Volume 10, Issue 9, September – 2025 International Journal of Innovative Science and Research Technology
ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/25sep1355

Advancements in CRISPR-Cas–Based Biosensors for Detecting Water Pollutants and Contaminants

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Publication Date: 2025/09/29

Abstract: These CRISPR-Cas biosensors offer high specificity due to guide RNA programming and exceptional sensitivity often boosted by isothermal amplification, with multiple readout options. This review highlights recent progress in using CRISPR-Cas biosensors to detect vital water pollutants, such as pathogenic microbes, toxins, heavy metals, and organic micropollutants. It examines how these biosensors have transitioned from lab prototypes to field-ready devices, evaluates their effectiveness across various water samples, and discusses the challenges and future opportunities for this innovative technology. The aim is to provide readers with a current overview of the latest advances in CRISPR-Cas biosensor systems, deepen their understanding, and inspire further development of portable CRISPR-Cas technologies.

Keywords: *CRISPR-Cas Biosensors, Water Pollutants and Contaminants, Bacteria, Viruses, Heavy Metals, Biotoxins.*

How to Cite: Ishmeal Kwaku Duah (2025) Advancements in CRISPR-Cas–Based Biosensors for Detecting Water Pollutants and Contaminants. *International Journal of Innovative Science and Research Technology*, 10(9), 2072-2078. https://doi.org/10.38124/ijisrt/25sep1355

I. INTRODUCTION

Water is a limited and essential resource vital for human health, ecosystems, and economic growth. Yet, global water supplies face increasing threats from various chemical and biological pollutants. Traditional water quality testing methods, such as ICP-MS for heavy metals, HPLC for organic compounds, and qPCR or cell culture for microbes, are highly precise and sensitive. However, they tend to be costly, time-consuming, and require advanced lab facilities and skilled personnel [1-4]. This dependence on centralized labs causes delays from sample collection to data analysis, which hampers quick responses to contamination and makes real-time monitoring difficult.

The development of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) Systems has fundamentally transformed molecular diagnostics. Originally identified as an adaptive immune mechanism in bacteria and archaea, CRISPR-Cas has evolved into a powerful gene-editing technology. Recently, the discovery that some Cas nucleases, especially Cas12 and Cas13, possess collateral cleavage activity has highlighted their potential for nucleic acid detection. This process involves the Cas enzyme, which, upon detecting its specific target sequence, becomes hyperactivated and non-selectively cleaves nearby single-stranded DNA (ssDNA) or RNA reporters [5-14]. This mechanism forms the basis of a new generation of biosensors.

These CRISPR-Cas-based biosensors combine high specificity, thanks to guide RNA programming, with exceptional sensitivity, often enhanced by isothermal amplification, and provide various readout formats. This review summarizes recent advances in using CRISPR-Cas-based biosensors to detect key water pollutants, including pathogenic microbes, toxins, heavy metals, and organic micropollutants. I explore how these biosensors have evolved from laboratory prototypes to field-ready devices, assess their performance in different water samples, and discuss the challenges and future prospects of this innovative technology.

II. CRISPR-CAS-BASED BIOSENSOR DETECTION PRINCIPLES

Most CRISPR-based biosensors rely on the target-activated, non-specific collateral cleavage activity of specific Cas nucleases. The two leading enzymes in this area are Cas12a and Cas13a.

Cas12a is an RNA-guided DNA endonuclease that, upon binding to its target dsDNA near a PAM, becomes activated. This activation enables its “collateral” or “trans-cleavage” activity, leading it to cut nearby single-stranded DNA (ssDNA) molecules indiscriminately. Using ssDNA reporters tagged with a fluorophore and a quencher, the resulting cleavage produces a detectable fluorescent signal.

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Cas13a is an RNA-guided endonuclease that targets single-stranded RNA (ssRNA). When it binds to its specific ssRNA target, the Cas13a-crRNA complex activates a strong collateral ribonuclease activity, which cleaves nearby non-target ssRNA molecules. Like Cas12a, this activity can be used to produce a signal with ssRNA reporters.

III. BACTERIA AND VIRUSES

Waterborne pathogens, including bacteria, viruses, and protozoa, pose a direct and severe threat to public health. Rapid and accurate detection is crucial for preventing outbreaks of diseases like cholera, typhoid, and gastroenteritis. CRISPR-based sensors have been developed to detect a variety of pathogenic bacteria and viruses effectively. A study presented a CRISPR/Cas13a-based system combined with chemiluminescence resonance energy transfer (CRET) for sensitive, on-site detection of pathogenic bacteria in real samples (Fig. 1). When the hybrid double strand of aptamers S. aureus recognized the target bacteria, Staphylococcus aureus (S. aureus), the released cRNA bound with CRISPR/Cas13a to form a complex that cleaved the RNA in the probe comprising horseradish peroxidase (HRP)-modified gold nanoparticles (AuNPs) linked by RNA (AuNPs-RNA-HRP). This led to an increased chemiluminescence signal through the CRET "OFF" phenomenon after the addition of luminol. The strategy successfully identified S. aureus in drinking water and milk, with detection limits of 20 and 30 cfu/mL, respectively, and recovery rates ranging from 90.07% to 105.50%. Additionally, incorporating an immunochromatographic test strip (ICTS) enabled on-site detection of as low as 102 cfu/mL of S. aureus in drinking water and milk using a smartphone, roughly ten times more sensitive than previous AuNPs-based colorimetric ICTS, offering a practical and sensitive method for detecting S. aureus in real samples [15]. Zhu et al. developed a rapid, highly sensitive, and visual method for detecting E. coli O157:H7 by combining Recombinase-Aided Amplification (RAA) with CRISPR/Cas12a technology. The system first amplifies samples using RAA, allowing for detection limits of ~1 CFU/mL with fluorescence and 1×10^2 CFU/mL with the lateral flow assay. These sensitivities are significantly better than traditional real-time PCR (10^3 CFU/mL) and ELISA (10^4–10^{14} CFU/mL) [16]. Fluorescence-based methods are known to have high sensitivity compared to the colorimetric method [17]. The method proved effective in real-world samples, such as milk and drinking water. Notably, the entire process—from extraction to detection—can be completed in 55 minutes under optimized conditions, faster than most other sensors, which can take hours or days. Signal detection is either visualized with a handheld UV lamp via fluorescence or by a naked-eye lateral flow assay, depending on the DNA reporters. Given its speed, high sensitivity, and minimal equipment needs, this approach offers a promising tool for on-site detection of trace pathogens. Patnaik et al. developed a CRISPR-based diagnostic method utilizing nucleic acid pre-amplification for the detection and monitoring of Bacillus anthracis Sterne. They employed the Strand Invasion-Based Isothermal Amplification (SIBA) platform along with Cas12a (a CRISPR endonuclease) to create the versatile CRISPR-SIBA diagnostic system. SIBA served as the isothermal pre-amplification step, while the Cas12a collateral trans-cleavage reaction enhanced the system's specificity. The detection's effectiveness was tested in complex wastewater samples containing Bacillus anthracis Sterne. Targets included previously identified genes Prophage 3, Cya, and Pag. The amplification method reliably provided specific detection within 40 minutes, with a sensitivity of 100 colony-forming units. Endpoint fluorescence from CRISPR collateral cleavage reactions achieved a detection limit of 10^5 to 10^6 CFUs [18]. A study integrated propidium monoazide (PMA) with recombinase polymerase amplification (RPA) and the CRISPR/Cas12a system to develop a rapid, visual detection method for viable Salmonella, targeting the fimY gene. The DNA from viable Salmonella was amplified and detected visually within 60 minutes, effectively excluding dead cells. The study evaluated the assay's specificity and sensitivity, with results indicating high specificity and no cross-reactions with other pathogens. Using PMA did not affect the sensitivity of the RPA-CRISPR/Cas12a system or the fluorescence signal. The system successfully detected viable Salmonella in wastewater at a minimum limit of 10 CFU/mL. Overall, this integrated PMA-RPA-CRISPR/Cas12a method enables the quick and visual detection of viable Salmonella in wastewater at concentrations as low as 10 CFU/mL. By combining PMA with RPA-CRISPR/Cas12a, the system provides a valuable tool for sensitive, efficient, and straightforward detection of viable Salmonella in wastewater [19]. A portable paper-based device leveraging CRISPR/Cas12a and RT-LAMP was developed for SARS-CoV-2 detection in wastewater, offering high sensitivity and specificity. It used three RT-LAMP primer sets and guide RNAs to target the N, E, and S genes, facilitating precise detection through base pairing. The CRISPR/Cas12a trans-cleavage activity enabled visualization with fluorescence or lateral flow probes, using carboxyfluorescein-ssDNA-Black Hole Quencher-1 and carboxyfluorescein-ssDNA-biotin, respectively. The integrated paper device can detect these genes simultaneously, with detection limits of 25, 310, and 10 copies/mL, respectively. It provides semiquantitative results across a range of 0 to 310 copies/mL, based on different LODs for each gene. Validation studies showed 97.7% sensitivity and 82% semiquantitative accuracy in wastewater testing. This marks the first semiquantitative endpoint detection approach utilizing varied LODs, representing a promising point-of-use method for wastewater-based SARS-CoV-2 surveillance [20]. Cheng et al. reported a universal one-pot fluorescent detection method for epidemic pathogens, providing results in 15-20 minutes. This approach employed heparin sodium to fine-tune Cas12's cis-cleavage ability by interfering with the Cas12a-crRNA interaction, leading to increased fluorescence from isothermal amplification products. It is compatible with both traditional and suboptimal PAMs, as well as various Cas12a subtypes such as LbCas12a, AsCas12a, and AapCas12b. The method exhibits over 95% sensitivity and specificity in detecting monkeypox pseudovirus, influenza A virus, and SARS-CoV-2 from saliva or wastewater, outperforming qPCR or RT-qPCR [21]. The cost of heparin sodium for a thousand tests is only $0.01 to $0.04. Overall, this rapid, universal one-pot

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/25sep1355

technique utilizes heparin sodium and holds promising potential for point-of-care testing. Sun et al. developed a detection method based on RPA-CRISPR/Cas13a, aiming to provide a practical prevention strategy for public health threats from future viral pandemics. Using NoV GII.4 as a test case, they compared the virus detection capabilities of this new method with those of traditional PCR in various water environments. They discussed the challenges of CRISPR technology in quantitative detection. The method can produce results in 30 minutes and shows no cross-reactivity with other strains [22].

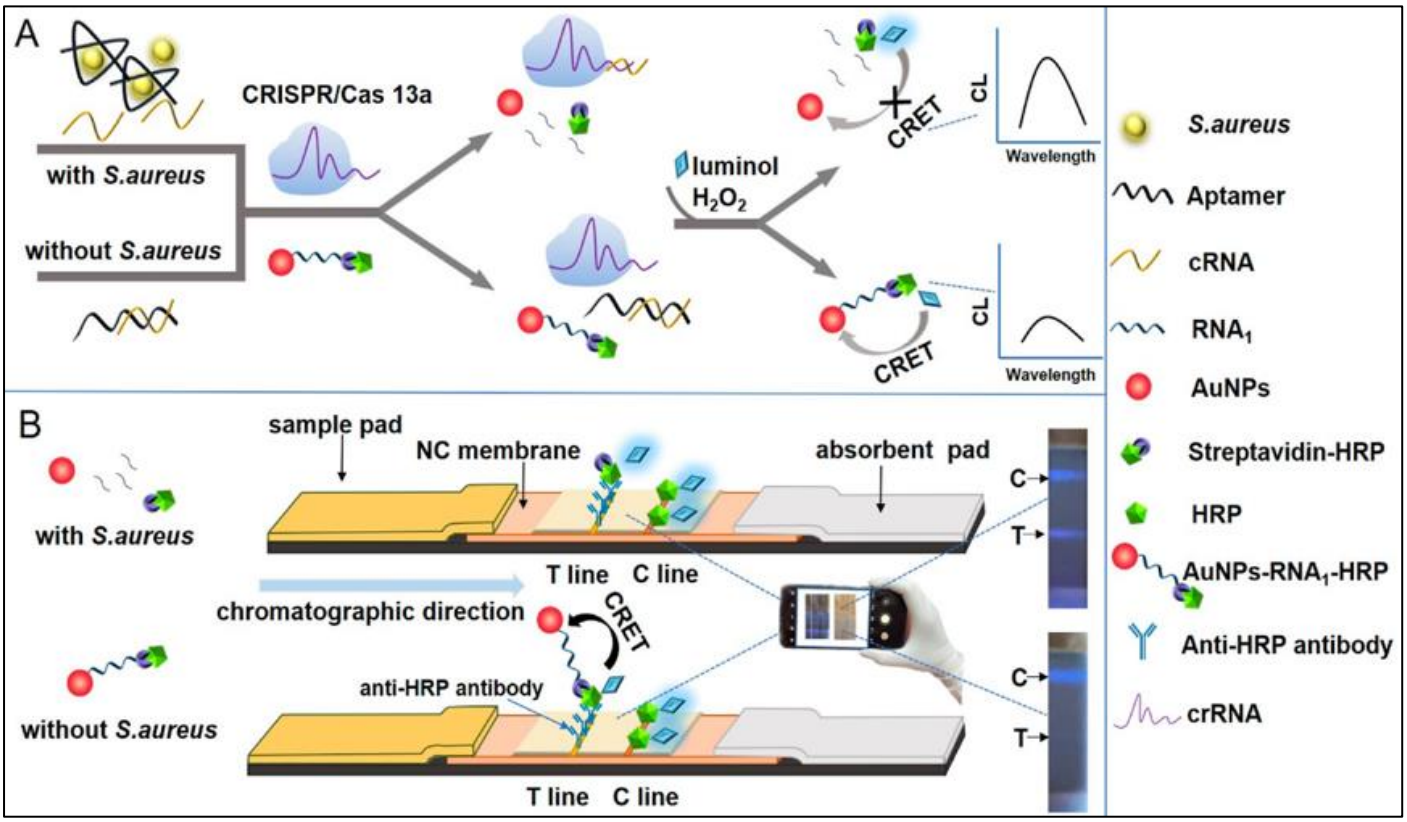

Fig 1 Illustration of (A) CRISPR/Cas13a-Assisted CRET and (B) ICTS-Based ICTS-CRISPR/Cas13a-CRET for Detecting *S. aureus*. Reproduced from Reference [15] with Permission from the American Chemical Society (Copyright 2024)

IV. DETECTION OF BIOTOXINS

Harmful algal blooms (HABs), particularly those caused by cyanobacteria, produce potent toxins called cyanotoxins that contaminate drinking and recreational water sources. Among these, microcystins are some of the most common and toxic. Kang et al. developed an MC-LR-Casor aptasensor platform based on CRISPR-Cas12a for on-site, sensitive detection of microcystin-LR (MC-LR) (Fig. 2). The MC-LR aptamers, after hybridizing with blocker DNA, were attached to magnetic beads (MBs) to create the MB aptasensor. When MC-LR was present, it interacted with the aptamers, triggering the release of blocker DNA. Using the programmable nature of the CRISPR-Cas system, the released DNA was designed to activate a Cas12a-crRNA complex, which rapidly cleaved single-stranded DNA reporters. Signal detection was possible via fluorometers or lateral flow strips, with signals proportional to MC-LR levels. Thanks to the CRISPR-Cas12a amplification, the system achieved high sensitivity, with detection limits around 3×10^{-6} µg/L (fluorescence) or 1×10^{-3} µg/L (lateral flow) [23]. The platform also demonstrated excellent selectivity and good recovery rates, suitable for analyzing real water samples. The entire assay required only two incubation steps at constant temperature and could be visualized with flow strips. This method provides a simple, convenient option for in situ MC-LR monitoring, showing great potential for future environmental surveillance. A new dual-amplification system utilizing CRISPR-Cas12a and horseradish peroxidase (HRP) was created for color-based detection of MC-LR [24]. This system combines the nuclease activity of CRISPR-Cas12a with the redox activity of HRP. HRP attached to magnetic beads via ssDNA (MB-ssDNA-HRP) triggers a color change in the TMB-H2O2 chromogenic solution. When MC-LR binds specifically to its aptamer, it releases a complementary DNA (cDNA) that activates CRISPR-Cas12a's trans-cleavage activity. Activated Cas12a then cleaves the ssDNA linker on MB-ssDNA-HRP, reducing HRP on the beads. As a result, the UV–Vis absorbance of the HRP-driven reaction decreases. This dual-signal amplification enables the colorimetric detection of MC-LR from 0.01 to 50 ng/mL, with a limit of detection (LOD) of 4.53 pg/mL. The method's practical application was confirmed by testing spiked real water samples, achieving recoveries from 86.2% to 118.5% and RSDs between 8.4% and 17.6%. This research offers new ideas for designing effective signal amplification platforms and presents a simple, accessible colorimetric method for detecting trace amounts of MC-LR. A dual-mode biosensor

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/25sep1355

combining fluorescent and colorimetric detection was developed using CdTe QDs and CRISPR/Cas to rapidly and highly sensitively identify microcystin-leucine-arginine (MC-LR). The sensor primarily activated Cas12a's trans-cleavage activity through nucleic acid amplification methods, such as bidirectional rolling circle amplification (B-RCA). This process enables signal conversion, releases Ag+ from a cleaved hairpin, quenches QD fluorescence, and produces a detectable signal. Capable of both fluorometric and colorimetric assays, it allows for quick field testing. The biosensor shows improved selectivity, higher sensitivity, and better accuracy, with a detection range of 0.05 to 500 nM and a minimum detection limit of 2.137 pM, outperforming traditional techniques [25]. It effectively detects MC-LR in environmental samples. Overall, this approach demonstrates the potential of nucleic acid detection technology for tracing environmental pollutants.

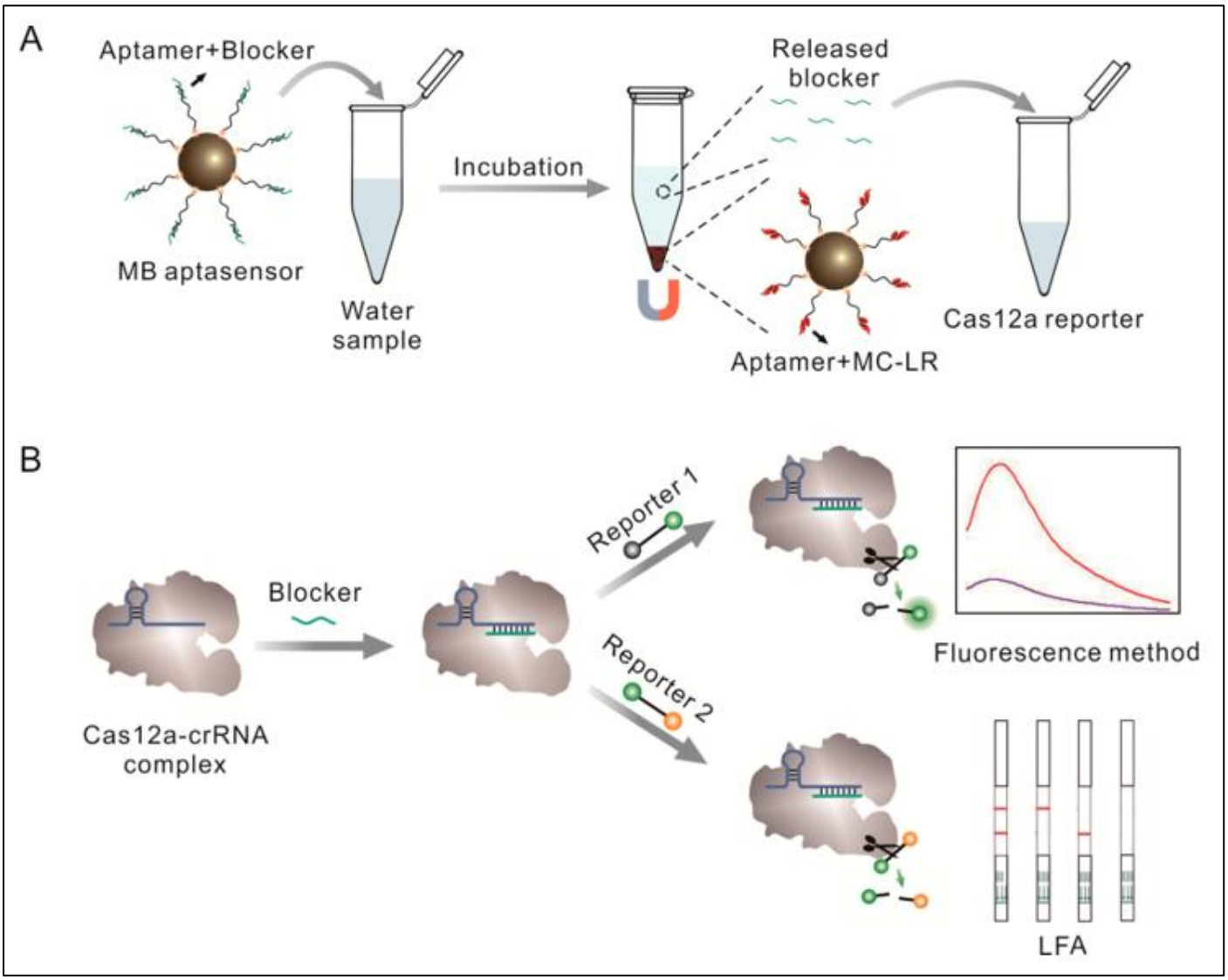

Fig 2 (A) Procedures for Blocker DNA Releasing and Magnetic Separation of MB Aptasensors. (B) Procedures for CRISPR-Cas12a Activation and Signal Readout. Reproduced from Reference [23] with Permission from the American Chemical Society (Copyright 2022)

V. HEAVY METALS

Expanding CRISPR-based detection to include non-biological chemical pollutants such as heavy metals is a major challenge and an exciting area of research. Heavy metals such as mercury (Hg^{2+}), lead (Pb^{2+}), and cadmium (Cd^{2+}) are highly toxic and persistent pollutants in the environment. Kong et al. introduced a CRISPR-based technique called CRISPR-Hg for detecting Hg^{2+} (Fig. 3). The method uses a CRISPR/Cas12a system activated by PCR products, which then produces fluorescence signals through trans-cleavage activity. CRISPR-Hg demonstrates excellent selectivity and specificity, with a detection limit of 10 pM and low interference from background signals. It has been successfully used to detect Hg^{2+} in real samples such as water, soil, and mushrooms. Additionally, a portable device was developed that allows fluorescence readout via a smartphone within 30 minutes. This affordable, highly selective, and visually clear method offers broad potential for Hg^{2+} monitoring in food safety and public health [26]. Zhou

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/25sep1355

et al. described a simple, highly sensitive visual detection method for MT, a biomarker indicating heavy metal ion pollution in water from fish [27]. The method works by MT binding to Hg2+ in hairpin DNA probes, causing the hairpin to unfold into ssDNA. These ssDNAs then hybridize with partial dsDNA duplexes through strand displacement, producing specific dsDNA sequences. Cas12a/crRNA recognizes these sequences and activates its enzyme activity to cyclically cleave ssDNA linkers in blue gold nanoparticle aggregates, causing a change in their color to red for visual detection. Due to Cas12a/crRNA's signal amplification, as little as 25 nM of MT can be visually detected by the naked eye. The method also exhibits high selectivity for MT over other proteins and successfully detects MT in the livers and kidneys of crucian carp from a local supermarket. pollution.

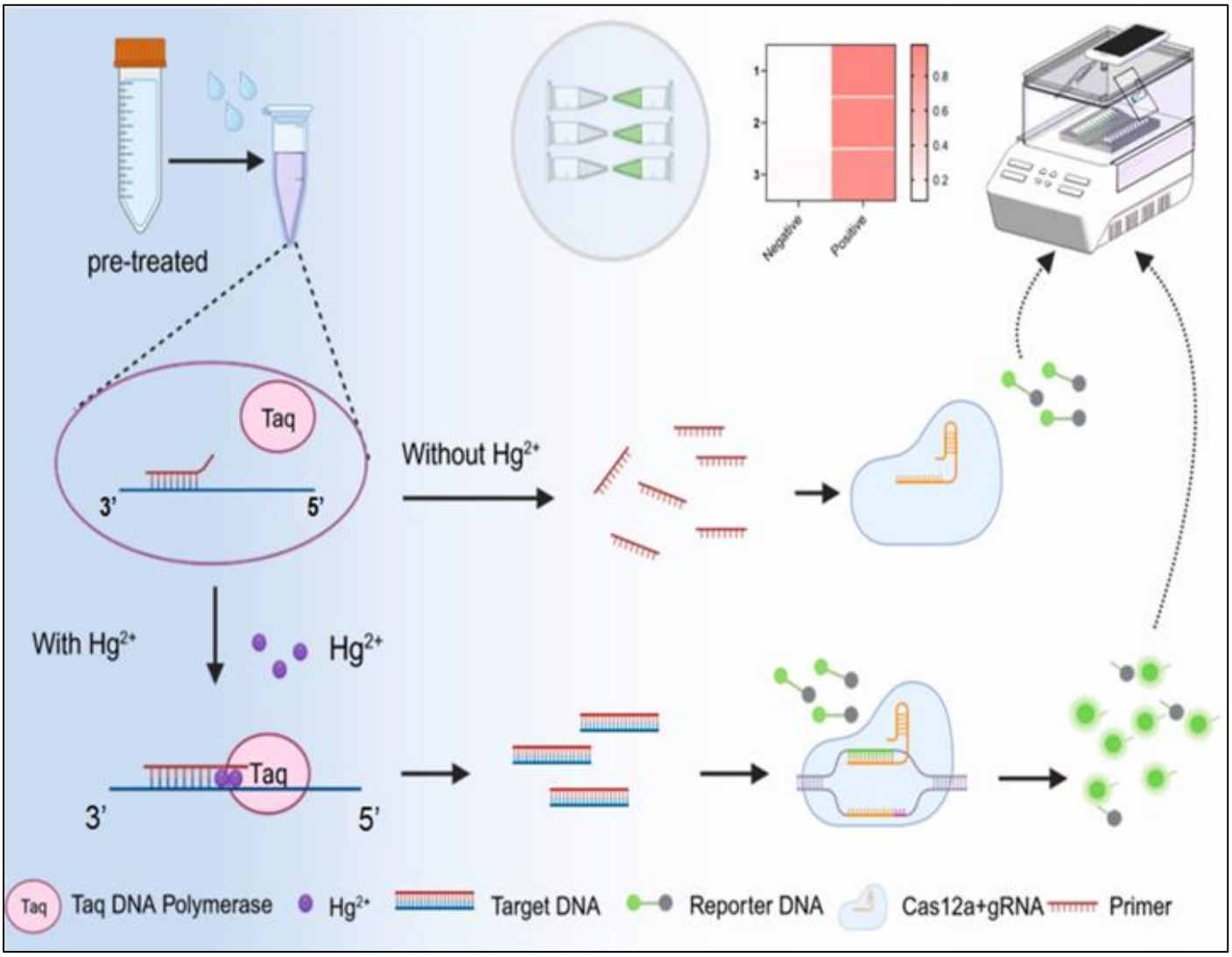

Fig 3 Schematic of Visual Detection for Hg2+ Based on T-Hg2+-T Triggered PCR Coupled with CRISPR/Cas12a (CRISPR-Hg). Taq, Taq DNA Polymerase; gRNA, Guide RNA. Reproduced from Reference [26] with Permission from Elsevier (Copyright 2024)

VI. RESEARCH GAPS AND FUTURE DIRECTION

Despite progress, several hurdles limit the widespread use of CRISPR-based water sensors. Although detection is fast, sample preparation steps such as filtration, concentration, and nucleic acid extraction can still slow down on-site testing. Automating these processes in self-contained cartridges is a key future target, as noted in the patent analysis [Patent-Search]. Furthermore, complex water samples like wastewater and surface water contain organic matter, salts, and other substances that can inhibit reactions. Although many studies have reported success with these matrices, more dependable, universal methods to counteract inhibition are necessary. The main challenge is detecting small molecules that lack natural nucleic acids. While aptamer- and DNAzyme-based techniques are promising, creating effective recognition elements for heavy metals, pesticides, PFAS, and pharmaceuticals remains a major research focus. Direct detection of inorganic ions is also a significant existing gap.

The future of water sensing using CRISPR-Cas technologies is likely to feature fully integrated, automated systems. These systems will incorporate automated sample preparation, isothermal amplification, and CRISPR detection

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/25sep1355

all within a single disposable cartridge. Data will be read and transmitted through a smartphone or IoT-enabled device. Moreover, research into novel Cas enzymes with diverse characteristics, such as enhanced thermostability or smaller size, like Cas14 or Cas12f, will expand the spectrum of diagnostic choices.

VII. CONCLUSION

CRISPR–Cas–based biosensors have shifted from a theoretical idea to a robust, practical technology for water quality monitoring. Recent advancements show these platforms can identify a wide range of pollutants, including pathogenic bacteria and viruses, biotoxins, heavy metals, and organic compounds, with sensitivity and precision often comparable or superior to traditional laboratory methods. The main benefits of CRISPR-based detection include speed, affordability, portability, and adaptability. Multiple studies have demonstrated their effectiveness, and they are now evolving into commercially available products. Although issues like sample preparation, multiplexing, and detection of non-nucleic acid targets still pose challenges, innovation is progressing rapidly. Once these obstacles are addressed, CRISPR-based sensors are set to become essential tools in the global effort to protect water resources.

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