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论文题目：CRISPR-Cas-Based Detection of Bacterial Nucleic Acids in Environmental Water Samples

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# CRISPR-Cas-Based Detection of Bacterial Nucleic Acids in Environmental Water Samples

**Li Sheng, Guanhong Liu**

## Abstract

Bacteria are widely distributed in natural environments, and their abundance and composition are closely related to local environmental conditions. Detecting bacterial DNA in water can help us to understand the microorganisms present in different water environments.

Over nearly one year, we learned and practiced molecular biology and biochemistry techniques, including molecular cloning, protein expression and purification and PCR etc. We then used these techniques to develop a CRISPR-Cas12a method for detecting bacterial DNA in environmental water samples.Water samples were collected from multiple sites at Beijing 101 Middle School, *Yuanmingyuan*, and the Olympic Forest Park. Some sites were sampled more than once to test the method with water collected at different times and locations. Because the water contained sediment and suspended material, we tested different ways to concentrate microorganisms before DNA extraction. Sequential filtration followed by collection on a 0.22-μm membrane was used to concentrate microorganisms from the water. The extracted DNA was then amplified by PCR.

For CRISPR-based DNA detection, we prepared AsCas12a protein and crRNAs targeting selected 16S rRNA gene markers. We tested a general gut-associated bacterial marker, a human gut-associated marker, and two different avian gut-associated markers. The Cas12a–crRNA complex was used to recognize the target DNA containing the PAM sequence within the 16S rRNA gene, and an ssDNA fluorescent reporter was used to generate the detection signal.

We tested water samples collected from multiple sites at three locations and found different patterns of bacterial DNA markers among the samples. These patterns were generally consistent with differences in the surrounding environments and human and animal activities at the sampling sites. To improve the reliability of the results, we carried out repeated sampling and adjusted the sampling sites and collection methods based on the first round of experiments. Our results suggest that AsCas12a-based detection can be used to detect selected bacterial DNA markers in urban environmental waters.

Our study shows that the molecular biology methods we learned can be combined into a workflow for detecting selected bacterial DNA in environmental water. The results also show that sample processing and target selection can strongly affect the detection results. More samples and bacterial markers will be needed to better understand the microbial communities in these water environments.

**Key words**: CRISPR-Cas12a , Bacterial DNA Detection , Microbial source tracking , PCR , Environmental water samples.

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# 1. Introduction

## 1.1 Detecting Bacteria in Water Is Essential

Bacteria and bacteriophages are abundant and widely distributed in natural environments and they are important part of aquatic ecosystems (Whitman et al., 1998; Newton et al., 2011). Their abundance and composition vary with water conditions, nutrient availability, and human and animal activities. Detecting bacteria in water is essential to understand the microbial community and changes in the local environment. It is also important for water quality monitoring and for identifying bacteria associated with human or animal sources. Human and animal activity near bodies of water leaves a detectable bacterial trace. In this study, we compared the signals of certain bacteria that indicates nearby human and animal activity. We developed a CRISPR-Cas12a-based method which was applied to three different environments: a high-school campus, a historical landmark, and a local park. The high school campus has a relatively fixed group of people interacting with the environment, and the physical isolation of waters may contribute to a unique composition. *Yuanmingyuan* has a more dynamic interaction between the evironment and people due to the changing number and origin of visitors and the connection to outside water systems. Similar to *Yuanmingyuan*, the local park has a large number of visitors and connection to the outside, but with more direct interactions such as shallow shores for duck-feeding. Apart from direct contact, water infiltration in the soil may indicate activity that occurs over a wider area. Possible forms include farming and underground pipe leakages. All three sites had birds present.

## 1.2 DNA detection by CRISPR-Cas12a

CRISPR-Cas12a is a nuclease derived from bacteria (Zetche et al., 2015). It is most widely used in clinical diagnostics, specifically in fields such as infectious disease detection, cancer biomarker detection, genotyping and SNP detection (Broughton et al., 2020; Tsou et al., 2020; Li et al., 2018). Cas12a uses a crRNA to recognize a complementary target DNA sequence (Yamano et al., 2016). Cas12a scans DNA for a T-rich PAM (protospacer adjacent motif) sequence, and it unwinds the DNA if such PAM is present (Zetsche et al., 2015; Yamano et al., 2016). Then, its crRNA guide attempts to base pair with the unwound strand. If the base-paring is successful, the entire enzyme is activated specifically in the RuvC domain responsible for cleavage (Swarts & Jinek, 2019; Strohkendl et al., 2024). In its natural functions, the RuvC domain conducts cis-cleavage (Zetsche et al., 2015; Swarts & Jinek, 2019), cutting the target and nontarget DNA. However, in artificial settings, RuvC conducts trans-cleavage as well (Chen et al., 2018; Swarts & Jinek, 2019), cutting any ssDNA in contact regardless of any traits. This non-specific cleavage is exploited for signal amplification, where a short strand of ssDNA, with a fluorophore and quencher attached to each end, is used as an indicator to magnify the activation of Cas12a that is otherwise undetectable (Chen et al., 2018). When intact, the

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ssDNA's short length allows the quencher to inhibit the fluorescent signal of the fluorophore; but after the cut by the RuvC domain of the activated Cas12a, the signal is no longer inhibited and thus detectable (Li et al., 2008; Chen et al., 2018). The Cas12a's programmable crRNA guide allows for detection of any target sequence (Zetsche et al., 2015), hence we explored its application in detecting genomes that indicate human and animal activity near waters. A schematic overview of the Cas12a-based detection mechanism is shown in Figure 1_1.

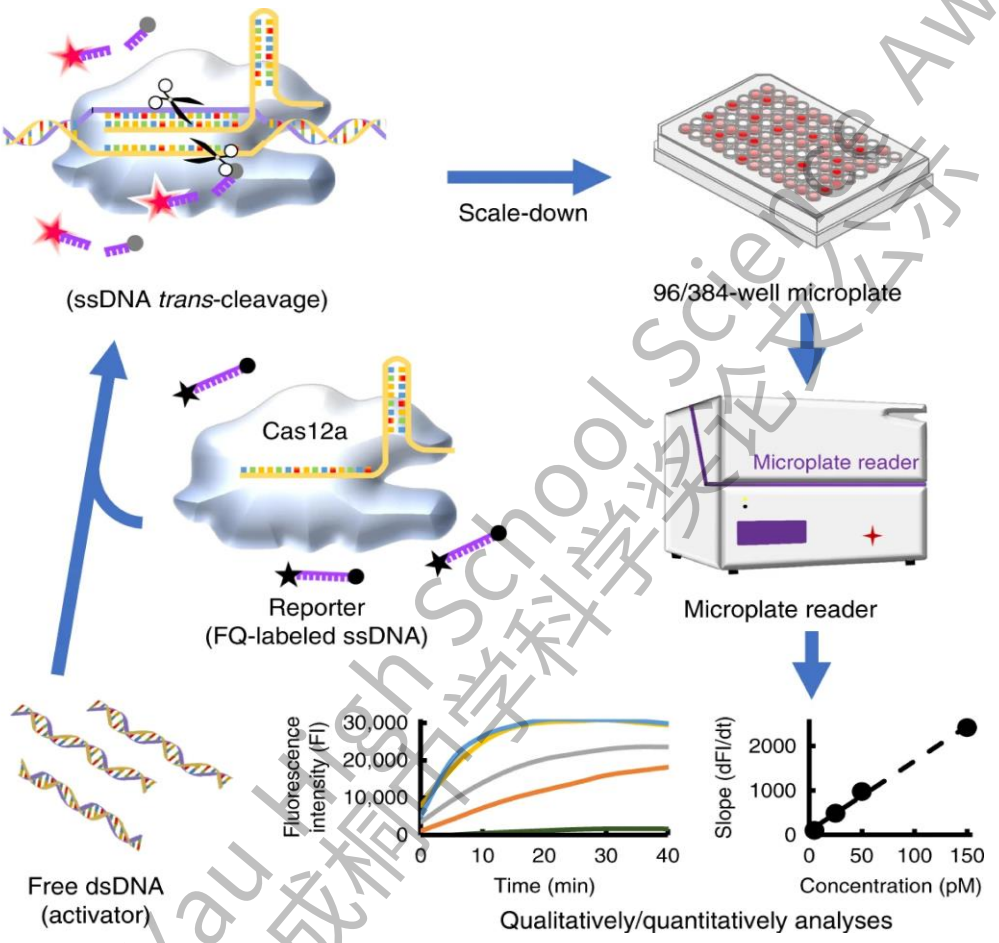



*Figure 1_1. The principle of Cas12a nucleic acid detection (Adapted from Liang et al., 2019)*

If the extracted double-stranded DNA contains the target DNA harboring the correct PAM and protospacer complentary to the guide of crRNA, it can serve as activators to activate the ssDNA transcleavage activities of Cas12a bound to its cognate crRNA. Consequently, the fluo-reporter will be cleaved. The fluor cannot be quenceched by the quencher anymore, and the fluorescence intensity can be read by a microplate reader (Chen et al., 2018). Based on this principle, we can detect the specific DNA from environmental samples.

### 1.3 Nucleic acid extraction and processing

The extraction of nucleic acids is a mature and common procedure, and thus commercial extraction kits are available for our use. Taking the kit that we used for example, the extraction of DNA first requires

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cell lysis and protein denaturation, especially DNase (Boom et al., 1990); then the washing of residual proteins, salts, cellular debris and other impurities involving ethanol-based buffers; and lastly the air-drying of ethanol to prevent disturbance of later steps (Schrader et al., 2012; TIANGEN Biotech, 2025).

### 1.4 What We Did in This Study

As students from Beijing 101 Middle School, we wanted to test whether CRISPR-based nucleic acid detection could also be used to study the water environments around us. We collected water samples from Beijing 101 Middle School, *Yuanmingyuan*, and the Olympic Forest Park. We expressed and purified AsCas12a, selected bacterial DNA markers associated with human and animal sources, and tested these markers in water samples from the three sites. Our results show that the Cas12a-based method can detect selected bacterial DNA markers in urban environmental water samples.

## 2. Methodology

### 2.1 Cloning, expression and purification of Cas12a proteins

#### 2.1.1 Cloning of Cas12a genes into pET28a vectors

Full-length ORFs encoding *Acidaminococcus sp.* Cas12a protein was synthesized from Sangon Biotech and were cloned into the expression vector containing a $His_6$-sumo tag at the N-terminus through Gibson Assembly (Gibson et al., 2009; Malakhov et al., 2004). The protein expression plasmids are kanamycin resistant. The corrected plasmid verified by sequencing was transformed into *E. coli* BL21 (DE3) competent cells.

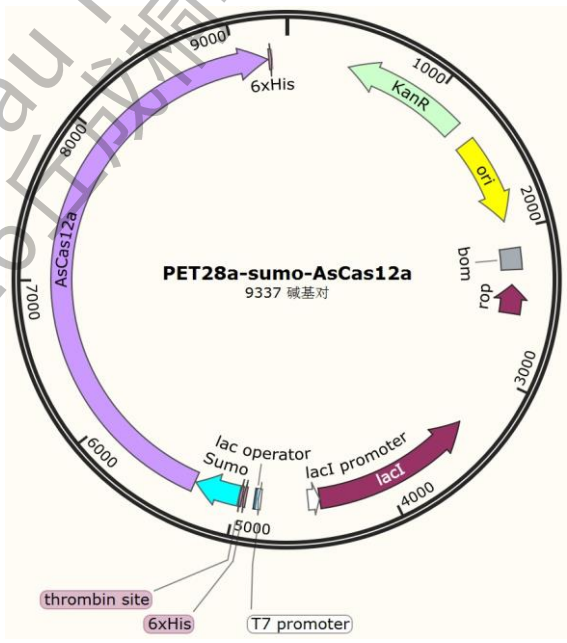



*Figure 2_1. The plasmid constructed to overexpress AsCas12a protein*

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### 2.1.2 Overexpression of Cas12a proteins

After incubation at 37℃ for around 12 h, one colony was inoculated to 80 ml LB medium and cultured for 12 h, and then scaled up to 750 mL LB medium. All media contain 50 μg/ml Kanamycin. When the bacteria grown to OD600 =0.6-0.8, a final concentration of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression at 18℃ for 12 h.

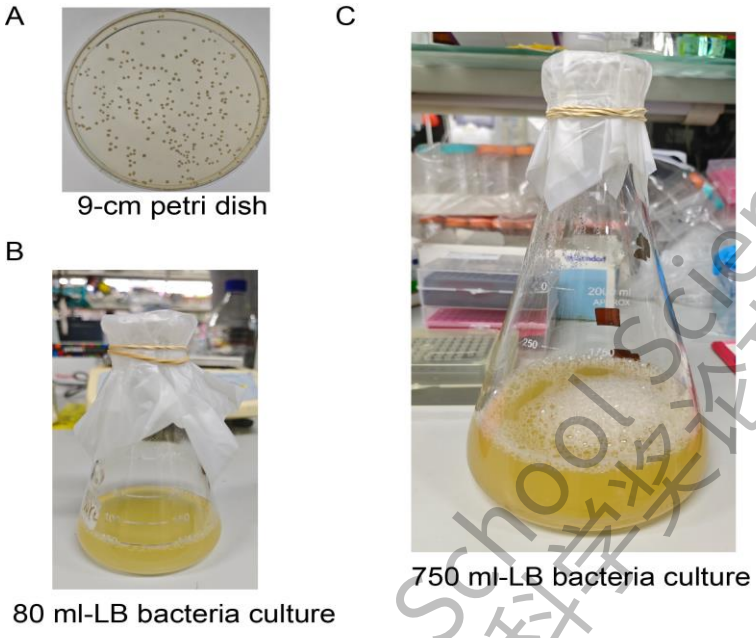



*Figure 2_2. The colony and media of BL21 (DE3) cells oeverexpressing Cas12a proteins*

### 2.1.3 Purification of Cas12a proteins

Cells were harvested by a centrifuge at a speed 4,000 rpm for 20 min, and then resuspended in lysis buffer containing 20 mM Tris-HCl (pH 7.5) and 0.5 M NaCl. Subsequently, cells were disrupted by ultrasonic cell disruptor in ice-water mixture with a power 200 W. The program is 3s on and 8s off for 200 times. Then, the samples were processed with high speed centrifugation 18000 rpm for 1 h at 4℃ to separate supernatant from cell pellet (Martin et al., 2023).

**First Nickel column**

Afterward, the supernatant was loaded onto a nickel gravity column containing ~8 mL of $Ni^{2+}$-Sepharose resin (GE Healthcare), and the flow-through (FT) was collected. The resin was then washed and eluted with lysis buffer using an increasing imidazole gradient: 20 mM (200 mL), 50 mM (200 mL), 70 mM (40 mL), 250 mM (40 mL), and 500 mM (20 mL).

**Dialysis, His-sumo tag cleavage and second nickel column**

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The fractions with high purity and amount were selected for dialysis and $His_6$-sumo tag cleavage. The $His_6$-sumo tags were cleaved from AsCas12a proteins by His-tagged ubiquitin-like protein 1 (Ulp1) protease during dialysis against buffer containing 20 mM Tris-HCl (pH 7.5), 0.2 M NaCl for 2h at 4°C (Malakhov et al., 2004; Lee et al., 2008).

Cas12a protein was further purified by a second nickel column to remove the $His_6$-Sumo tag. Two columns with ~2 ml resin were used for each Cas12a proteins. The imidazole gradient wash for each column was as follows: flow-through (FT); 50 mM imidazole (20 mL); 300 mM imidazole (20 mL) (Lee et al., 2008).

**Heparin column**

Next, the Flow-through fractions of AsCas12a were loaded into Heparin columns for further purification. Buffer A (20 mM Tris pH7.5, 0.2 M NaCl) and Buffer B (20 mM Tris pH7.5, 1.2 M NaCl) were used to elute Cas12a protein bound to the column. The gradient wash program is 0 to 100%B in 30 min, with 2mL/min flow rate. A 40%B gradient was set in the middle (Martin et al., 2023).

In the end, all fractions collected were verified by SDS-PAGE and stained with Coomassie Blue R250 (Laemmli, 1970). The target fractions were pooled and then concentrated to a high concentration. The final proteins were aliquoted and flash-frozen to -80°C.

## 2.2 crRNA preparation and DNA systhesis

**(1) Oligo DNA template preparation for crRNA in vitro transcription**

The templates for crRNA in vitro transcription was amplified using two primers, which contains a 25-30 bp overlap part (Milligan et al., 1987). First, anneal the two primers (20 μM each) by incubation at 95℃ for 5 min, and then cooled down with a decrease rate -0.1℃/min to 30℃. Second, mix 5 μL annealed product and 5 μl Phanta Mix DNA polymerase. Incubation at 72℃ for 5 min reaction as the template for next step. Next, set up two 50 μl reaction system:

*F primer (20 μM) 12 μL*

*R primer (20 μM) 12 μL*

*template 1 μL*

*2*Phanta Mix 25 μL*

PCR program:

*Initial denaturation at 95°C for 3 min*

*30 cycles of:*

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*Denaturation at 95°C for 15 s*

*Annealing at 60°C for 15 s*

*Extension at 72°C for 30 s*

*Final extension at 72°C for 3 min*

**(2) In vitro transcription and purification of crRNA.**

The crRNAs were synthesized by in vitro transcription using a T7 RNA polymerase and PCR amplified oligo double-stranded DNA templates (Milligan et al., 1987). Transcription reactions were performed at 37°C for 6 h with 100 mM HEPES-KOH (pH 7.9), 30mM $MgCl_2$, 30 mM DTT, 3 mM each NTP, 2 mM spermidine, 0.1 mg/mL T7 RNA polymerase, and 10 ng/μL linearized plasmid DNA template (Ho-Xuan et al., 2020; New England Biolabs, n.d). The RNAs were then purified by gel electrophoresis on a 16% denaturing (8 M urea) polyacrylamide gel and the parts containing the RNA were excised (Petrov et al., 2013). The excised gel was smashed and soaked in soaking buffer containing 0.38 M NaAc pH5.2, 0.8 mM EDTA pH8.0, 0.2% SDS for 12 h at 4℃. Thereafter, the RNA solution was subjected to ethanol precipitation. Finally, RNA was resuspended in RNA refolding buffer (50 mM KCl, 5 mM $MgCl_2$) and refolding using a thermocycler as follows: Inbubation at 75℃ for 5 min, and the cooled down to 30℃ with a decrease rate -0.1℃/min, and stay at 70, 65, 60, 55, 50, 45, 40, 35 and 30℃ for 1 min (Strohkendl et al., 2024).

**(3) Preparation of DNA**

All short DNAs used in this study were purchased from TsingKe Biotech. All DNAs were dissolved in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$. For double-stranded DNA, equimolar amounts of two complementary strands were pre-denatured at 95°C for 10 min and then annealed at room temperature (Strohkendl et al., 2024)

The Fluo-reporter (5' -6FAM-TTATT-BHQ-1-3') was purchased from Genscript Biotech. It is a single-stranded DNA (ssDNA) probe with the 5’ labeled with 6FAM fluorphore and the 3’ labeled with BHQ-1 (Black Hole Quencher 1) quencher. The fluorescence of 6FAM is quenched by BHQ-1 when the reporter is intact. In contrast, after the ssDNA was cleaved, BHQ-1 cannot quench 6FAM anymore, and the fluorescence signal will increase (Chen et al., 2018; Rossetti et al., 2022).

Target DNA sequences are listed in the following:

NTS-1B: TAGGAGTTTGGACCGTGTCTCAGTTCCAATGTGGGGGACCTTCC

TS-1B: GGAAGGTCCCCCACATTGGAACTGAGACACGGTCCAAACTCCTA

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NTS-2C: AGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGATAGT

TS-2C: ACTATCTAATGGAACGCATCCCCATCGTCTACCGGAAAATACCT

NTS-5B: ATCGCTTTTGGATGGACCCGCGGCGTATTAACTAGTTGGTAGGG

TS-5B: CCCTACCAACTAGTTAATACGCCGCGGGTCCATCCAAAAGCGAT

DNA primers used to prepare the templates of crRNA1B, 2C and 5B:

1B-F: GACTTTAATACGACTCACTATAGGAATTTCTACTCTTGTAGATGACCGTG

1B-R:

AAAAAACCACATTGGAACTGAGACACGGTCATCTACAAGAGTAGAAATTC

2C-F: GACTTTAATACGACTCACTATAGGAATTTCTACTCTTGTAGATCGGTAGA

2C-R: AAAAAAGGAACGCATCCCCATCGTCTACCGATCTACAAGAGTAGAAATTC

5B-F: GACTTTAATACGACTCACTATAGGAATTTCTACTCTTGTAGATGATGGAC

5B-R: AAAAAATAGTTAATACGCCGCGGGTCCATCATCTACAAGAGTAGAAATTC

### 2.3 Water sampling

All extractions took place at around 0.5-1 m from the shore. Water is sampled from surface to avoid collection of large particles with consistent sample size for each batch. The samples where left unprocessed for a maximum time of 90 minutes before undergoing preliminary processing.

### 2.4 Preliminary processing of sample

Two methods of preliminary processing were proposed, stepwise filtration and stepwise centrifugation (Deiner et al., 2015; Goldberg et al., 2016). The first method was to use a 10-um filtration to remove large inorganic particles, algal aggregates, other multicellular organisms, and other debris. The liquid then goes through a 0.22 μm (filtration complex, where bacteria is trapped in the filtering membrane and dissolved into water via repetitive rinsing with 40 mL of $ddH_2O$)(Liang & Keeley, 2013; Goldberg et al., 2016). The second method was to use a 4000rpm centrifuge for 10 minutes and 25 minutes consecutively to ensure that the bacteria is settled along with other particles in the pellet. The supernatant is discarded. 40 mL of $ddH_2O$ is added and stirred with the pellet before another round of centrifuge with 300 rpm for 5 minutes. Bacteria remaining in the supernatant while other heavier particles are condensed in the pellet. The supernatant is extracted. The two methods both produce a remaining liquid around 40 mL that goes under centrifuge with 4000 rpm for 15 minutes to spin down the bacteria pellet. The supernatant is

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removed and the pellet goes to the next procedure.

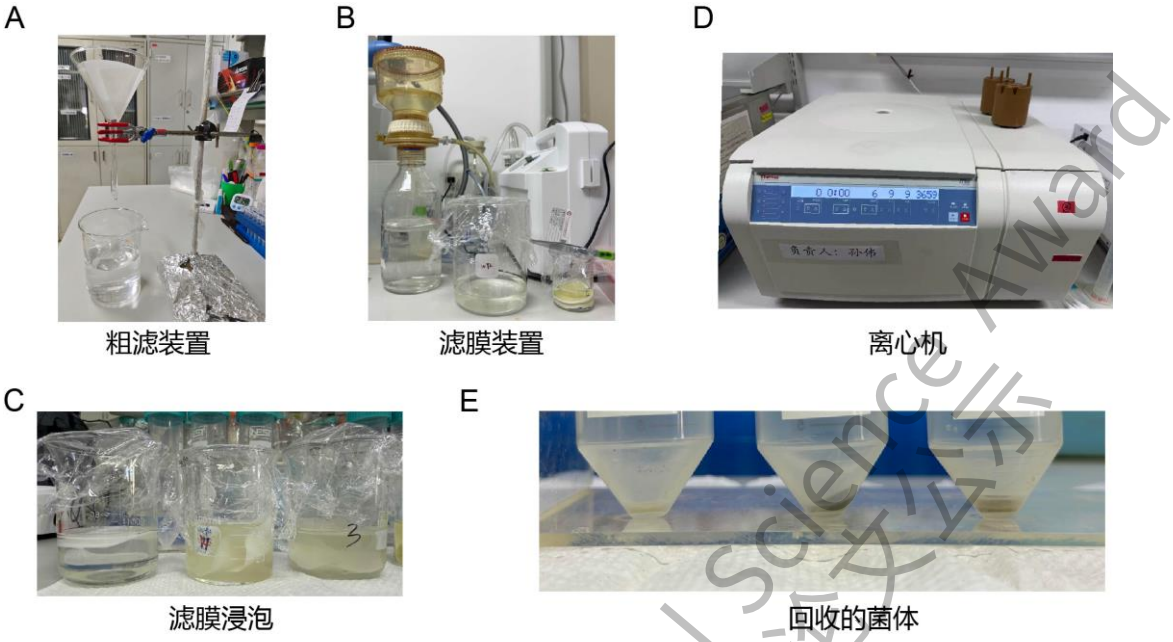



*Figure 2_3. The water preprocessing devices.*

### 2.5 Extraction of DNA

For the extraction of bacterial DNA, we used a Bacteria DNA Kit produced by Tiangen Biotech. We followed the provided instructions, except for the last step, applying TE (elution) buffer into the spin column. dd$H_2O$ was used for final DNA elution instead of the supplied TE buffer. This modification was intended to minimize EDTA carryover into subsequent $Mg^{2+}$-dependent PCR reactions. Although TE buffer is compatible with many PCR applications, excessive EDTA may reduce the availability of $Mg^{2+}$ required for DNA polymerase activity (Schrader et al., 2012; TIANGEN Biotech, 2025).

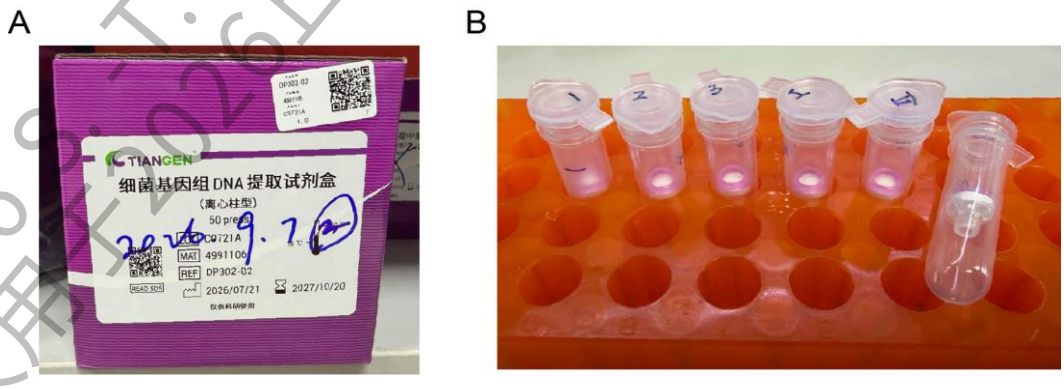



*Figure 2_4. The kit used for genomic DNA extraction.*

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## 2.6 PCR

### 2.6.1 PCR reaction system

To amplify the target fragments, Polymerase Chain Reaction (PCR) was performed using 2*Phanta Master Mix (Vazyme) DNA polymerase (Vazyme Biotech, 2023). Two primer pairs, 1F/1R, 2F/2R and 5F/5R, were used for the amplification of each sample. The PCR reaction was carried out in a 20 μL total volume in PCR tubes. The 20 μL reaction system is as follows:

*2*Phanta Master Mix 10 μl*

*F primer (10 μM) 1 μL*

*R primer (10 μM) 1 μL*

*genomic DNA template 50 ng*

*addition of $ddH_2O$ to a total volume of 20 μL*

### 2.6.2 PCR program

The PCR thermal cycling conditions were programmed as follows (Figure 2_5)(Vazyme Biotech, 2023):

Initial denaturation at 95°C for 3 min

*35 or 40 cycles of:*

*Denaturation at 95°C for 15 s*

*Annealing at 60°C for 15 s*

*Extension at 72°C for 30 s*

*Final extension at 72°C for 3 min*

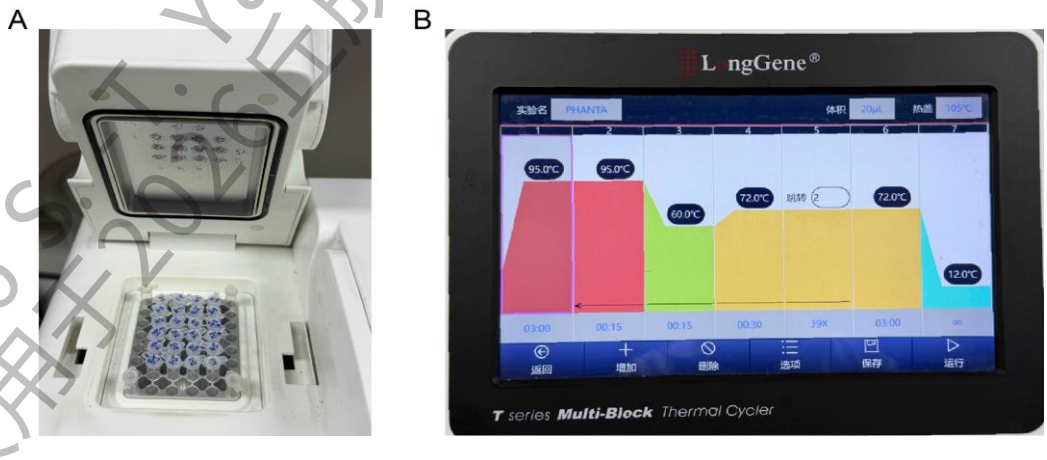



*Figure 2_5. The inside and the dsplay panel of the thermocycler*

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### 2.6.3 PCR primer pairs used for amplification of trarget fragments of 16S rRNA gene

**(1) GenBac3** (Dick & Field, 2004; Siefring et al., 2008)**:**

1F: GGGGTTCTGAGAGGAAGGT

1R: CCGTCATCCTTCACGCTACT

(2) **HF183** (Green et al., 2014):

2F: ATCATGAGTTCACATGTCCG

2R: CTTCCTCTCAGAACCCCTATCC

**(3) CPQ_056** (Stachler et al., 2017):

3F: CAGAAGTACAAACTCCTAAAAAACGTAGAG

3R: GATGACCAATAAACAAGCCATTAGC

**(4) GFD** (Green et al., 2012)**:**

4F: TCGGCTGAGCACTCTAGGG

4R: GCGTCTCTTTGTACATCCCA

**(5) AV4143** (Ohad et al., 2016)**:**

5F: TGCAAGTCGAACGAGGATTTCT

5R: TCACCTTGGTAGGCCGTTACC

### 2.7 Fluorescence reaction

The fluorescence detection assay was conducted based on the trans-cleavage activity of the CRISPR-Cas12a system (Chen et al., 2018; Swarts & Jinek, 2019). First, the reaction buffer was prepared, containing 20 mM Tris (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$.

For the Cas12a-crRNA complex formation, 20 μL of 100 nM Cas12a and 20 μL of 100 nM crRNA were mixed and incubated at room temperature for 15 minutes (Strohkendl et al., 2018). Following this, 20 μL of 2 μM fluorescence reporter was added to the mixture and mixed gently (Chen et al., 2018; Liu et al., 2022).

To trigger the trans-cleavage reaction, the PCR products were diluted 3-fold using the reaction buffer. Subsequently, 20 μL of these diluted PCR products were added to the prepared 60 μL Cas12a-crRNA-reporter mixture. The final reaction system was incubated at 37°C for 20 minutes in the dark (Chen et al., 2018; Liu et al., 2022).

Finally, the reaction products were transferred to a black 96-well plate, and fluorescence signals were measured using a microplate reader (EnSpire, Perkin Elmer). The excitation wavelength was set to 485 nm, and the emission wavelength was 520 nm. The intensity of the fluorescence indicated the cleavage of the ssDNA fluo-reporter, and confirming the presence of target DNA sequences in the detected samples

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(Chen et al., 2018).

## 3. Results

### 3.1 Establishment of an AsCas12a-Based DNA Detection System

#### 3.1.1 Expression and purification of Cas12a proteins

The AsCas12a gene was cloned into a pET expression vector with an N-terminal $His_6$-Sumo tag. After the construct was confirmed by DNA sequencing, the plasmid was transformed into *E. coli* BL21(DE3) cells for protein expression. The cells were grown to an $OD_{600}$ of approximately 0.6, and protein expression was induced by adding IPTG to a final concentration of 0.1 mM. After induction for 12 h at 18°C, the cells were harvested by centrifugation and used for subsequent protein purification.

The harvested cells were resuspended in lysis buffer and lysed. After removing the cell debris by centrifugation, the soluble proteins were purified by $Ni^{2+}$ affinity chromatography twice followed by heparin chromatography (Malakhov et al., 2004; Lee et al., 2008). The Cas12a proteins were eluted from the first $Ni^{2+}$ affinity column with buffer containing 250 mM imidazole (Figure 3_1A and B). The fractions containing Cas12a were collected, and 400 μL 1mg/mL Ulp1 protease was added to remove the N-terminal $His_6$-Sumo tag. The proteins were then subjected to further purification by the second $Ni^{2+}$ affinity column to remove the $His_6$-Sumo tag. Most proteins flew through the second nickel column, and some were washed in the wash buffer containing 50 mM imidazole.

Subsequently, both the FT and the 50 mM imidazole wash fractions were mixed and loaded into a heparin column for further purification. AsCas12a proteins can bind to the heparin column, and no protein flows through the column. When increasing the concentration of sodium chloride (2mL/min, 0-100%B in 30 min), the main peak of AsCas12a was eluted around a conduction value of 52.46 mS/cm, which is corresponding to 42%B buffer B. SDS-PAGE shows that Fractions (15-31) are of high purity. Therefore, fractions (15-31) were pooled and concentrated with concentrator of 50 kDa cutoff (Figure 3_1C).

After these three purification steps, the AsCas12a proteins showed high purity and good sample homogeneity and were used for subsequent experiments. Finally, the concentration of AsCas12a proteins is about 15.4 mg/mL. Proteins were aliquoted to 30 μL/tube, and flash-frozen to -80°C for use.

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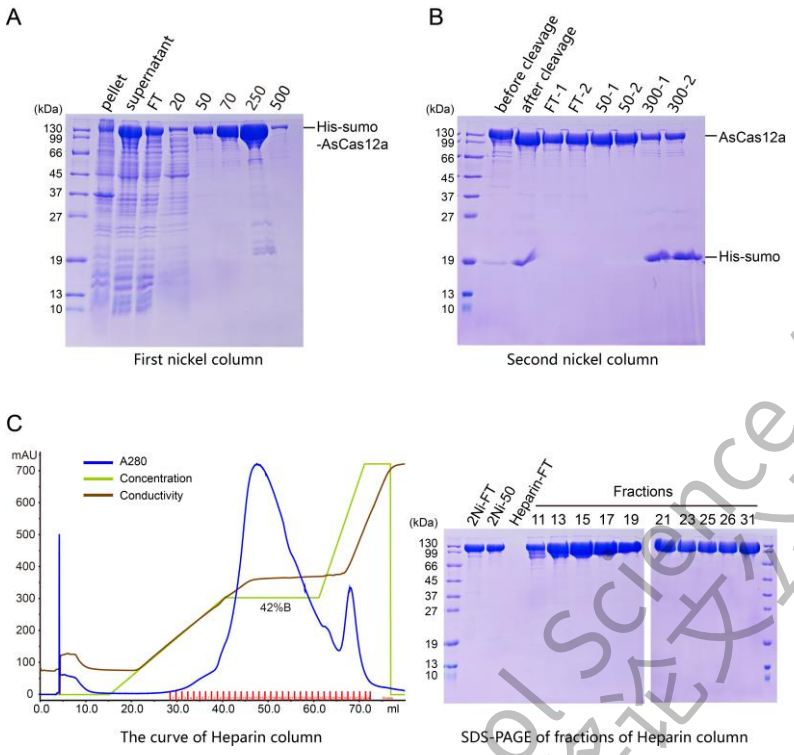



*Figure 3_1. Purification of AsCas12a proteins. (A) First nickel column; (B) Second nickel column; (C) Heparin column purification.*

### 3.1.2 crRNA preparation and DNA synthesis

Two oligonucleotides were designed and synthesized based on the target sequence (Figure 3_2A) and the sequence requirements for AsCas12a crRNA (Figure 3_2B) (Ageely et al., 2021). The oligonucleotides were used to generate DNA templates by PCR. After amplification, the DNA template for the in vitro transcription was obtained infered from the agarose gel, which is longer than the F primer control (Figure 3_2C).

DNA template for crRNA transcription contained a T7 promoter at the 5'-prime, and terminated with a continuous poly-T facilitating transcription termination (Calvopina-Chavez et al., 2022). The final crRNAs for 1B, 2C and 5B are of 51 nt lengths (Figure 3_2B). The first two nucleotides of each crRNAs are 5'-GG which can promote T7 transcription (Milligan et al., 1987). Finally, Urea-PAGE shows that the three types of crRNAs are prepared correctly (Figure 3_2C).

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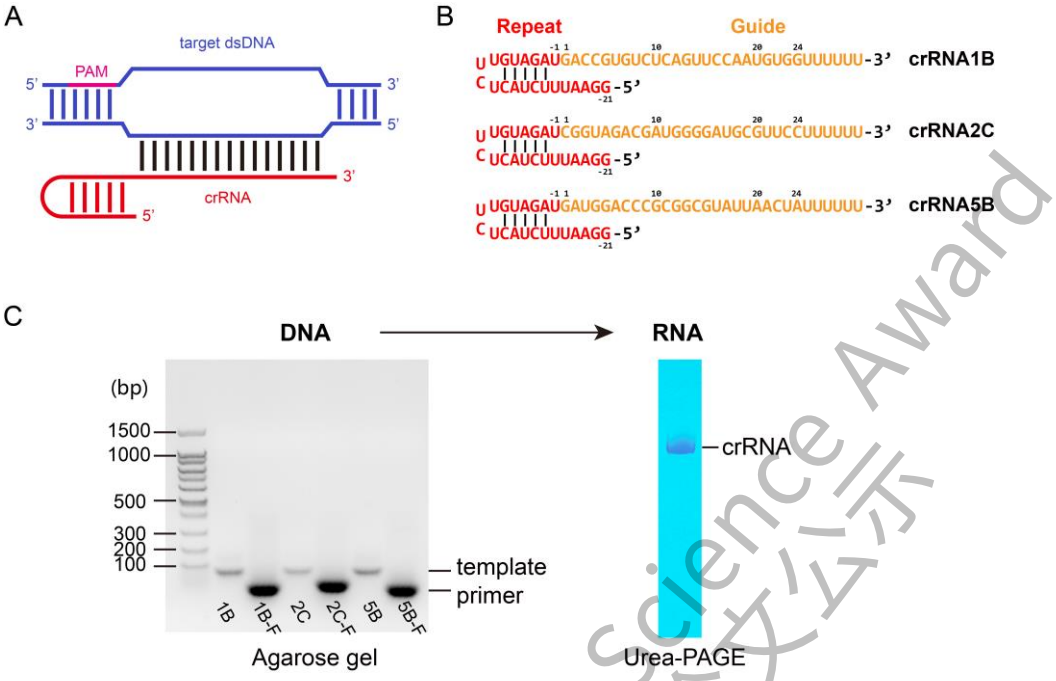



*Figure 3_2. Preparation of crRNA and dsDNAs.*

*(A) Schemes of crRNA and target double-stranded DNA. (B) Schemes of three types of crRNAs used in this study. (C) Agarose gel of DNA templates for crRNA in vitro transcription and Urea-PAGE of a representative crRNA.*

### 3.1.3 Selection of Target Markers

We selected five microbial markers that have previously been used in qPCR assays for the detection of fecal-associated microorganisms in environmental water: GenBac3, HF183/BacR287, CPQ-056, GFD, and Av4143. These markers represent different microbial sources, including a general fecal-associated bacterial marker, a human-associated bacterial marker, a human-associated bacteriophage marker, and two avian-associated bacterial markers.

GenBac3 is a general Bacteroidales marker used to detect fecal-associated bacteria from different animal sources (Siefring et al., 2008; Shanks et al., 2010). HF183/BacR287 targets human-associated Bacteroidales, including *Bacteroides dorei* (Green et al., 2014; Haugland et al., 2010). CPQ-056 targets crAssphage, a bacteriophage commonly associated with the human gut (Stachler et al,. 2017). GFD and Av4143 are avian-associated markers targeting bacterial groups related to *Helicobacter* spp. and *Lactobacillus* spp., respectively (Rytkönen et al., 2021; Shen et al., 2013; Liang et al., 2021; Ohad et al,. 2016; Green et al., 2012).

For each marker, we selected a target sequence suitable for AsCas12a recognition and designed the corresponding crRNA. A synthetic DNA containing a known AsCas12a target was used as the positive control, while pure water or irrelevant plasmid was used as the negative control. The markers and Cas12a targets used in this study are listed in Table 3_1.

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| Target | Marker | What it represents |
|---|---|---|
| General gut-associated bacteria | **GenBac3** | General Bacteroidales-associated signal |
| Human gut-associated bacteria | **HF183/BacR287** | Human-associated *Bacteroides/Bacteroidales* marker |
| Human-associated phage | **crAssphage (e.g. CPQ_056)** | Phage marker strongly associated with human fecal material |
| Avian-associated bacteria | **GFD** | Avian-associated *Helicobacter* marker |
| Second avian marker | **AV4143** | Specific avian/bird-associated signal |
| Positive control | **N.A.** | Target dsDNA that is complementary to the guide region and harbors the PAM |
| Negative control | **N.A.** | Pure water |

*Table 3_1. The markers and Cas12a targets used in this study.*

The published primer pairs for the five markers were used for PCR amplification, as described in Methods Section 2.6.3. The expected amplicon sizes for GenBac3, HF183/BacR287, CPQ-056, GFD, and Av4143 were 129, 126, 126, 123, and 245 bp, respectively. For each PCR amplicon, we searched for a suitable AsCas12a target containing a 5′-TTTV PAM and selected the adjacent protospacer sequence for crRNA design. Each crRNA was designed with a guide sequence complementary to its target DNA.

Before testing environmental water samples, we first checked whether the AsCas12a detection system worked with synthetic target DNA as a positive control. crRNA1B and crRNA5B were selected for the initial test, and their corresponding double-stranded DNA targets were synthesized. Both target DNAs activated reporter cleavage by their respective AsCas12a–crRNA complexes and produced clear fluorescence signals. The raw fluorescence measurements are shown in Table 3_2, and the corresponding data are presented as a bar graph in Figure 3_3. These results confirmed that the designed crRNAs could recognize their target sequences and that the AsCas12a reporter assay was working under our experimental conditions (Chen et al., 2018).

| crRNA1B | 5 min | 10 min | 15 min | 20 min | 25 min | 30 min | 35 min | 40 min |
|---|---|---|---|---|---|---|---|---|
| target dsDNA | 1576 | 1878 | 2314 | 2850 | 3315 | 3792 | 4314 | 4671 |
| Blank | 269 | 248 | 292 | 273 | 269 | 273 | 272 | 297 |
| | | | | | | | | |
| crRNA5B | 5 min | 10 min | 15 min | 20 min | 25 min | 30 min | 35 min | 40 min |
| target dsDNA | 852 | 1067 | 1322 | 1624 | 1935 | 2277 | 2707 | 2940 |
| Blank | 266 | 272 | 315 | 256 | 285 | 291 | 266 | 284 |

*Table 3_2. The raw data of target dsDNA activating AsCas12a bound to crRNA1B and crRNA5B in a time course.*

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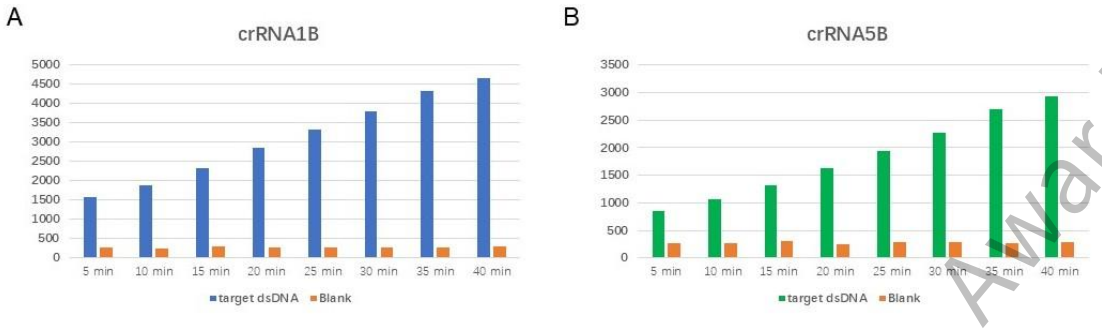



*Figure 3_3. Target dsDNA can activate the AsCas12a-crRNA to cleave fluo-reporter robustly*

## 3.2. Collection and Preparation of Water Samples

### 3.2.1 Selection of Sampling Sites

Water samples were collected from *Yuanmingyuan*, Beijing 101 Middle School, and the Olympic Forest Park. These sites differ in their surroundings and patterns of human and animal activity. *Yuanmingyuan* has extensive park waters with abundant vegetation and waterfowl, together with frequent visitor activity.The campus ponds at Beijing 101 Middle School are closed to *Yuanmingyuan* and exposed to regular student activity and are also home to birds and other animals. The Olympic Forest Park has larger, more open water bodies surrounded by urban green space. Sampling from these three sites allowed us to test the bacterial DNA detection method using water from different environmental settings.

Water sampling was carried out in two rounds. In the first round, two samples (A and B) were collected from Beijing 101 Middle School and used to compare methods for collecting and concentrating bacteria. At the same time, five samples were collected from Yuanmingyuan and designated Y1–Y5. After the first round, we adjusted the sampling plan and carried out a second round of sampling. Three samples were collected from different sites at Beijing 101 Middle School (sampel S1-S3), and three additional samples were collected from Yuanmingyuan and designated Y6–Y8.

For the first sampling round in *Yuanmingyuan*, the five sites differ in human and animal activity as well as in their local water conditions (Figure 3_4). Sites 1 and 4 are isolated water bodies, whereas site2, 3, and 5 are connected. Site 2, a narrow water channal, had man-made shores with frequent activity of tour boats. Significant bird activity had been observed at sites 2, 3 and5. Samples collected from these five sites were designated Y1-Y5, respectively.

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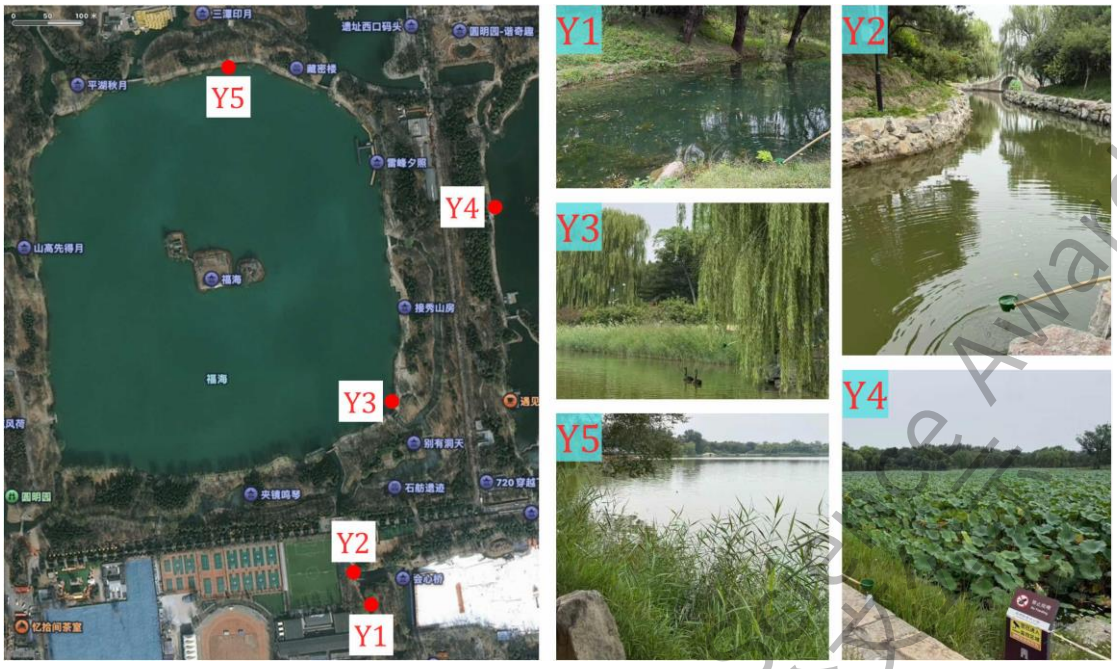



*Figure 3_4. Sites of first sampling round in Yuanmingyuan*

For the second sampling round in *Yuanmingyuan*, the three sites were designated Y6-Y8 (Figure 3_5). All sampling sites are in the same water system. Bird and human activity was observed near the sites across all samples.




*Figure 3_5. Sites of second sampling round in Yuanmingyuan*

For the first sampling round at 101 Middle school, two sites were designated A and B. Both sites belong to a body of water with a purification system, large masses of lotus plants in the center, dwelling of birds, and activities of humans. For the second sampling round at Beijing 101 Middle School, water samples were collected from three separate ponds, with one sample taken from each pond. These samples were designated S1 , S2, and S3.

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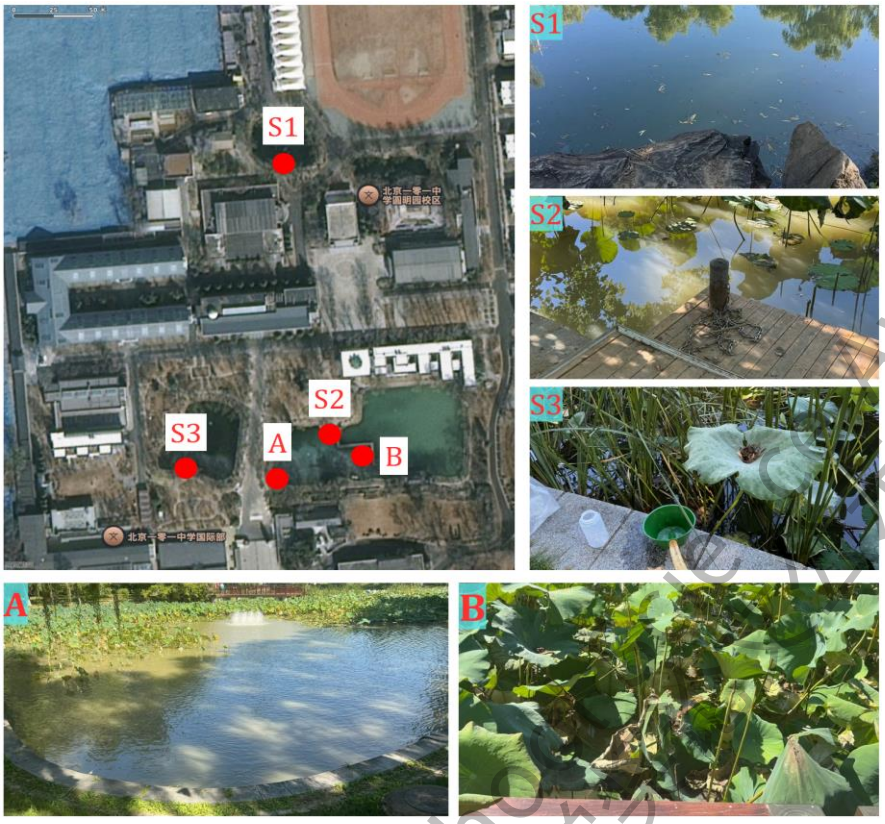



*Figure 3_6. Sites of sampling round in 101 Middle School*

S1 was collected from a pond next to a main road in the northen side of the campus where there is no bird activity. S2 and S3 were sampled from the south side of the campus where birds including geese and ducks are active. The two samples came from separate bodies of water. Several ducks and geese were present at site 2, a purification system and a waterwheel were also observed at this site. S3 was collected from a pond with tall and well-grown lotus plants, close to students' outdoor study tables and activity space, where students also eat near the shore. The water at this site was relatively still. Site 3 had a more artificial shore with stone, site 2 had a wooden platform, and site 1 has less sign of human construction.

Four sampling sites were selected from different areas of the park. Water samples collected from these sites were designated O1–O4. O1 and O2 are connected, and O1 is downstram of O2. There are many ducks in O1 and the water is turbid and smells not good. The water from O2 is relatively clear, and there are some waterfowls in addtion to ducks. There are many tourists along the river of O1 and O2. O3 is far away from O1 and O2, and there are some ducks, and the water is rich in green algae. O4 is a little pond, and the water seems to be some rain water. There were no any fowls observed and little human activity there.

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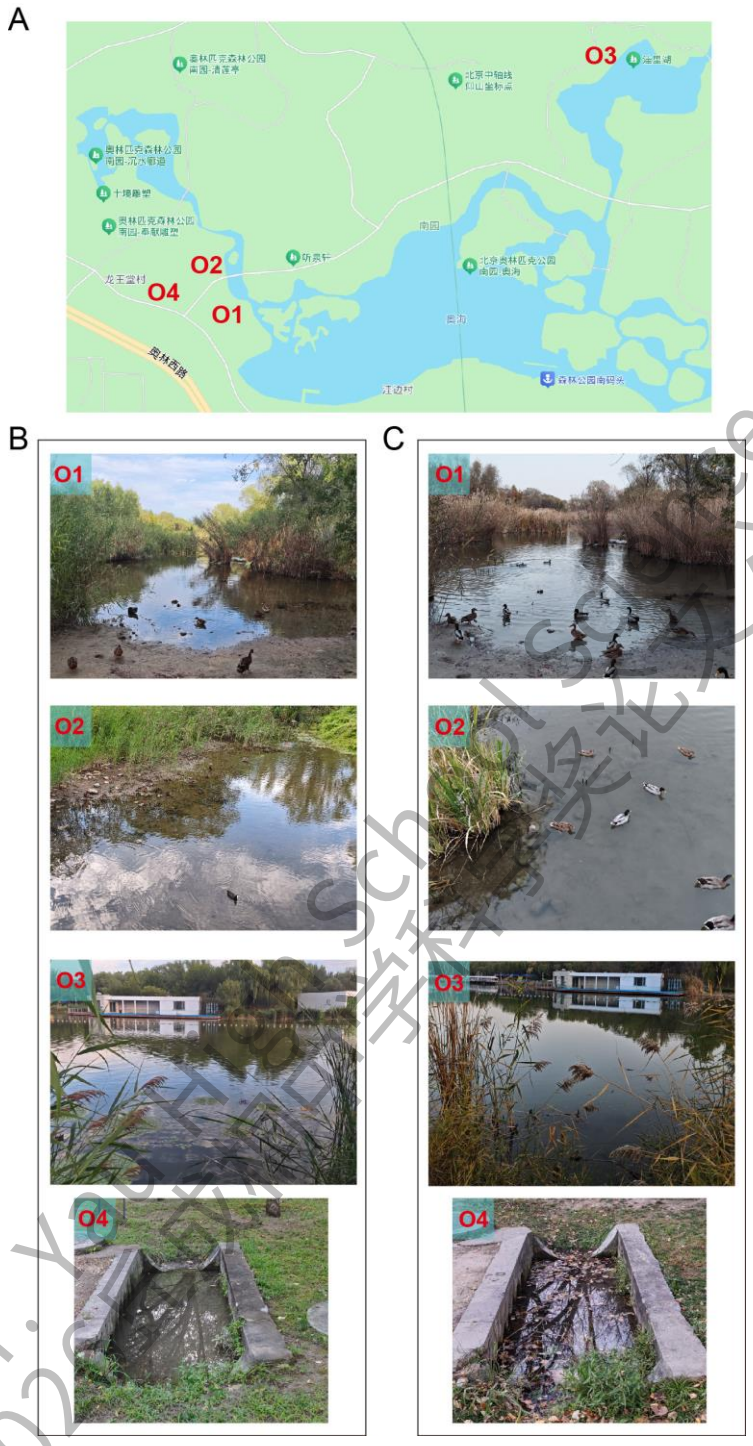



*Figure 3_7. Water samples from the Olympic Forest Park.*

*(A) The map labeled with four sites O1-O4 for water sampling. (B) The photos of sites O1-O4 taken in September. (C) The photos of sites O1-O4 taken in November.*

### 3.2.2 Processing and Concentration of Water Samples

**Optimization of Water Sample Processing**

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Each water sample normally had a volume of 1 L and contained sediment and floating debris. Some bacteria may also have been associated with these particles, making bacterial collection more difficult (Liang & Keeley, 2013). We therefore compared two methods for concentrating bacteria from the water samples.

For this comparison, 2 L of water was collected from each of two sites at Beijing 101 Middle School, designated samples A and B. Each sample was divided into two 1 L portions. One portion was processed by Method 1 (stepwise filtration) and the other by Method 2 (stepwise centrifugation), giving four samples: 1A and 1B for Method 1, and 2A and 2B for Method 2.

Method 1 used two consecutive filtration steps. The water was first passed through a coarse filter to remove sediment and floating debris. The filtrate was then passed through a 0.22 μm membrane to collect and concentrate most bacterial cells for DNA extraction (Deiner et al., 2015; Goldberg et al., 2016). We found that agitating the samples before filtration increased bacterial DNA recovery. Coarse filtration also reduced clogging of the 0.22 μm membrane and shortened the time needed to process the samples.

Method 2 used differential centrifugation. The samples were first centrifuged at low speed to remove sediment and larger particles, while most bacteria remained in the supernatant. The supernatant was then collected and centrifuged at a higher speed to pellet the bacterial cells. The resulting pellets were used for DNA extraction.

We compared the two methods based on the amount of DNA recovered and the intensity of the DNA bands. Samples 1A and 1B processed by Method 1 gave stronger DNA bands than samples 2A and 2B processed by Method 2 (Figure 3_8), indicating a higher DNA recovery with Method 1. Two bands were observed in the samples processed by Method 1. The lower band was consistent with genomic DNA, while the identity of the upper band was not determined.

Based on these results, Method 1 was used to process the remaining water samples. An additional washing step was included before DNA extraction to reduce impurities in the collected material.

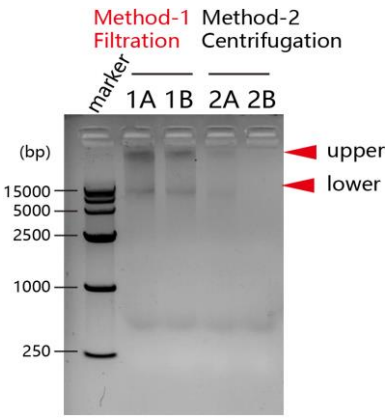



*Figure 3_8. The genome DNA extracted from the tested water samples processed with two methods*

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### 3.2.3 Extraction of bacterial genome DNA

Bacteria were collected from each water sample using the filtration method described above. The amount of bacterial material collected varied among the samples, as shown by the different amounts of bacterial pellets obtained after processing (Figure 3-9). DNA was then extracted from the collected bacteria and analyzed by gel electrophoresis. The DNA obtained from the three sampling locations is shown in Figure 3-10.

Genomic DNA was extracted from bacteria collected from the three sampling locations: Yuanmingyuan, the Olympic Forest Park, and Beijing 101 Middle School. The extracted DNA was analyzed by agarose gel electrophoresis, and a major DNA band was observed in most samples. Sample S2 from Beijing 101 Middle School, and sample Y5 and Y7 from Yuanmingyuan showed stronger DNA bands, suggesting that more bacterial DNA was recovered from these samples. Interestingly, sample S2 from campus was collected from relatively clear water that had passed through a filtration system. However, ducks and geese were commonly present around this sampling site. The relatively strong DNA band observed for S2 may therefore be related to the presence of these birds and the bacterial input associated with their activity.

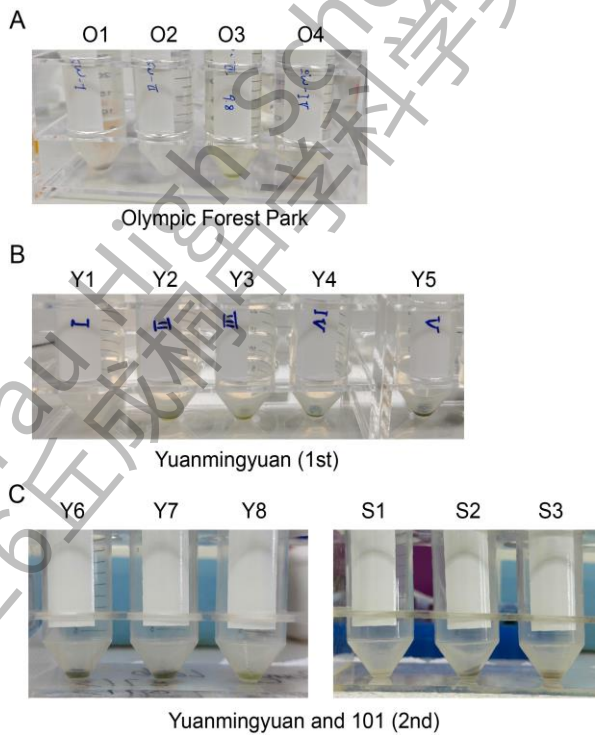



*Figure 3_9. Bacteria cell pellets after water preprocessing.*

For the three batches of water samples, we used method 1 to obtain the bacterial cells (Figure 3_9). Clear water leads to relatively less cell, and turbid water gives more cells. Some water contains dirt band green aalgaes. However, it will not interfer with the DNA extraction (Schrader et al., 2012; Goldberg et al., 2016).

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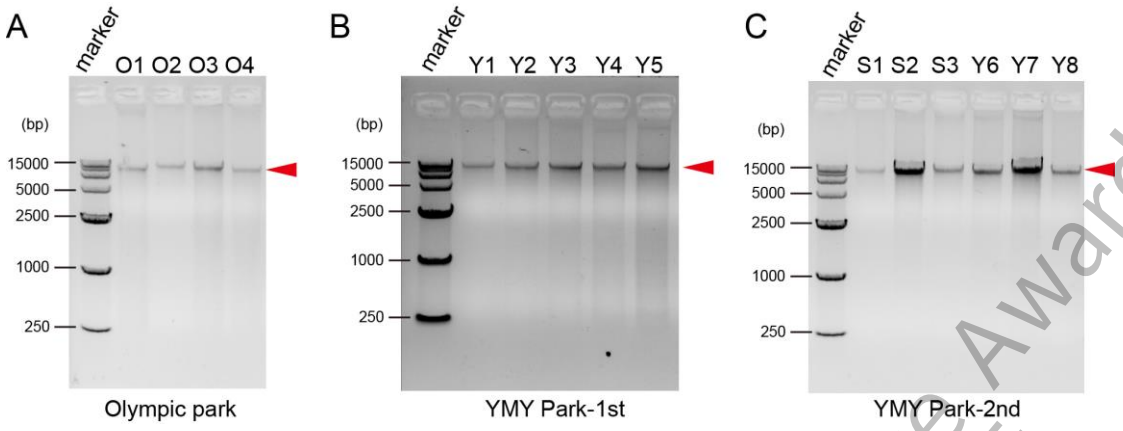



*Figure 3_10. Genome DNA extracted from the bacteria cells after water preprocessing.*

Using the commerical genome DNA extraction kit，we obtained the DNA from the three batches of water samples. Agarose gel showed that the yields of them differ very much (Figure 3_10). However, the purity is high, and the suspected band with protein contamination observed in Figure 3_8 (upper band) is missing. This indicates that we obtained high-quality genome DNA.

## 3.3 Detection of Bacterial DNA in Water Samples Using AsCas12a

### 3.3.1 PCR Amplification of Bacterial DNA from Water Samples

To further test whether our method could be used with real environmental water samples, we first analyzed water samples collected from the Olympic Forest Park in September. We first tested samples form Olympic Forest park （Figure 3_11). Primer 1 and 2 can amplify all or most of the samples. Similarly, both primer 4 and 5 can amplify all the samples, and they both detect domestic fowls and water fowl, therefore we only chose primer 5 for further PCR of other water samples. Primer pair 3 did not produce a clearly identifiable expected-size amplicon in the tested Olympic Forest Park samples. Potential reasons include low target abundance, incomplete recovery of crAssphage-associated DNA, PCR inhibition, or suboptimal amplification conditions. The cause of the absent amplification was not independently determined (Stachler et al., 2017; Schrader et al., 2012).

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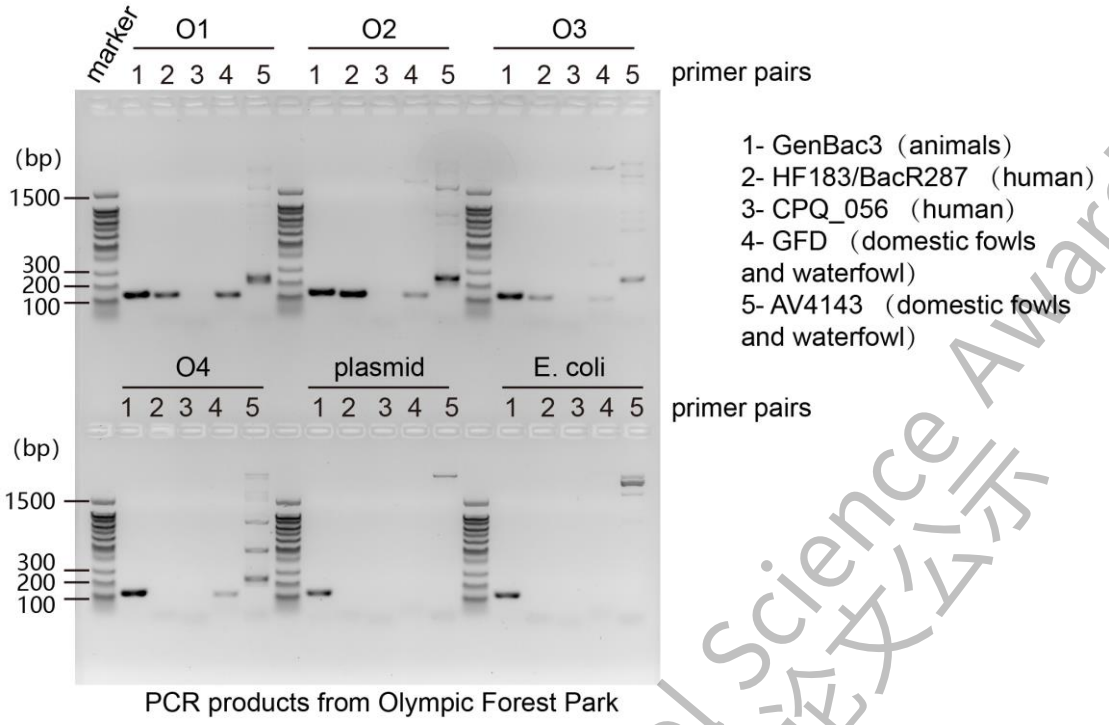



*Figure 3_11. PCR amplifications using genomic DNA templates from a batch of samples collected in September from Beijing Olympic Forest Park.*

Next, we extended our analysis to water samples collected from Yuanmingyuan and 101 Middle School (Figure 3_12). For the two batches of Yuanmingyuan samples, primers 1, 2, and 5 were used to amplify the target fragments. Primer 1 amplified the expected 129-bp target fragment from all samples, including the control water and plasmid DNA, indicating the broad detection range of this primer pair. In contrast, only samples Y5, S2 and S3 showed strong bands with primer 2, while almost no signal was detected in the water and plasmid controls, showing the higher specificity of this primer pair. For primer 5, the band intensities varied among the different water samples.

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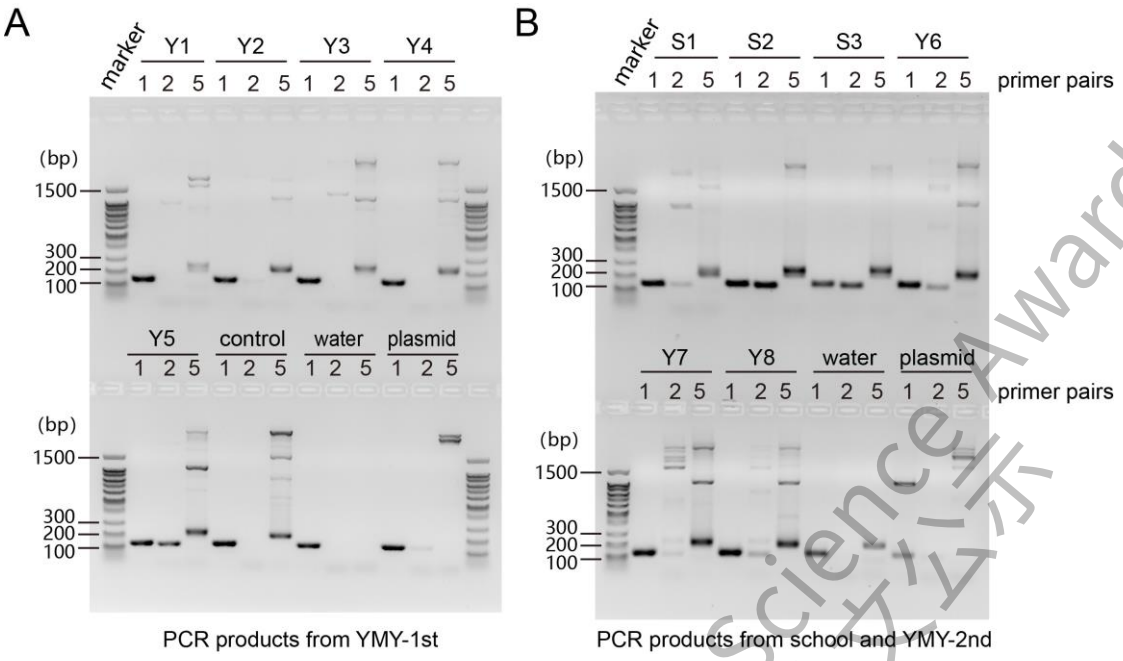



*Figure 3_12. PCR amplifications using genomic DNA templates from two batches samples of Yuanmingyuan Park and 101 high school.*

Finally, to test the reproducibility of our detections, and the impact of temperature on the signals, we collected a second batch of water samples from the Olympic Park in November (Figure 3_13). We used primer 1, 2 and 5 to amplify the two batches of samples collected in both September (O1, O2, O3 and O4) and November (O1-1, O2-2, O3-2 and O4-2). As a result, primer 1 can amplify all the samples. Primer 2 can amplify from O1, O2, O3 except O4. Primer 5 can amplify almost all samples, but gives weak bands for O4 and O4-2.

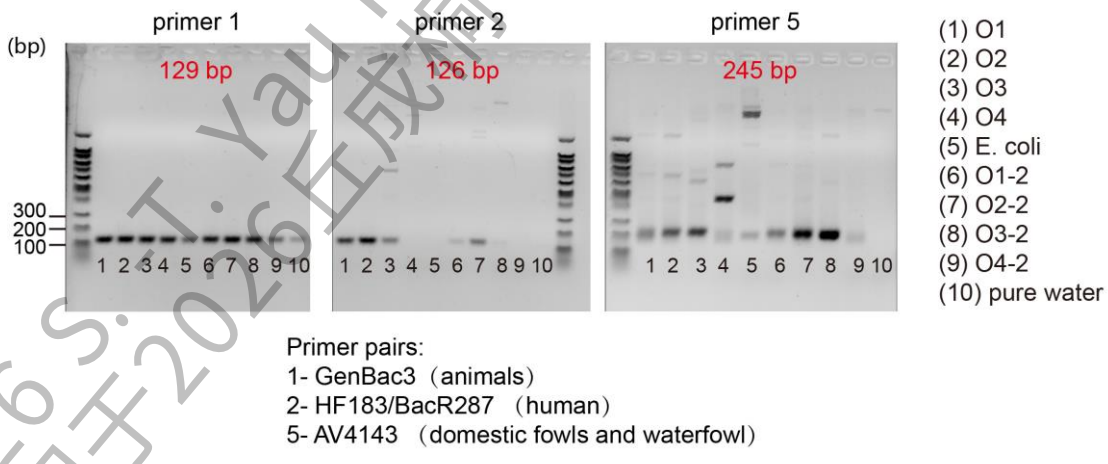



*Figure 3_13. PCR amplifications using genomic DNA templates from the September batch of samples and a new batch of water samples collected in November from Beijing Olympic Forest Park.*

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### 3.3.2 Cas12a-Based Fluorescence Detection of PCR Products

Cas12a, crRNA, and the fluorescent reporter DNA were first mixed and incubated under room temperature for 15 min. The PCR products were diluted threefold and then added to the Cas12a–crRNA–reporter mixture. The reactions were incubated at 37°C for 20 min, and the fluorescence signals were then measured using a microplate reader (Chen et al., 2018).

#### (1) Detection of two batches of Olympic Forest Park Water Samples

Water samples O1–O4 collected from the Olympic Forest Park were analyzed using the Cas12a-based fluorescence assay. The fluorescence measurements are shown in Table 3_3, and the corresponding results are presented as a bar graph in Figure 3_14 and Figure 3_15. Samples O1-O4 all can activate Cas12a, showing the presence of the target sequences in the PCR products, indicating the presence of animals. Interstingly, for crRNA2C which target PCR products from primers 2, no signal was detected for sample 4, indicating there is no much human pollution in the little pond, showing in Figure 3_7. Signals were observed for all four samples for crRNA5B targeting PCR products from primers 5, showing the acitivities of birds and domestic fowls.

All four samples from the Olympic Forest Park showed strong GenBac3 signals, indicating the presence of gut-associated bacteria. Samples O1–O3 also showed strong signals for the human-associated marker, while avian-associated signals were detected in all four samples (Figure 3_14). The Olympic Forest Park has a more natural environment, with extensive vegetation, abundant bird activity, and many visitors. The detection of both human- and avian-associated markers is therefore consistent with the surroundings of the sampling sites. Furthermore, the repeated signals for O1–O4 detections for the first batch of samples agree well. In addition, comparing the sample signals for September and November, we observed less activities of human, birds and waterfowls at site O3, as well as decreased fluorescence signals at O3 for crRNA2C and crRNA5B in November (Figure 3_15).

| RFU | O1 | O2 | O3 | O4 | plasmid | E. coli | target dsDNA | blank |
|---|---|---|---|---|---|---|---|---|
| crRNA1B | 34819 | 37404 | 39675 | 41905 | 33262 | 35338 | 67065 | 418 |
| crRNA2C | 16863 | 17478 | 15817 | 506 | 559 | 663 | 32877 | 640 |
| crRNA5B | 9532 | 7389 | 9653 | 8383 | 529 | 586 | 28099 | 458 |

| RFU | O1 | O2 | O3 | O4 | E. coli | O1-2 | O2-2 | O3-2 | O4-2 | Pure water | target dsDNA | blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| crRNA1B | 38829 | 41037 | 48642 | 51528 | 44151 | 46615 | 44100 | 51810 | 46509 | 38829 | 74710 | 304 |
| crRNA2C | 21002 | 16529 | 17352 | 545 | 596 | 14328 | 13566 | 5338 | 494 | 2664 | 29175 | 528 |
| crRNA5B | 9854 | 8476 | 7399 | 3207 | 516 | 34000 | 6810 | 4115 | 1148 | 298 | 38867 | 372 |

*Table 3_3. Raw data of the fluorescence signals of AsCas12a activated by samples from Olympic Foreset Park. The upper table includes the data of the first batch collected in September, and the lower table includes the repeated data of the first batch as well as the data of the second batch (O1-2, O2-2,*

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*O3-2, O4-2) collected in November.*

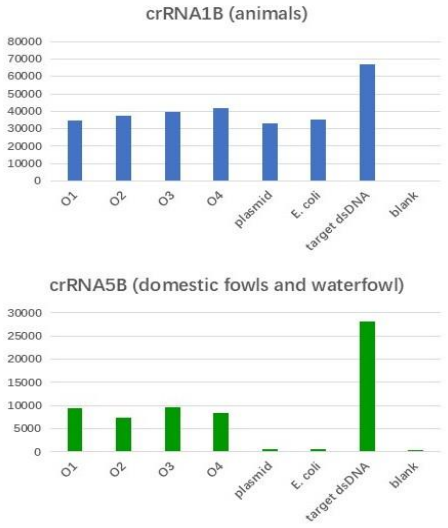



*Figure 3_14. First detection of the fluorescence signals of water samples from Olympic Forest Park.*

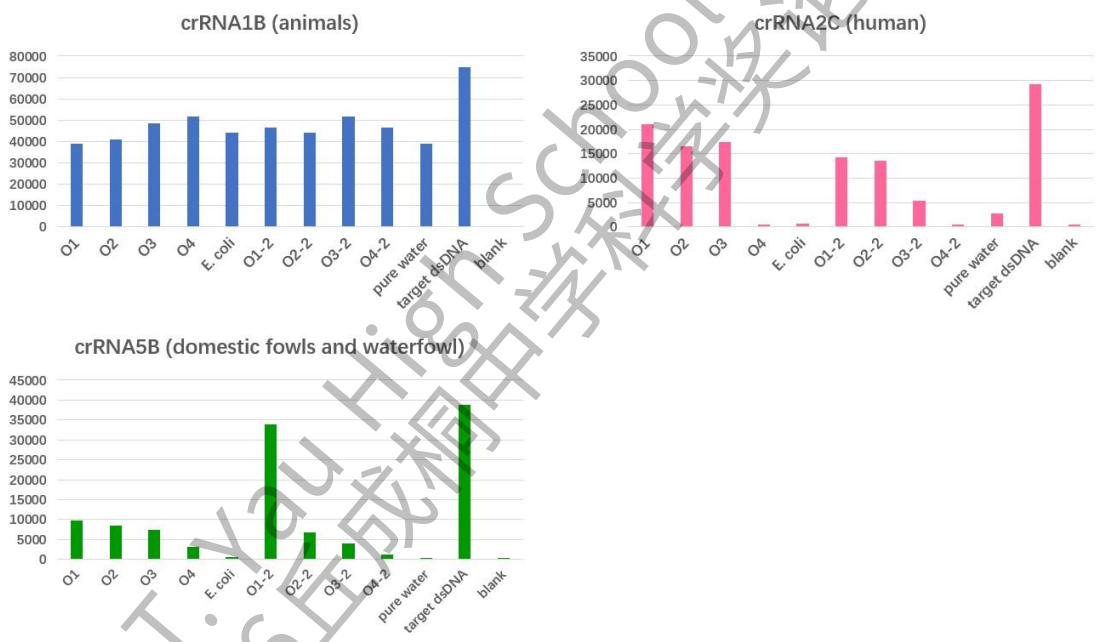



*Figure 3_15. Second Detection of the fluorescence signals of water samples from Olympic Forest Park.*

**(2) Detection of the First-Round Yuanmingyuan Water Samples**

We next analyzed the first batch of water samples collected from Yuanmingyuan (Y1–Y5) using the Cas12a-based fluorescence assay. The fluorescence measurements are shown in Table 3_4, and the corresponding results are presented in Figure 3_16. In the first batch of Yuanmingyuan samples (Y1–Y5), the general gut-associated bacterial marker GenBac3 produced relatively strong signals in most samples. In contrast, a strong signal for the human-associated marker HF183 was mainly observed in Y5. The avian-associated markers showed relatively weak signals across all five samples.

Interestingly, Y1 showed a different pattern from the other samples. This sample was collected from a

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relatively isolated site with few visitors, and the water was turbid with a noticeable unpleasant odor. Y1 showed a strong GenBac3 signal, but relatively weak signals for both the human- and avian-associated markers. This suggests that the gut-associated bacteria detected in Y1 may have come from sources not well represented by the human and avian markers used in our study.

| RFU | Y1 | Y2 | Y3 | Y4 | Y5 | water | plasmid | target dsDNA | blank |
|---|---|---|---|---|---|---|---|---|---|
| crRNA1B | 44950 | 49693 | 39455 | 43720 | 47845 | 51902 | 52648 | 87013 | 588 |
| crRNA2C | 641 | 4771 | 573 | 638 | 17858 | 564 | 9389 | 43127 | 744 |
| crRNA5B | 1019 | 2811 | 4577 | 2213 | 3601 | 444 | 535 | 47298 | 632 |

*Table 3_4. Raw data of the fluorescence signals of AsCas12a activated by samples from the first-round Yuanmingyuan Water Samples.*

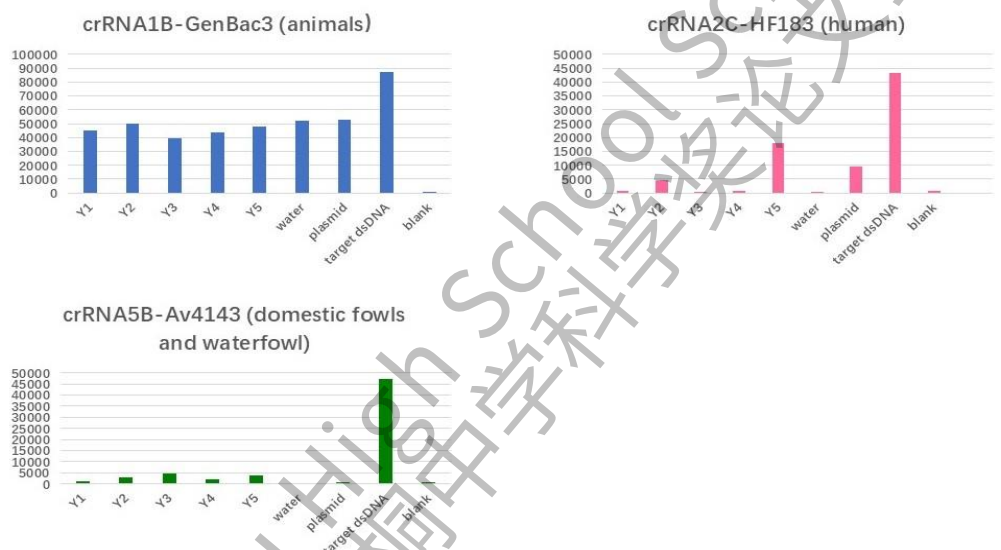



*Figure 3_16. Detection of the fluorescence signals of water samples from first-round Yuanmingyuan Water Samples*

### 3.3.3 Detection of the Second-Round Water Samples

We next analyzed the second batch of water samples collected from Yuanmingyuan (Y6–Y8), together with three samples collected from Beijing 101 Middle School (S1–S3), using the Cas12a-based fluorescence assay. The fluorescence measurements are shown in Table 3_5, and the corresponding results are presented in Figure 3_17.

In the second batch of Yuanmingyuan samples and three smaple from 101 Middle School, GenBac3, a general gut-associated bacterial marker, produced strong signals in most samples. The human-associated marker HF183 also showed relatively strong signals. In contrast, the avian-associated markers produced only weak signals. These results indicate that general and human-associated gut bacterial markers were more readily detected in these samples than the avian-associated markers.

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| RFU | Y6 | Y7 | Y8 | S1 | S2 | S3 | water | plasmid | target dsDNA | blank |
|---|---|---|---|---|---|---|---|---|---|---|
| crRNA1B | 34820 | 40710 | 38278 | 44200 | 39735 | 35033 | 30204 | 22574 | 67957 | 356 |
| crRNA2C | 8144 | 8792 | 9871 | 9892 | 12758 | 18570 | 633 | 6109 | 29164 | 704 |
| crRNA5B | 4104 | 4833 | 4459 | 3260 | 5623 | 7205 | 556 | 582 | 41348 | 389 |

*Table 3_5. Rawdata of the fluorescence signals of AsCas12a activated by samples from the second-round Yuanmingyuan Water Samples*

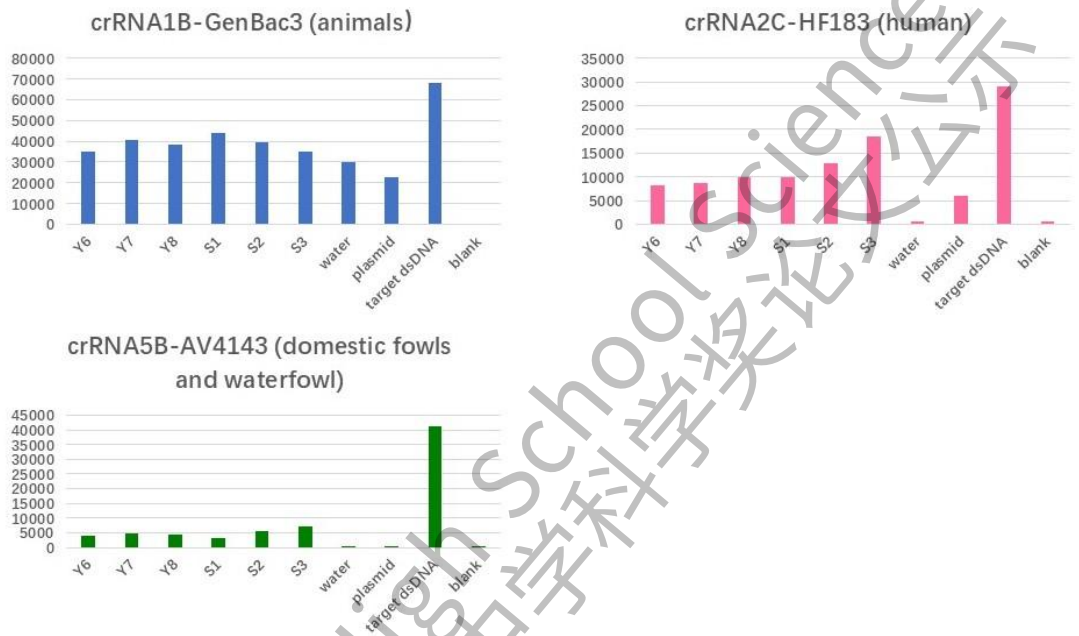



*Figure 3_17. Detection of the fluorescence signals of water samples from second-round Yuanmingyuan Water Samples*

## 4. Discussion

### 4.1 Development and Application of a CRISPR-Cas12a Assay for Urban Environmental Waters

In this study, we established a PCR-assisted CRISPR-Cas12a workflow for detecting selected microbial DNA markers in urban environmental waters. We cloned, expressed, and purified AsCas12a, prepared target-specific crRNAs, and selected microbial genetic markers for PCR amplification and Cas12a fluoro-detection. The assay was applied to water samples collected from three urban environments. The synthetic target experiments demonstrated the functionality of the prepared AsCas12a–crRNA complexes, while environmental samples produced varying fluorescence responses. This study provides an exploratory foundation for further development of Cas12a-based water monitoring with the verified ability to detect specific bacterial DNA markers in urban waters. Potential applications include:

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**(A) preliminary localization of underground sewage leakage or improper sewer connections**

By collecting water samples from strategically selected locations along interconnected drainage networks, elevated human-associated marker signals could help identify regions requiring further investigation, potentially reducing the need to inspect an entire pipeline uniformly. Previous research has demonstrated the use of microbial source tracking to locate sewage cross-connections in stormwater networks (Carson et al., 2024).

**(B) indirect monitoring of bird-associated activity in aquatic environments**

Avian-associated markers such as Av4143 may provide information about microbial inputs originating from bird feces (Ohad et al., 2016). Repeated measurements at selected sites could potentially reveal spatial or seasonal changes in bird-associated fecal inputs without requiring continuous direct observation of birds. This approach may be useful in urban wetlands, park lakes, and nature reserves.

**(C) early screening of waterborne bacterial pathogens**

By redesigning the PCR primers and crRNAs to recognize pathogen-specific genetic targets, the detection framework could potentially be adapted to organisms of public-health concern, such as Pseudomonas aeruginosa, an opportunistic pathogen associated with certain aquatic environments. Previous studies have demonstrated CRISPR-Cas12a-based detection of P. aeruginosa, supporting the technical feasibility of such an extension (Liu et al., 2023). In future applications, the method could provide targeted molecular screening of recreational waters, water storage systems, or other environments where particular pathogens are of concern.

Overall, these applications highlight the potential adaptability of the PCR–Cas12a platform to different environmental monitoring objectives. Future development may prioritize assay specificity, sensitivity, reproducibility, matrix interference, and appropriate quality controls before the system can be used for reliable microbial source attribution, infrastructure screening, or pathogen surveillance.

## 4.2 Consistency Between Signal and Site Characteristics

Fluorescent signals of crRNA1B are of strong magnitude across all samples because the six sampling sites are all under the influence of animals. Signals of crRNA2C are stronger in the three 101 middle school samples than in the three *Yuanmingyuan* samples. This is due to a higher level of human activity in the 101 campus than in *Yuanmingyuan* and the smaller scale of water bodies in the campus. Among the three 101 samples, S3 demonstrated higher signals of crRNA2C than S1. This is because students study and eat near the banks of S3, while they rarely stay around S1. S3 is more artificially constructed, with more surrounding buidings. crRNA5B signals are similar across the three *Yuanmingyuan* samples due to the direct connection between sites and the even occurrence of bird activity in the water system. Among, Y3 and Y5 still demonstrated more signal due to more bird activity in the *Fuhai* region.

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crRNA5B signals in S2 and S3 are higher than that of S1 due to the absence of bird activity in and near Y1. Despite more bird activity present at Y2 during sampling, its larger mass and the presence of a filtration system might have reduced the signal.

For the Olympic Forest Park samples, crRNA2C had similar signals except for O4, because site 4 was a remote, isolated body of accumulated rain. crRNA5B signals are similar across all Olympic Forest Park samples. The signals from the two O1–O4 detections from the first batch of samples align well, demonstrating good experimental reproducibility. In addition, as the environment temperature decreased, we observed less activities of human, birds and waterfowls in site O3, which is consistent with the changes that fluorescence signals at O3 for crRNA2C and crRNA5B decreased.

### 4.3 CRISPR-Cas12a Detection Does Not Require Bacterial Culture

Bacteria are commonly detected by culture-based methods. For example, bacterial culture is widely used in hospitals to identify bacteria from patient samples. However, culture-based methods are relatively slow and may take several days to culture bacteria. In addition, many environmental bacteria are difficult to grow under laboratory conditions (Staley & Konopka, 1985; Rappé & Giovannoni, 2003). In contrast, bacterial culture is not required in CRISPR-Cas12a detection. The total time cost is just several hours, much faster than canonical culture-based bacterial detection. These advantages enable the general use of this method for bacterial DNA detection.

### 4.4 Sample Processing for Urban Water Samples

The two filtration steps served different purposes. The large sediment particles and floating debris were removed during the first coarse filtration step, thus preventing rapid clogging of the 0.22-μm membrane. The second step concentrated bacteria and other microorganisms from the prefiltered water onto the 0.22-μm membrane (Goldberg et al., 2016). Together, these steps made it possible to process larger volumes of water more efficiently and reduced the time required for microbial concentration.

We found that agitating the samples before filtration increased bacterial DNA recovery. Coarse filtration also reduced clogging of the 0.22-μm membrane and made sample processing faster. These observations suggest that some bacteria may be associated with sediment or suspended particles (Liang & Keeley, 2013). Agitation before filtration may help release these bacteria into the water and improve their recovery.

A possible limitation of our experiments is that bacteria that remain tightly associated with large particles may be removed during coarse filtration, resulting in the loss of some bacteria (Liang & Keeley, 2013). The main concern is that bacteria associated with large particles may be lost when sediment is removed,

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whereas insufficient removal of sediment may interfere with filtration and DNA extraction (Deiner et al., 2015). Further optimization will be needed to balance microbial recovery with filtration efficiency. The combination and further comparison of filtration and centrifugation methods may help improve bacterial recovery while keeping the procedure simple (Goldberg et al., 2016).

### 4.5 Target Selection Affects Bacterial Detection

The amount of DNA recovered from each sample did not correlate directly with the Cas12a fluorescence signal. Samples with more total DNA did not necessarily produce stronger signals. This is expected because the extracted DNA represents the overall bacterial community, whereas our Cas12a assay detects only the specific bacterial markers selected in this study.

Interestingly, some relatively clear water samples, such as Y2, gave strong positive signals, whereas several turbid or foul-smelling samples showed only weak signals. A weak signal does not necessarily indicate fewer bacteria. Our targets were mainly selected from 16S rRNA gene sequences associated with human and avian gut bacteria and therefore represent only a small part of the bacterial community. Turbid or odorous water may contain other bacteria associated with organic matter and its decomposition that are not detected by these markers. This could explain why some samples contained relatively large amounts of DNA but produced weak Cas12a signals.

These results also show an important limitation of our current assay: the bacteria we can detect depend strongly on the markers we choose. Additional 16S rRNA gene targets would allow a broader range of bacteria to be detected. Sequencing the water samples could first identify the major bacterial groups present and help us choose more suitable Cas12a targets.

This could also extend the method beyond detecting gut-associated bacteria. For example, future work could identify bacterial groups associated with turbid or foul-smelling urban water and design Cas12a assays specifically for these organisms. Such assays could help investigate the microbial changes associated with poor water quality and unpleasant odors.

### 4.6 Limitations and Future Improvements

The main limitation of this study is the small number of bacterial markers tested. Human- and avian-associated markers cannot represent the full bacterial community in environmental water (Newton et al., 2011). This may explain why some samples with obvious differences in appearance did not show corresponding differences in our assay. We also tested a phage marker associated with human gut bacteria but did not detect a clear signal (Stachler et al., 2017). Two avian-associated markers were included, but signals were detected in only a few samples. The bacteria or phages may have been present at levels below our detection limit, or the markers we selected may not be suitable for these water samples.

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16S rRNA gene sequencing could help identify the major bacterial groups in these samples (Newton et al., 2011). We could then select new Cas12a targets based on the bacteria actually found in the water, rather than relying only on markers selected in advance. Testing more bacterial and phage markers would also give a better singals of the microorganisms present at different sites.

### 5. Conclusion

After comparing the two pretreatment methods—stepwise filtration and stepwise centrifugation—filtration was identified as the better option for pretreating the water samples in this study. Further, this study successfully established a Cas12a-based target nucleic acid detection system. Consequently, the detection signal intensity of the water samples was generally consistent with the level of human and animal activity in the area, confirming the practical feasibility of this CRISPR-based nucleic acid detection approach.

In conclusion, AsCas12a showed good activity and could detect selected microbial DNA in environmental water samples. By using different crRNAs, the method can be adapted to different DNA targets and may be useful for microbial detection in water. Further work is needed to improve sample processing and detection sensitivity and to test more microbial targets. With these improvements, Cas12a-based detection may become a useful method for monitoring microbial DNA in environmental water.

## List of instruments

1. Thermo Scientific Nanodrop2000
2. LongGene T Series multi lock Thermal Cycler for PCR
3. PerkinElmer Multimode Plate Reader for fluorescence reaction signal detection
4. Ultrasonic Cell Disruptor
5. Centrifuge
6. Nickel Gravity Column
7. Heparin Column
8. UV Gel Imager (Tanon MINI SPACE 3000)

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