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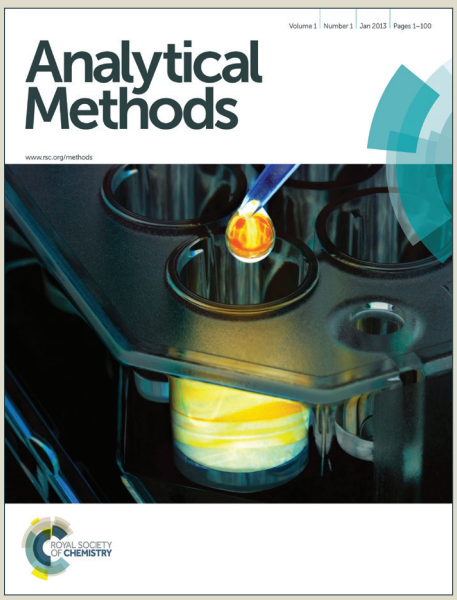

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Title:

Electrochemical Detection of Pathogenic Bacteria by Using Glucose Dehydrogenase Fused Zinc Finger Protein

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Abstract

We developed an electrochemical detection system for pathogenic bacteria by utilizing a glucose dehydrogenase-fused zinc finger protein (ZF-GDH), which could detect PCR products electrochemically without the need for DNA probe hybridization. Using ZF-GDH, we could specifically detect 10 copies of genomic DNA derived from *Escherichia coli* O157.

Introduction

Detecting and identifying pathogenic bacteria and viruses are important for the food industry, clinical diagnosis, and environmental control[1]. The polymerase chain reaction (PCR) is commonly used to detect these bacteria specifically, rapidly, and sensitively. Although quantitative PCR using DNA intercalators is the most commonly used method to quantify PCR products, it cannot distinguish specific PCR products from non-specific PCR products. Thus, to apply PCR to pathogen detection, a sequence-specific PCR product detection system is required.

We previously reported a DNA sequence-specific detection system using a zinc finger (ZF) protein[2-5]. A ZF protein can bind to double-stranded DNA (dsDNA) and specifically recognize its target sequence. The most common type of ZF protein is the C_2H_2 ZF protein comprising several tandem ZF domains, which consist of two β-strands and one α-helix that specifically recognize a 3-bp dsDNA sequence[6-9]. Because this type of ZF protein can be designed to bind to any sequence[10], C_2H_2 ZF proteins have been widely used as dsDNA recognition elements for artificial transcriptional factors, ZF nucleases, and dsDNA detection elements[11-13]. Using a ZF protein, we detected dsDNA without purifying single-stranded DNA or hybridizing DNA probes, which involve time-consuming and troublesome steps. Moreover, because a ZF protein has sequence specificity, we could check the sequences of PCR products and distinguish between specific and non-specific PCR products.

In our previous study, we constructed fusion proteins of Zif268 or Sp1 ZF proteins with firefly luciferase that maintained the activities of both luciferase and ZF[4]. We detected dsDNA PCR products by measuring luciferase activity with high sensitivity. In this study, we focused on glucose dehydrogenase (GDH) as the reporter enzyme in place of firefly luciferase. Because GDH activity can be measured electrochemically using miniaturized commercial devices, such as a blood glucose sensor, GDH is an attractive enzyme not only for glucose measurements but also for various applications as a reporter enzyme. We previously reported on various electrochemical sensing systems using GDH[14-17]. Because an electrochemical detection system can be combined with an on-chip PCR system, we considered that a miniaturized electrochemical detection system could be constructed to detect pathogenic bacteria. Thus, in this study, we constructed

ZF fused to GDH and applied this to pathogenic bacteria detection (Fig. 1).

Results and Discussion

We fused flavin adenine dinucleotide-dependent GDH from fungi, which is a monomeric GDH, to the C terminus of Zif268, one of the natural ZF proteins found in mice. Hereafter, this fusion protein is referred to as "Zif268-GDH." This fusion protein was recombinantly expressed in *Escherichia coli* BL21 (DE3) and purified using Strep-tag. Purified Zif268-GDH retained approximately 50% of its enzymatic activity compared with wild-type GDH.

We analyzed the binding specificity of Zif268-GDH for its target dsDNA, 5′-GCGTGGGCG-3′, using a micro titer plate assay. We immobilized dsDNA or non-target dsDNA on a plate via an avidin–biotin reaction and added Zif268-GDH. After washing, we measured GDH activity colorimetrically using 2,6-dichlorophenolindophenol (DCIP) and phenazine methosulfate (PMS). With target dsDNA immobilization, higher GDH activity was found than with non-target dsDNA. These results showed that Zif268-GDH retained both GDH activity and its binding to the Zif268 recognition sequence (data not shown).

Next, we performed electrochemical detection of a 49-bp synthetic oligonucleotide that contained the target sequence for Zif268. First, we immobilized different concentrations of this synthetic oligonucleotide on NeutrAvidin-coated beads via an avidin–biotin reaction and subsequently added Zif268-GDH (f.c. 100 nM) in the presence of skimmed milk to prevent non-specific Zif268 binding. After washing, we measured GDH activity electrochemically. During electrochemical measurements, we directly added the prepared beads sample on a DEP Chip (BioDevice Thechnology, Ishikawa, Japan) and used 1-methoxyphenazine methosulfate (m-PMS) as a mediator. After adding glucose solution (f.c. 100 mM), the response current was measured at a potential of 0.1 V (vs. Ag/AgCl). We defined the delta current as the difference in the current between before adding glucose and the plateau signal after adding 100 mM glucose. The delta current increased in a target dsDNA concentration-dependent manner, while only a minimal delta current was observed without target DNA (Fig. 2). The lowest detection limit was 10^{10} copies of target dsDNA (S/N $\geq$ 3). If the target region in 1 copy of genomic DNA was optimally amplified by 35 PCR cycles, then the amount of PCR product would be $>10^{10}$ copies, which indicated that this method had the potential to detect 1 copy of genomic DNA. This

sensitivity was comparable to that when using ZF-luciferase.

We also examined the sequence specificity of this electrochemical assay using Zif268-GDH. In addition to target DNA, we used mutated target DNA, 5′-GCGTGGTCG-3′, and random sequence DNA, 5′-ACTGATCCT-3′. As a result, we observed a higher delta current in the presence of target DNA than the currents when using mutated DNA and random DNA (Fig. 3). These results indicated that using Zif268-GDH, we could detect a target dsDNA electrochemically with high sensitivity and specificity.

Next, we investigated the detection limit and specificity of Zif268-GDH using *E. coli* O157 as a model pathogenic bacterium. To specifically detect *E. coli* O157, we used previously designed primers that amplified the Zif268-binding site in the fimbrial-like protein gene of *E. coli* O157[2]. We prepared 10–10^5 copies of genomic DNA purified from *E. coli* O157 and amplified the Zif268 target region in the genomic DNA using 35 PCR cycles. After amplification, we detected the PCR products electrochemically in the same manner as with the synthetic oligonucleotide.

The observed delta current increased with an increased amount of template genomic DNA. Even in the presence of 10 copies of genomic DNA, there was a higher delta current than with a negative control (Fig. 4), which indicated that bacterial genomic DNA could be electrochemically detected using Zif268-GDH. The lowest detection limit was 10 copies of *E. coli* O157 genomic DNA, which was comparable to that when using Zif268-luciferase. This result was consistent with the results for synthetic oligonucleotide detection. We consider that this sensitivity is sufficient to detect pathogenic bacteria because only 10^2 bacterial copies, such as for *E. coli* O157, will cause an infectious disease[18].

When we used a high copy number of genomic DNA of >10^3 copies, we did not observe any increase in the signal that was dependent on the amount of genomic DNA. This indicated that 35 amplification cycles for a higher genome copy number was not suitable for classifying these copy numbers. However, we did observe a copy number-dependent signal increase when using 10–10^3 copies. This dynamic range may be sufficient for applying this method to pathogenic bacteria detection.

Next, we evaluated the specificity of this assay system for detecting bacterial genomic DNA. As negative

controls, we used the genomic DNA of *Salmonella enterica* subspecies *enterica* and *E. coli* BL21 (DE3). A high delta current was observed with the target genomic DNA, whereas the delta current in the presence of non-target genomic DNA alone was as low as that of the negative control that did not contain any genomic DNA. Moreover, for a sample that contained 10^4 copies of *E. coli* O157 genomic DNA and 10^4 copies of *S. enterica* subspecies *enterica* genomic DNA combined, there was also a high delta current, which indicated that even 10^4 copies of non-target genomic DNA did not affect the ability to detect the target genomic DNA (Fig. 5). These results indicated that the sensitivity and specificity of ZF-GDH were comparable to those of ZF-luciferase. In addition, ZF-GDH had certain advantages because we could detect pathogenic bacteria electrochemically with simple equipment using ZF-GDH.

Conclusions

We developed an electrochemical detection system using ZF-GDH for a bacterial genome detection system. Using this method, we could detect *E. coli* O157, a model pathogenic bacterium, with high sensitivity and specificity. Of note, the lowest detection limit was 10 copies of bacterial genomic DNA, which was comparable to that for ZF-luciferase. This method was simple and rapid because it did not require bacterial culture and could detect dsDNA directly using Zif268-GDH.

Because this is an electrochemical detection method, the entire system can be small and simple compared with a fluorescence or luminescence based detection system. Moreover, because we used GDH as the labeling enzyme, we can construct portable pathogenic bacteria sensors by utilizing the same platform used with commercial blood glucose sensors.

Because GDH activity did not decrease dramatically after fusing it with ZF, we should be able to construct various types of ZF-GDH fusion proteins, including those for artificial ZF proteins. Using an artificial ZF protein that recognizes a desired target genomic region, we can design detection systems for various types of pathogenic bacteria.

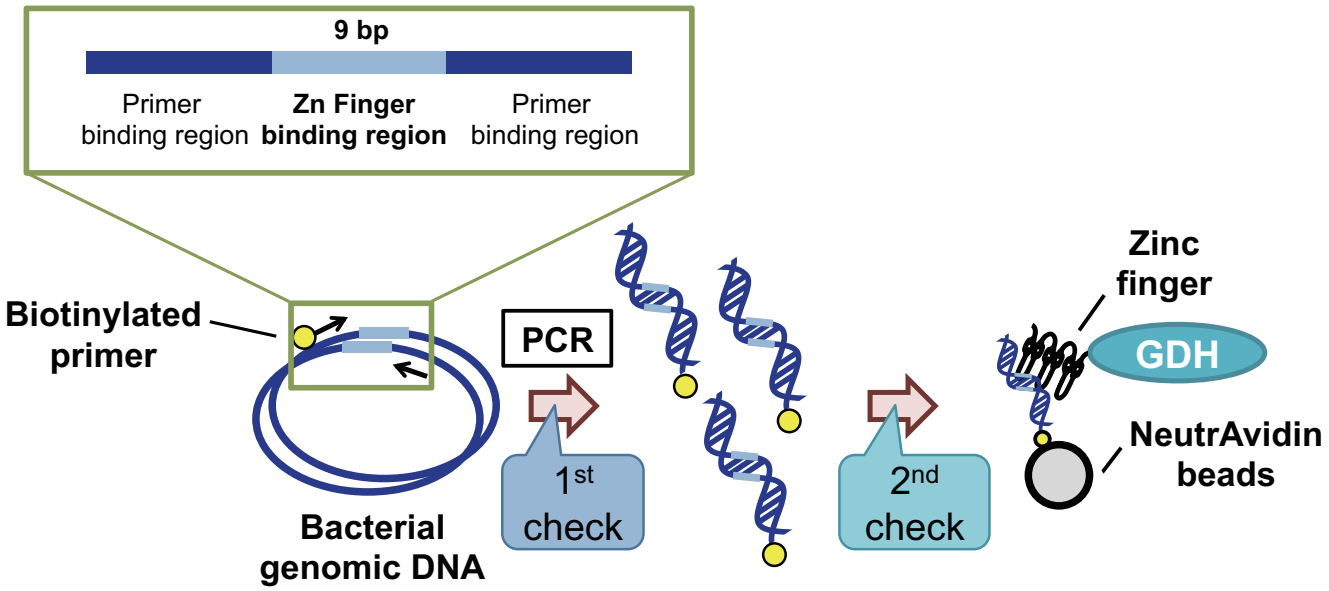

Fig. 1 Schematic of our detection system. The system that we designed in this study involved two steps: 1. PCR amplification of a target genomic region of pathogenic bacteria that included a ZF recognition sequence and 2. electrochemical detection of the PCR products using ZF-GDH.

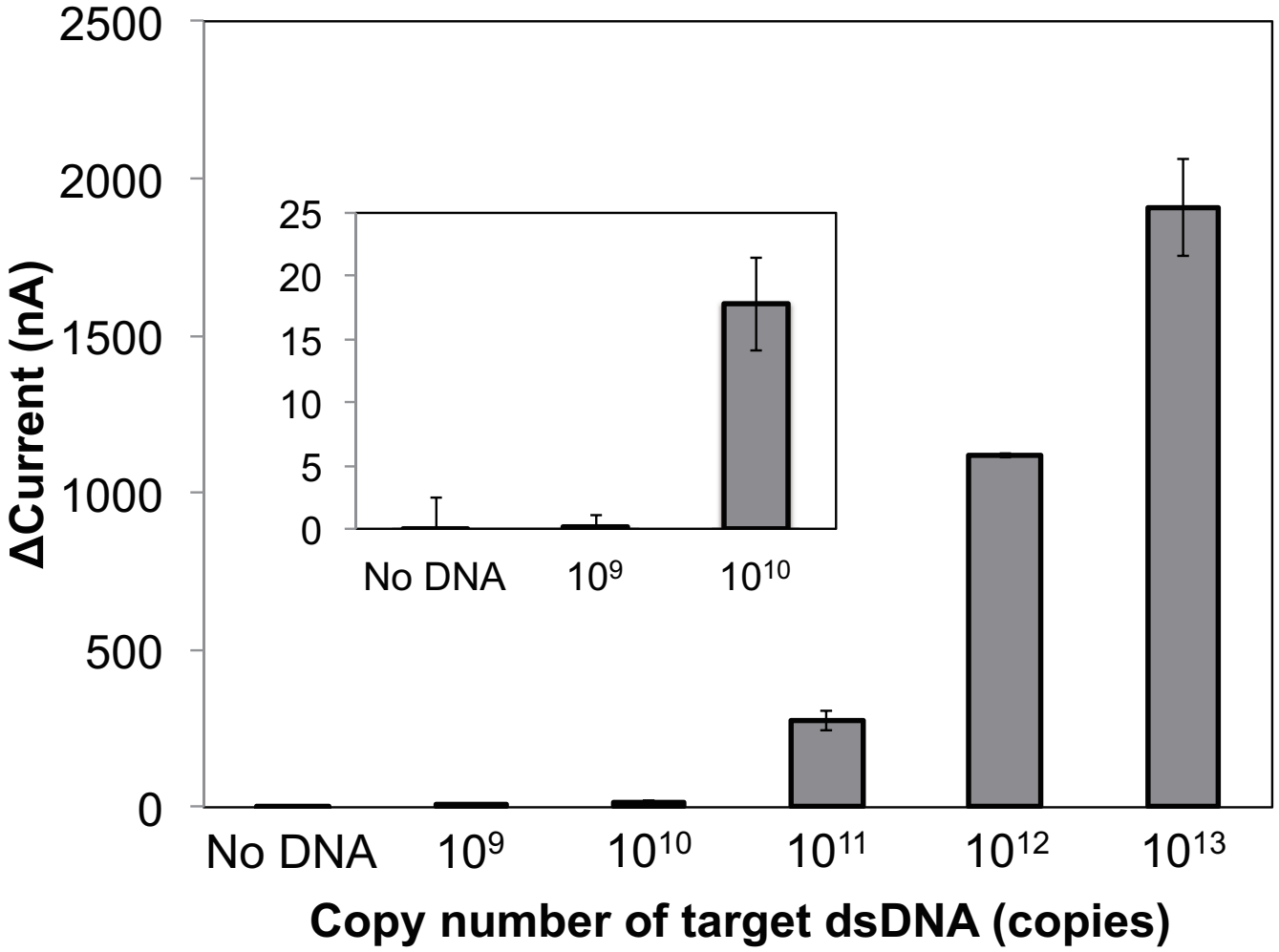

Fig. 2 Electrochemical detection of synthetic oligonucleotides that included the Zif268 recognition region. Delta current is the difference in current between before adding glucose and the plateau signal after adding 100 mM glucose. Error bars indicate standard deviations (n = 3).

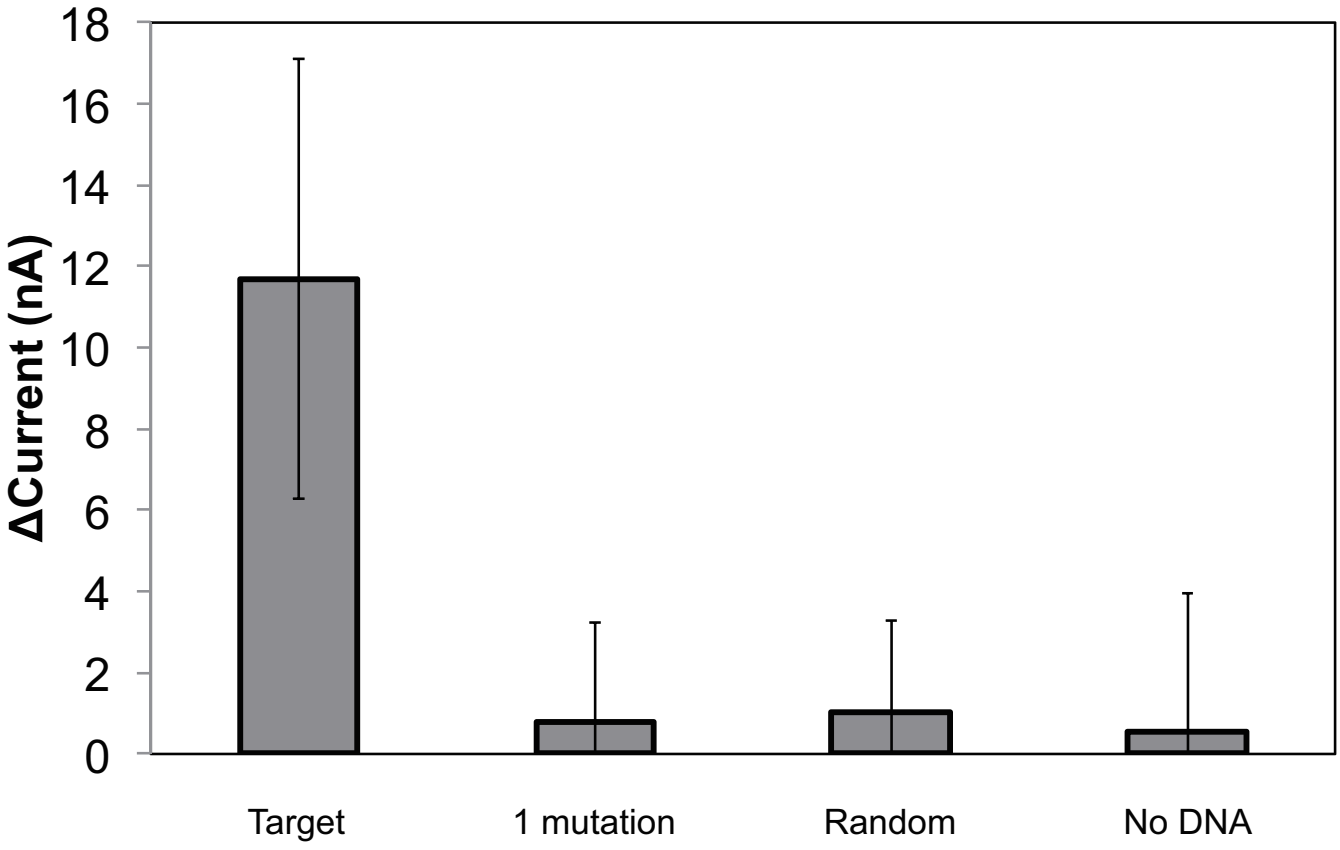

Fig. 3 Sequence specificity of Zif268-GDH with our electrochemical assay. A total of 10^{10} copies of synthetic oligonucleotides were analyzed. Error bars indicate standard deviations (n = 3).

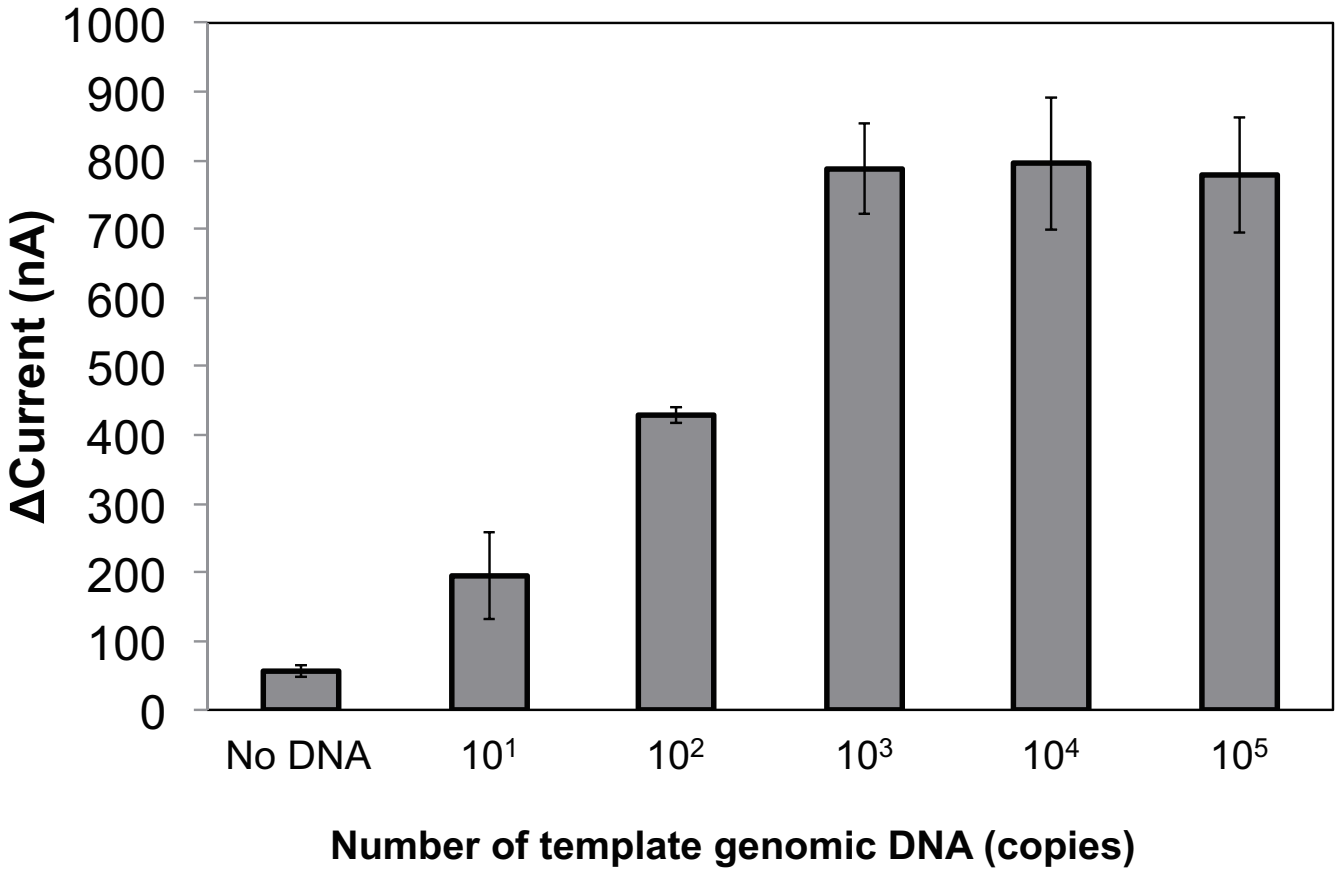

Fig. 4 Calibration curve for detecting *E. coli* O157 using Zif268-GDH. Different copy numbers of genomic DNA purified form *E. coli* O157 were added to PCR solution and the PCR products were analyzed. Results are the averages of three independent assays and error bars indicate standard deviations.

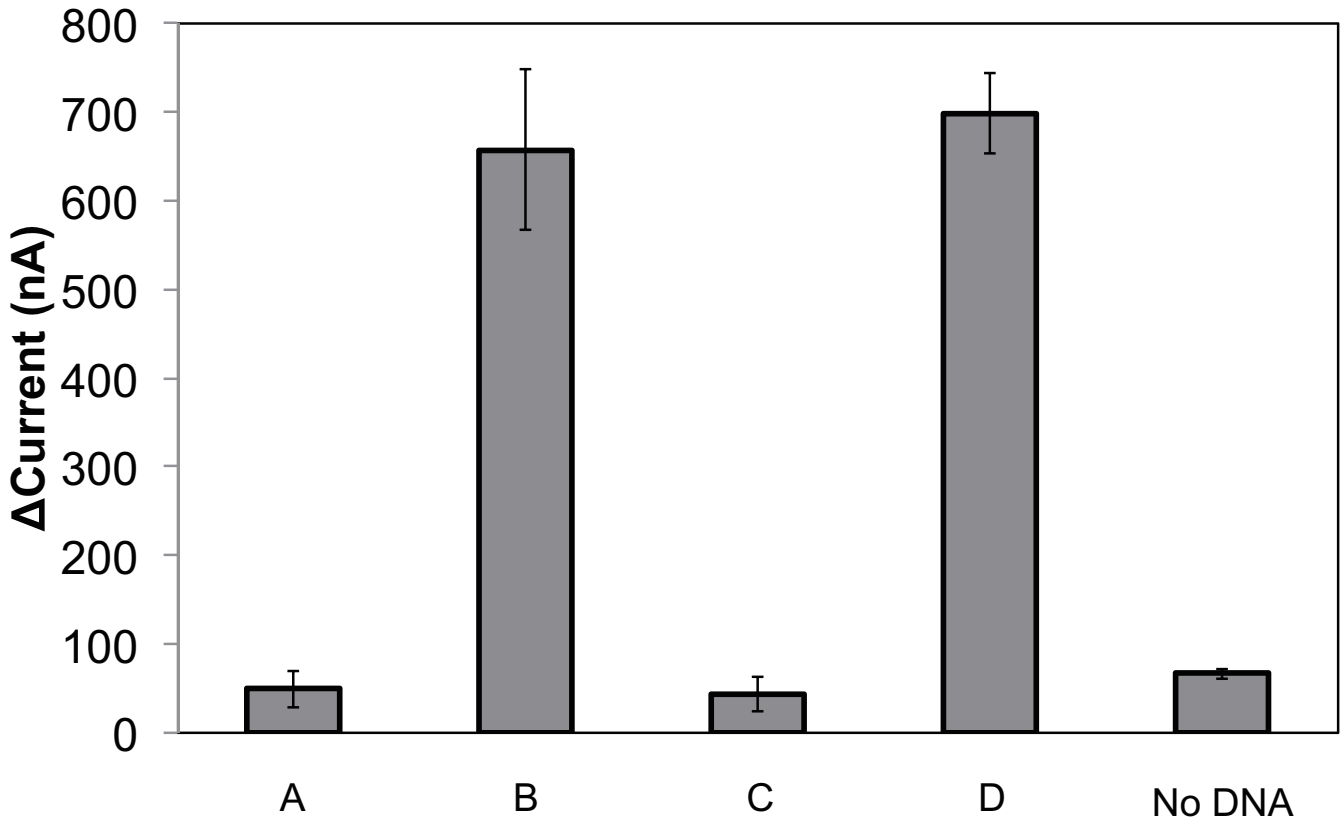

Fig. 5 Specificity of PCR product detection. A total of 10^4 copies of genomic DNA purified from either (A) *E. coli* BL21 (DE3), (B) *E. coli* O157, or (C) *Salmonella enterica* subspecies *enterica* were added to PCR solution. After the target region was amplified using specific PCR primers for *E. coli* O157, PCR products were analyzed using ZF-GDH. (D) A mixture of 10^4 copies of genomic DNA from *E. coli* O157 and 10^4 copies of genomic DNA from *Salmonella enterica* subspecies *enterica* were analyzed using Zif268-GDH in the same manner.

References

1. O. Lazcka, F. J. Del Campo and F. X. Munoz, *Biosensors & bioelectronics*, 2007, 22, 1205-1217.
2. Y. Osawa, K. Ikebukuro, H. Motoki, T. Matsuo, M. Horiuchi and K. Sode, *Nucleic acids research*, 2008, 36, e68.
3. Y. Osawa, K. Ikebukuro, T. Kumagai, H. Motoki, T. Matsuo, M. Horiuchi and K. Sode, *Biotechnology letters*, 2009, 31, 725-733.
4. K. Abe, T. Kumagai, C. Takahashi, A. Kezuka, Y. Murakami, Y. Osawa, H. Motoki, T. Matsuo, M. Horiuchi, K. Sode, S. Igimi and K. Ikebukuro, *Analytical chemistry*, 2012, 84, 8028-8032.
5. W. Yoshida, A. Kezuka, Y. Murakami, J. Lee, K. Abe, H. Motoki, T. Matsuo, N. Shimura, M. Noda, S. Igimi and K. Ikebukuro, *Analytica chimica acta*, 2013, 801, 78-83.
6. I. Sanchez-Garcia and T. H. Rabbitts, *Trends in genetics : TIG*, 1994, 10, 315-320.
7. A. Klug and J. W. Schwabe, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 1995, 9, 597-604.
8. S. A. Wolfe, L. Nekludova and C. O. Pabo, *Annual review of biophysics and biomolecular structure*, 2000, 29, 183-212.
9. S. Iuchi, *Cellular and molecular life sciences : CMLS*, 2001, 58, 625-635.
10. C. O. Pabo, E. Peisach and R. A. Grant, *Annual review of biochemistry*, 2001, 70, 313-340.
11. I. Ghosh, C. I. Stains, A. T. Ooi and D. J. Segal, *Molecular bioSystems*, 2006, 2, 551-560.
12. J. G. Mandell and C. F. Barbas, 3rd, *Nucleic acids research*, 2006, 34, W516-523.
13. T. Sera, *Advanced drug delivery reviews*, 2009, 61, 513-526.
14. K. Ikebukuro, C. Kiyohara and K. Sode, *Anal Lett*, 2004, 37, 2901-2909.
15. K. Ikebukuro, C. Kiyohara and K. Sode, *Biosensors & bioelectronics*, 2005, 20, 2168-2172.
16. Y. Nonaka, K. Abe and K. Ikebukuro, *Electrochemistry*, 2012, 80, 363-366.
17. Y. Nonaka, W. Yoshida, K. Abe, S. Ferri, H. Schulze, T. T. Bachmann and K. Ikebukuro, *Analytical chemistry*, 2013, 85, 1132-1137.
18. Y. Hara-Kudo and K. Takatori, *Epidemiology and infection*, 2011, 139, 1505-1510.
19. K. Ikebukuro, Y. Kohiki and K. Sode, Biosensors & bioelectronics, 2002, 17, 1075-1080.