

Construction of DNA-NanoLuc luciferase conjugates for DNA aptamer-based sandwich assay using Rep protein

Masayasu Mie  · Takahiro Niimi · Yasumasa Mashimo · Eiry Kobatake

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Abstract

Objective We developed a DNA-NanoLuc luciferase (NnaoLuc) conjugates for DNA aptamer-based sandwich assay using the catalytic domain of the replication initiator protein derived from porcine circovirus type 2 (pRep).

Results For construction of DNA aptamer and NanoLuc conjugate using the catalytic domain of Rep from PCV2. pRep fused to NanoLuc was genetically constructed and expressed in *E. coli*. After purification, the activities of fused pRep and NanoLuc were evaluated, and DNA-NanoLuc conjugates were constructed via the fused pRep. Finally, constructed DNA-NanoLuc conjugates were applied for use in a DNA aptamer-based sandwich assay. Here, pRep was used not only for conjugation of the NanoLuc to the detection aptamer, but also for immobilization of the capture aptamer on the plate surface.

Conclusion We have demonstrated that DNA-NanoLuc conjugates via the catalytic domain of PCV2 Rep

could be applied for DNA aptamer-based sandwich assay system.

Keywords DNA aptamer · DNA–protein conjugate · Rep · NanoLuc luciferase · DNA aptamer-based sandwich assay

Introduction

Construction of DNA–protein conjugates is important for a number of biotechnological applications, such as biosensing (Lee et al. 2015; Sano et al. 1992; van Buggenum et al. 2016; Vivekananda and Kiel 2006), imaging (Jungmann et al. 2014), and DNA-templated nanofabrication (Koussa et al. 2014; Nakata et al. 2012; Sagredo et al. 2016). Especially, conjugates of DNA aptamer and reporter protein are useful for construction of DNA aptamer-based assay system. However, conventional conjugation methods require cumbersome preparation or reaction procedures.

To overcome such problems, we previously developed a method for site-specific labeling of single-stranded DNA (ssDNA) to a genetically engineered POI through the fused Gene A* from bacteriophage phiX174 (Mashimo et al. 2012). Gene A* is a member of the replication initiator protein (Rep) family. Gene A* exhibits the ability to cleave ssDNA at specific sequence positions, which is then followed by

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M. Mie (✉) · T. Niimi · Y. Mashimo · E. Kobatake
Department of Life Science and Technology, School of Life Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-Ku, Yokohama 226-8502, Japan
e-mail: mie.m.aa@m.titech.ac.jp

conjugation of the protein and the 5' phosphate of the cleaved ssDNA (Hanai and Wang 1993; Sanhueza and Eisenberg 1984). Using this method, a protein fused with Gene A* can be covalently linked to ssDNA enzymatically, without the need for any chemical modifications of the ssDNA. Recently, Gordon's group published a similar strategy using HUH-tags (Lovendahl et al. 2017). They tested several HUH-tags, including the catalytic domain of porcine circovirus type 2 Rep (pRep), for formation of covalent DNA–protein conjugates. While the function of Gene A* is similar to that of pRep, expression of Gene A* in *E. coli* is problematic. Moreover, the size of Gene A* (MW = 38,700) is much larger than pRep (MW = 13,400). Therefore, we focused on pRep for construction of DNA and NanoLuc luciferase (NanoLuc) conjugates in the present study.

Herein, we constructed DNA-NanoLuc conjugates using the catalytic domain of Rep from PCV2. pRep fused to NanoLuc was genetically constructed and expressed in *E. coli*. After purification, the activities of fused pRep and NanoLuc were evaluated, and DNA-NanoLuc conjugates were constructed via the fused pRep. Finally, we demonstrated the application of the DNA-NanoLuc conjugates for use in a DNA aptamer-based sandwich assay. Here, pRep was used not only for conjugation of the reporter protein to the detection aptamer, but also for immobilization of the capture aptamer on the plate surface (Fig. 1).

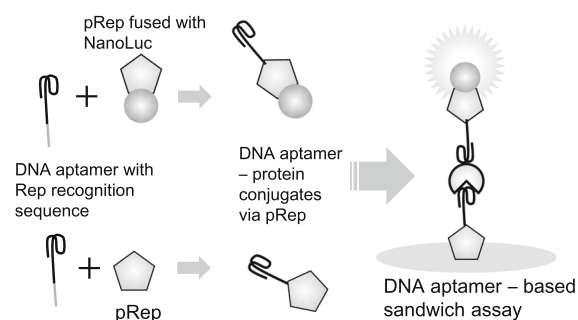


Fig. 1 Schematic representation of the DNA aptamer-based sandwich assay with DNA–protein conjugates through the catalytic domain of the replication initiator protein. The detection aptamer was conjugated to the NanoLuc via fused pRep. The capture aptamer was conjugated to the pRep and immobilized on the hydrophobic plate

Materials and methods

Materials

The gene of the catalytic domain of PCV2 Rep comprising residues 1–116 (pRep), with codon optimization for *E. coli* expression, was synthesized and cloned into the pUCFa vector by FASMAC (Japan). The optimized sequence is shown in Supplementary Fig. 1. Restriction enzymes and ligase were purchased from Takara Bio Inc. (Japan).

Plasmid construction

For expression of pRep with His-tag (His-pRep), the pET-His-pRep plasmid was constructed as follows. The pRep fragment derived from pUCFa-pRep by digestion with *Nco* I and *Xho* I was inserted into pET-His(*Nco* I) previously modified in our laboratory, and digested with the same restriction enzymes.

The pET-His-NanoLuc-pRep plasmid used to express the pRep protein fused to the C-terminal end of NanoLuc was constructed as follows. The NanoLuc fragment derived from the pUC18-NanoLuc plasmid previously constructed in our laboratory was digested by *Nco* I and *Eco* R I and inserted into suitably digested pET-His-pRep.

The pET28-pRep-NanoLuc-His plasmid used to express the pRep protein fused to the N-terminal end of NanoLuc was constructed as follows. The NanoLuc fragment derived from pUC18-NanoLuc by digestion with *Sal* I and *Xho* I was cloned into suitably digested pET-His-pRep. The constructed plasmid was digested with *Nco* I and *Xho* I to obtain the pRep-NanoLuc gene fragment. The resultant pRep-NanoLuc fragment was cloned into the pET28-Eluc-His plasmid previously constructed in our laboratory, and digested with *Nco* I and *Xho* I.

The pET-His-NanoLuc plasmid used to express His-tagged NanoLuc (His-NanoLuc) was constructed according to the same procedure used to construct pET-His-pRep.

Protein expression and purification

His-pRep, His-NanoLuc-pRep, pRep-NanoLuc-His and His-NanoLuc proteins were expressed in *E. coli* BL21(DE3) transformed with pET-His-pRep, pET-His-NanoLuc-pRep, pET28-pRep-NanoLuc-His and

pET-His-NanoLuc, respectively. Transformed cells, except for cells transformed with pET28-pRep-NanoLuc-His, were cultured in LB media with 50 µg/mL ampicillin at 37 °C until the OD₆₆₀ reached ~ 0.6. Cells transformed with pET28-pRep-NanoLuc-His were cultured in LB media with kanamycin. Protein expression was induced by addition of isopropyl-β-D(-)-thiogalactopyranoside (IPTG, final concentration 1 mM). After 6 h of culture at 25 °C, cells were harvested by centrifugation. The cell pellets were re-suspended in phosphate-buffered saline (PBS), and disrupted by sonication followed by His-tag metal-ion affinity chromatography purification. The purified samples were dialyzed against PBS using a Slide-A-lyzer dialysis cassette (Thermo Scientific) and analyzed by SDS-PAGE. The concentrations of the purified proteins were determined with a BCA protein assay kit (Thermo Scientific).

Evaluation of pRep activity

Purified proteins were mixed with the ssDNA oligonucleotide genome25 (5′-AAGTATTACCAGCGCACTTCGGCAG-3′), either with or without FITC modification at the 3′-end, in reaction buffer (PBS containing MgCl₂; final concentration: 2.5 mM). Both FITC-modified and unmodified genome25 were synthesized by FASMAC (Japan). The underlined sequence, AAGTATTAC, denotes the Rep recognition sequence. To investigate the protein-to-DNA ratio, purified proteins (8 µM) were mixed with varying concentrations of FITC-modified genome25 (0–20 µM) in 10 µL of reaction buffer. After incubation for 30 min at 37 °C, the samples were analyzed by 12% SDS-PAGE, stained with Coomassie Brilliant Blue (CBB), and visualized using a fluorescence scanner.

Evaluation of NanoLuc activity

Purified proteins (50 pM), namely His-NanoLuc-pRep, pRep-NanoLuc-His and His-NanoLuc, diluted in PBS, were injected into 96-well white polystyrene microplates. Equal volumes of the NanoLuc substrate, namely Nano-Glo luciferase Assay System (Promega), were added to the samples. After incubation for 1 min at room temperature, bioluminescence was measured using an LMaxII microplate reader (Molecular Devices).

DNA aptamer-based sandwich assay

The thrombin DNA aptamers, TBA15 (5′-AAGTATTACGGTTGGTGTGGGTTGG-3′) (Bock et al. 1992) and TBA29 (5′-AAGTATTACAGTCCGTGGTAGGGCAGGTTGGGGTGACT-3′) (Tasset et al. 1997) containing the Rep recognition sequence respectively, were used in the DNA aptamer-based sandwich assay. Equal amounts (1 µM each) of His-pRep and TBA15 containing the Rep recognition sequence were mixed in 100 µL of binding buffer, followed by incubation for 10 min at 37 °C. TBA15-conjugated His-pRep was immobilized on a 96-well white flat bottom polystyrene high bind microplate (Corning #3922). After blocking, 70 µL of varying concentrations of thrombin solution (0–100 nM) was added to the wells, followed by incubation for 1 h at room temperature. Wells were washed with PBS-T, and then 70 fmol of TBA29-conjugated His-NanoLuc-pRep was added to each well, followed by incubation for 1 h at room temperature. Wells were then washed with PBS-T, followed by addition of 100 µL of the NanoLuc substrate to each well. After incubation for 1 min at room temperature, bioluminescence was measured using a LMaxII microplate reader.

Results and discussion

Expression and purification of pRep fusion proteins

The replication initiator protein (Rep) of porcine circovirus type 2 (PCV2) is comprised of 314 amino acids. The functions of Rep during the replication process include recognition of specific sequences (*i.e.*, AAGTATT[^]AC) and cleavage of ssDNA at a specific sequence position (indicated by [^]), followed by formation of a covalent linkage to the 5′ phosphate of the cleaved ssDNA. The recognition sequence, as well as both the structure and function of the catalytic domain of PCV2 Rep (pRep), comprising residues 1–116, were previously reported by Vega-Rocha et al. (Vega-Rocha et al. 2007).

In our previous study, we developed a method for construction of DNA–protein conjugates using the Gene A* protein derived from phiX174 phage (Mashimo et al. 2012). While the function of Gene A* is similar to that of pRep, expression of Gene A* in

E. coli is problematic. Moreover, the size of pRep (MW = 13,400) is much smaller than Gene A* (MW = 38,700). Therefore, we focused on pRep for construction of DNA-NanoLuc conjugates in the present study. First, pRep fused with a His-tag at its N-terminal end (His-pRep) was expressed in *E. coli*. The His-pRep was expressed in both the soluble and insoluble fractions (data not shown). For expression of Gene A*, the addition of glucose to the culture medium was required to decrease leaky expression. Without the addition of glucose, *E. coli* harboring the plasmid encoding Gene A* gene is unable to grow, even prior to induction of protein expression. In contrast, *E. coli* harboring the plasmid for expression of His-pRep was able to grow even without inhibition of leaky expression. Expressed His-pRep was purified from the soluble fraction using the His-tag. To confirm the activity of pRep, 5'-FITC-labeled ssDNA, *i.e.*, genome25, encoding the PCV2 Rep recognition sequence (AGTATTAC) was added to purified His-pRep (8 μ M). Following the reaction, the samples were analyzed by SDS-PAGE (Fig. 2). Addition of 5'-FITC-labeled genome25 (0–20 μ M) resulted in a shift

of the bands to higher molecular weights. The fluorescence signals derived from the shifted bands were confirmed by fluorescence imaging. These results demonstrate that His-pRep exhibits the ability to covalently bind to ssDNA.

Evaluation of fusion protein activities

Next, pRep was fused to NanoLuc, and the activities of the resulting fusion proteins were analyzed. To evaluate whether the DNA binding ability of pRep and the activity of the fused NanoLuc are retained following fusion, plasmids were constructed for expression of pRep fused to the C-terminal and N-terminal ends of NanoLuc (His-NanoLuc-pRep and pRep-NanoLuc-His, respectively). These fusion proteins containing His-tag were expressed in *E. coli*, and subsequently purified. We first evaluated the DNA binding ability of pRep. Each of the fusion proteins were mixed with various concentrations of genome25 ssDNA. After incubation, samples were analyzed by SDS-PAGE (Fig. 3a). Similar to His-pRep, the bands of each protein were shifted to higher molecular weights by formation of DNA-NanoLuc conjugates. These results demonstrate that a protein of interest (POI) could be fused to both the N-terminal and C-terminal ends of the pRep without significant reduction of DNA binding activity of pRep.

The NanoLuc activities of the fusion proteins were also evaluated (Fig. 3b). In the absence of DNA conjugation, the NanoLuc activities of His-NanoLuc-pRep and pRep-NanoLuc-His were decreased, but retained \sim 30% activity compared to that of His-NanoLuc. The NanoLuc activities of DNA-conjugated fusion proteins were nearly the same as those in the absence of DNA conjugation. The NanoLuc activity of DNA-conjugated pRep-NanoLuc-His was slightly higher than the unconjugated fusion protein. We speculate that the surrounding of the fusion proteins changed by conjugation to DNA. Taken together, these results demonstrate that pRep can be employed for the construction of DNA-NanoLuc conjugates.

Construction of DNA aptamer-based sandwich assay system

We evaluated the application of DNA-NanoLuc conjugates for a DNA aptamer-based sandwich assay system. Here, pRep was used not only for conjugation

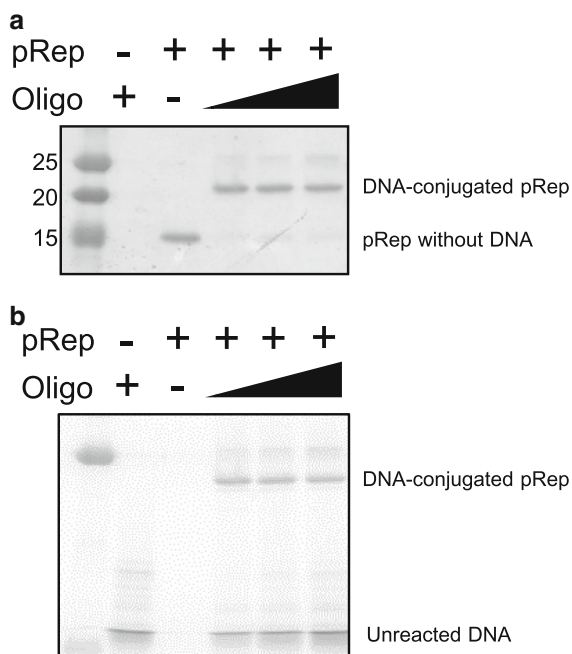


Fig. 2 Evaluation of His-pRep activity. Purified His-pRep (8 μ M) was mixed with different concentrations of FITC-modified ssDNA substrate (genome25: 5, 10, 20 μ M). SDS-PAGE analysis stained with Coomassie Brilliant Blue (**a**), and visualized using a fluorescence scanner (**b**)

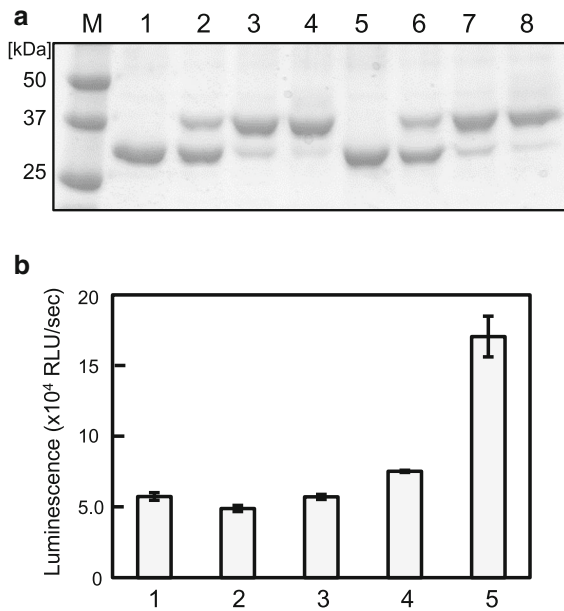


Fig. 3 Construction of DNA-NanoLuc. **a** DNA conjugation activities of His-NanoLuc-pRep (lanes 1–4) and pRep-NanoLuc-His (lanes 5–8). His-NanoLuc-pRep was mixed with 0 μ M (lane 1), 2.5 μ M (lane 2), 5.0 μ M (lane 3) and 25 μ M (lane 4) of genome25. pRep-NanoLuc-His was mixed with 0 μ M (lane 5), 2.5 μ M (lane 6), 5.0 μ M (lane 7) and 25 μ M (lane 8) of genome25. Lane M, SDS Broad Range Marker. **b** NanoLuc activity of DNA-NanoLuc conjugates: lane 1, His-NanoLuc-pRep without DNA conjugation; lane 2, pRep-NanoLuc-His without DNA conjugation; lane 3, His-NanoLuc-pRep with DNA conjugation; lane 4, pRep-NanoLuc-His with DNA conjugation; lane 5, His-NanoLuc. Each value represents the mean of 3 replicates ($n = 3$)

of the reporter protein to the detection aptamer, but also for immobilization of the capture aptamer on the plate surface. In general, immobilization of a capture aptamer first requires modification with biotin (Tuleuova et al. 2010), amine (Lee et al. 2015), etc. Modification with biotin is one of the more commonly used methods, with the resulting biotin-modified aptamers immobilized on an avidin-coated surface. Herein, pRep was used for immobilization of DNA. His-pRep conjugated with ssDNA was added to the hydrophobic plate surface, followed by incubation. After blocking, His-NanoLuc-pRep conjugated with a complementary ssDNA was added for detection of immobilized DNA by bioluminescence. Luminescence was found to increase with increasing concentration of genome21-conjugated His-pRep (Supplementary Fig. 2). These results demonstrate

that pRep can be applied for immobilization of DNA on the hydrophobic surface.

We then constructed a DNA aptamer-based sandwich assay system using the well-studied 15- and 29-mer thrombin binding aptamers, namely TBA15 and TBA29, respectively. TBA15 was used as the capture aptamer, while TBA29 was used as the detection aptamer. After immobilization of TBA15-conjugated His-pRep, we evaluated the binding abilities of the DNA aptamer conjugated to the protein via pRep (Supplementary Fig. 3). When TBA29-conjugated His-NanoLuc-pRep was added to the wells after the addition of a non-target protein, namely bovine serum albumin (BSA), the luminescence derived from NanoLuc was nearly the same as that of the negative control, in which no protein was added prior to addition of TBA29-conjugated His-NanoLuc-pRep. In contrast, when thrombin was added prior to addition of TBA29-conjugated His-NanoLuc-pRep, the luminescence intensity was markedly increased compared to the samples with BSA. Finally, various concentrations of thrombin were also added to the assay, followed by addition of TBA29-conjugated His-NanoLuc-pRep. As shown in Fig. 4, the luminescence derived from NanoLuc was found to increase with increasing thrombin concentration. The limit of detection of thrombin using the DNA-NanoLuc conjugates was

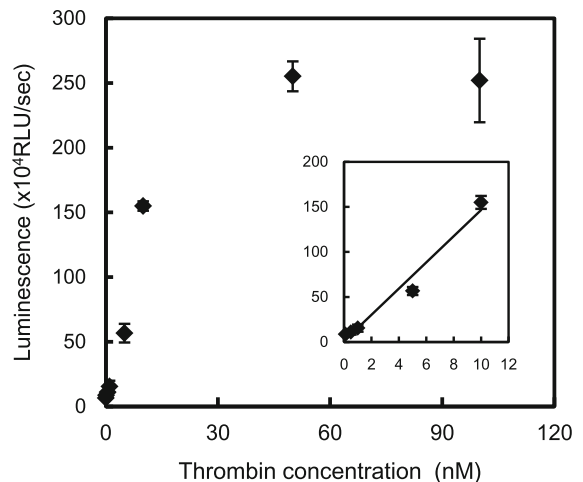


Fig. 4 Thrombin concentration dependency of DNA aptamer-based assay system. Thrombin binding aptamers, TBA15 and TBA29, were used as the capture and detection aptamer respectively. TBA15 was conjugated to pRep for immobilization, while TBA29 was conjugated to the NanoLuc via fused pRep for detection. Each value represents the mean of 3 replicates ($n = 3$)

determined to be 0.1 nM. These results highlight the utility of DNA-NanoLuc conjugates for use in a DNA aptamer-based sandwich assay system.

Conclusions

Taken together, we constructed of DNA-NanoLuc conjugates using the catalytic domain of PCV2 Rep for DNA aptamer-based sandwich assay. Without DNA modification, the DNA binding reaction of pRep occurs quickly and efficiently, even after fusion with a NanoLuc. DNA and NanoLuc conjugated via fused pRep retained their abilities even after conjugation. The resulting DNA-NanoLuc conjugates were used in a DNA aptamer-based sandwich assay. These results highlight the considerable potential of pRep for construction of DNA aptamer-based sandwich assay system.

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Supplementary Information Supplementary Figure 1—The gene of the catalytic domain of PCV2 Rep comprising residues 1–116 (pRep). The amino acid sequence of the pRep was shown as red. The sequence of the pRep optimized for E.coli expression was shown as black (Opt).

Supplementary Figure 2—Immobilization of DNA-protein conjugates on the hydrophobic plate surface.

Supplementary Figure 3—Evaluation of specific binding abilities of Thrombin DNA aptamer conjugated to protein.

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