Yi., et al. DNAzyme-templated exponential isothermal amplification for sensitive detection of lead pollution and high-throughput screening of microbial biosorbents. *Science of The Total Environment*, 2023, 863, 160899.3