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Paired Design of dCas9 as a Systematic Platform for the Detection of Featured Nucleic Acid Sequences in Pathogenic Strains

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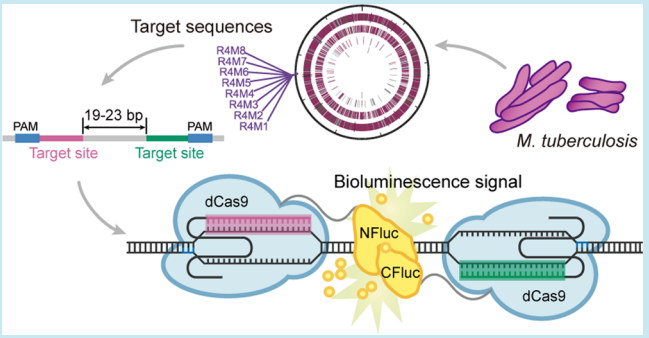
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Supporting Information

ABSTRACT: We developed an *in vitro* DNA detection system using a pair of dCas9 proteins linked to split halves of luciferase. Luminescence was induced upon colocalization of the reporter pair to a ~44 bp target sequence defined by sgRNAs. We used the system to detect *Mycobacterium tuberculosis* DNA with high specificity and sensitivity. The reprogrammability of dCas9 was further leveraged in an array design that accesses sequence information across the entire genome.



KEYWORDS: *dCas9, paired design, split luciferase, tuberculosis, DNA detection, in vitro pathogenic detection*

Fast and reliable detection of featured nucleic acid (NA) sequences in microbial genomes, from a potentially large pool of nonspecific DNA, bears important application in pathogen detection, molecular diagnostics, as well as food and environmental safety monitoring. The past ten years have witnessed tremendous development in hybridization-based NA detection methods such as the ever-evolving molecular beacon (MB) design.[1,2] At its heart, a probe with predesigned sequence is covalently linked to a quenched reporter moiety. Upon binding to the complementary target DNA, conformational change of the beacon unleashes the reporter, which in turn gives off a luminescent or electrochemical signal. Despite its elegant design, two major hurdles have kept MB from wide applications in and outside of laboratories: (1) the choice of the probe sequence is often subject to strong constraints of secondary structures and energetic favorability, and (2) sensitivity is less desirable due to incomplete quenching and unstable signal output of the normally adopted fluorophores, while linking stable and ultrasensitive enzymatic proteins to the probes remains a tedious and costly process.[3,14] As a result, the development of each new probe requires fabrication and optimization steps that are both costly and time-consuming.[15] To overcome these limitations, one solution is to decouple the probing sequence and the reporter moieties, so that an independent, universal reporter system can be quickly reprogrammed by the probe to target arbitrary DNA sequences.

Due to its outstanding efficiency and programmability, the Type II CRISPR-Cas system from *Streptococcus pyogenes* has emerged as an exciting new tool for precise *in vivo* genome editing in a large number of species.[4] While the wild-type Cas9 protein is a nuclease, the engineered nuclease-deficient Cas9

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(dCas9) has greatly expanded its functional repertoire by acting as an interface between the guiding RNA that determines sequence specificity and the various actuator domains that perform desired functions such as transcription regulation and imaging.[5−7,17] In this work, we propose and demonstrate dCas9's potential for a powerful *in vitro* NA detection platform. In our Paired dCas9 (PC) reporter system, dCas9 linked to the N- and C-terminal half of the firefly luciferase enzyme (NFluc and CFluc) are programmed by single guide RNAs (sgRNA) complementary to the upstream and downstream segments of a target DNA sequence, respectively. When substrate DNA containing the two segments in proximity are detected and bound by a pair of dCas9, luminescence is generated from the catalytic activity of luciferase in its entirety (Figure 1a).

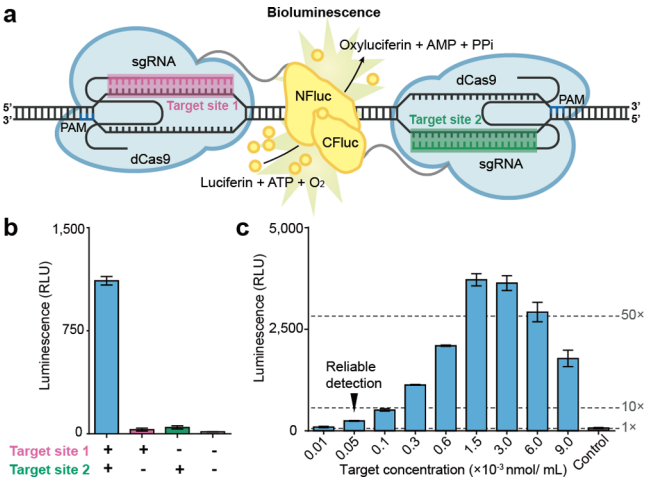


Figure 1. RNA-guided featured DNA detection by the paired dCas9 (PC) reporter system. (a) dCas9 is linked to one of the two split fragments of the firefly luciferase (NFluc or CFluc) to form the universal reporters. Specificity is imparted by sgRNAs complementary to two segments (target sites 1 and 2) in the target DNA sequence. The binding of the dCas9:sgRNA duo to their respective target sites brings the half enzymes into proximity, and the integral enzyme catalyzes the bioluminescent reaction. (b) Specificity of the PC reporter system. Stable luminescent signals were measured in reaction systems containing 3×10^{-3} nmol/mL DNA substrate with both, one, or none of the two target sites. (c) Sensitivity of the PC reporter system. DNA containing both target sites was added to the reaction system at indicated concentrations. The control group contained no DNA. Fold changes were relative to the control group. In both (b) and (c), error bars show standard error of three experimental replicates.

The split firefly luciferase reporters used in this study was previously screened for studying protein−protein interactions.[8] We independently verified that the complemented form, mediated by FRB-rapamycin-FKBP complex assembly, generated a 5-fold increase in luminescence compared to the free forms of FRB-NFluc and FKBP-CFluc, in consistence with previously reported signal-to-noise ratios (see Supplemental Figure S1). To our knowledge, the split luciferase system has not been used in NA hybridization based detection designs, where the mediating complex is formed through weaker interactions than those happening on closely contacting protein interfaces. Therefore, we characterized its reporter performance in our paired dCas9 design, where NFluc and CFluc are fused to the N-terminus of dCas9 *via* 2 × GGGGS flexible linkers. Target specificity was conferred by incubating for 10 min each reporter half with sgRNAs complementary to one of the two segments spaced at 20 bp apart in a target DNA sequence. Both reporters were then added to reaction mixture containing D-luciferin and DNA with no target sequence, only one segment in the target sequence, or both segments in the target sequence. Maximal luminescence was reached after 2−3 min, and was stable for the next 20 min. Compared to DNA lacking both target segments, the reporter system generated a 80-fold luminescence increase in the presence of the full length target sequence, whereas the presence of a single target segment led to a mere 2−3-fold increase (Figure 1b). Without further optimization, the signal-to-noise ratio of the PC reporter system is readily comparable to that of a carefully optimized classic molecular beacon (20−126).[16] Sensitivity of the PC reporter was tested on serially diluted target DNA samples. The reporter was able to detect reliably 5×10^{-5} nmol/mL specific DNA. However, the reporter was saturated at 6×10^{-3} nmol/mL specific DNA, beyond which point, luminescence decreased due to distribution of the reporter halves to target segments located on different DNA molecules and thus a lack of pairing (Figure 1c).

As the large dCas9:sgRNA complex assembles on target DNA and promotes local unwinding of the double helix in a directional way,[9] when two such complexes are formed in close proximity, steric hindrance and tension in the DNA backbone may destabilize or even prevent the conjugation of the linked luciferase halves and subsequently, diminish or abolish its enzymatic activity. To test this effect, we constructed chimeric proteins with NFluc and CFluc linked to the N- or C-terminus of dCas9 (Supplemental Figure S2). Each of the four possible pairs of reporter configurations were incubated with two sgRNAs targeting the same upstream and downstream segments but oriented differently relative to each other depending on where the 5′-NGG-3′ PAM sequences were located in target DNA. Gains in luminescence upon target recognition ranged from 2- to 35-fold (Figure 2a). ANOVA test suggested that reporter configuration was the major determinant of the signal output (p-value < 10^{-13}), while PAM orientation was not a significant factor (p-value = 0.16), which relaxes the constraint on target selection—as long as two PAM sequences are present on either strand of DNA, their adjacent 20 bp sequences could serve the purpose of recognition. The largest signal increase was observed, though, when both Fluc halves were linked to the N-terminus of dCas9 and the two target segments were on opposite strands in a tail-to-tail (PAM-out) orientation. This finding qualitatively agrees with a previous study where split FokI endonucleases were linked to a pair of dCas9 for genomic editing with enhanced specificity.[10] Similarly, the spacing between the upstream and downstream target segments may play a role in determining reporter efficiency. Indeed, by varying this spacer length on substrate DNA, we found a periodic pattern of luminescence gain, with peak signals happening when the two dCas9 reporters sat *in cis* per helix turn, but significantly dampened as the distance exceeded three helix turns (Figure 2b). The results suggest a 21 ± 2 bp optimal spacing between the two target segments.

By preincubating the PC reporters with two sgRNAs, we can program the system to target virtually any sequence of interest in a fast and streamlined fashion. We demonstrate one example using the PC reporter to detect the genome of *Mycobacterium tuberculosis* (*Mtb*), which is the major pathogenic agent for human tuberculosis. We chose the target sequence in the 16S rRNA gene region of the *Mtb* H37Rv genome. In the presence of purified *Mtb* H37Rv genomic DNA isolated from a tuberculosis patient by one of the authors,[21] a 52-fold increase in luminescence was observed in comparison with equal amounts of the

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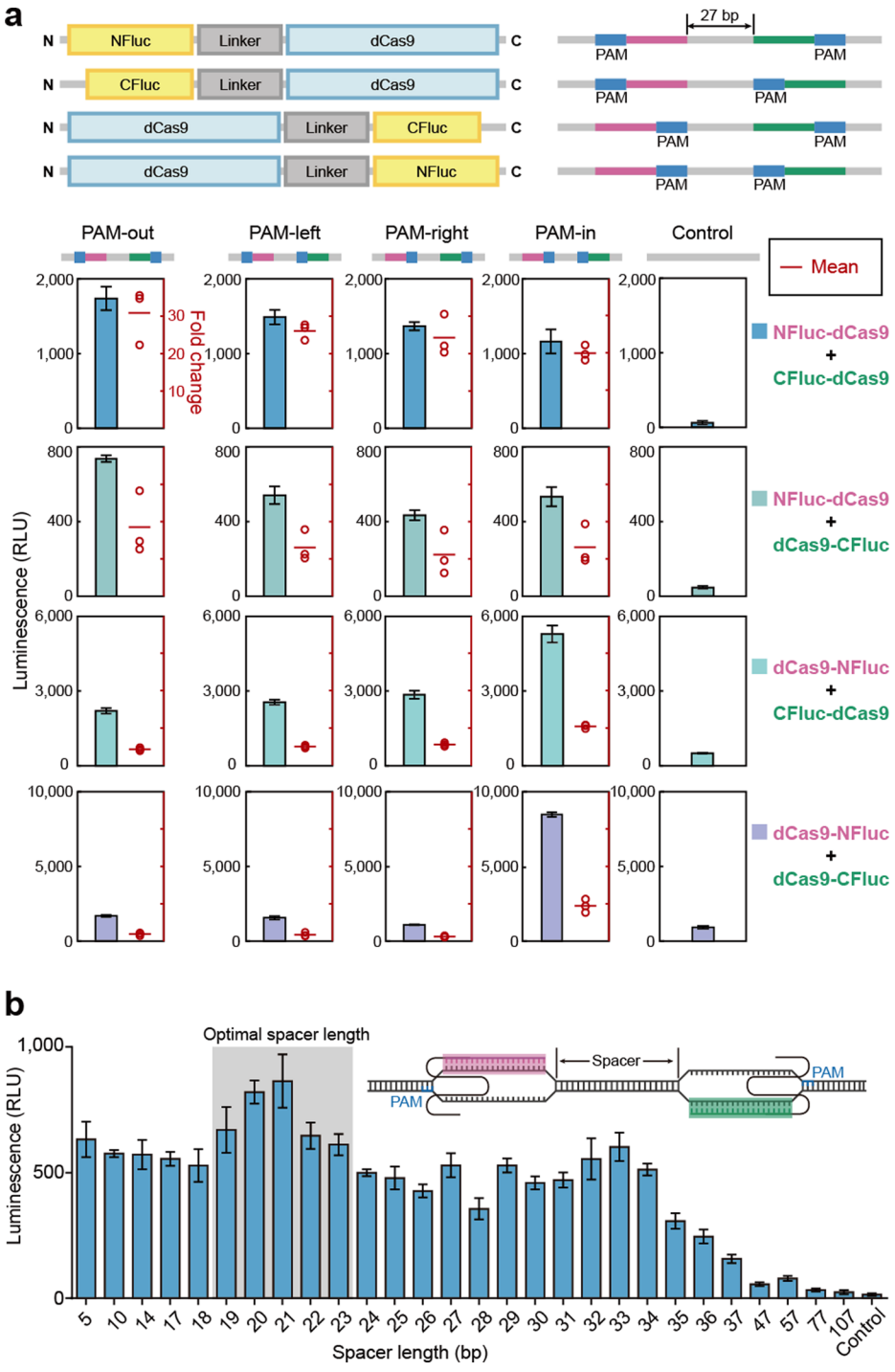


Figure 2. Construction and optimization of the PC reporter system. (a) Performance of the PC reporter in 16 different configurations. The universal reporters were constructed by linking NFluc and CFluc to the N- or C-terminus of dCas9 and used in four different combinations (rows). The substrate DNA contained identical target sites that adopted four different PAM orientations (columns). In all panels, bars indicate the absolute luminescent intensities (left *y*-axis); while red circles in the 16 experiment groups show the fold change in luminescence over the corresponding control groups (right *y*-axis). (b) The effect of spacer length on the performance of the PC reporter. The distance between two target sites varied from 5 bp to 107 bp on the substrate DNA, and the shaded box encloses the range of spacer length with ideal performance. The control group contained nonspecific DNA. N-terminally linked reporters were used in these experiments. In both (a) and (b), substrate DNA concentration was 3×10^{-3} nmol/mL, and error bars show standard error of three experimental replicates.

nonspecific *E. coli* genomic DNA (Figure 3a). Serially diluted *Mtb* genomic DNA samples were amplified through 35 rounds of PCR and tested with the PC reporters. Equivalently one genome/500 μL pre-PCR ($\sim 5 \times 10^{-13}$ g/mL PRC product) was detected with 11.6-fold luminescence gain in the post-PCR reaction mixture, demonstrating excellent sensitivity for the test. Signal intensity increased with substrate concentration in a power law fashion before the reporters were saturated (Figure 3b).

The unique programmability and ease of handling of our PC reporter system can be leveraged in parallelized array designs applied for the detection of pathogen DNA in clinical and other nonlaboratory samples, where minor variations in DNA

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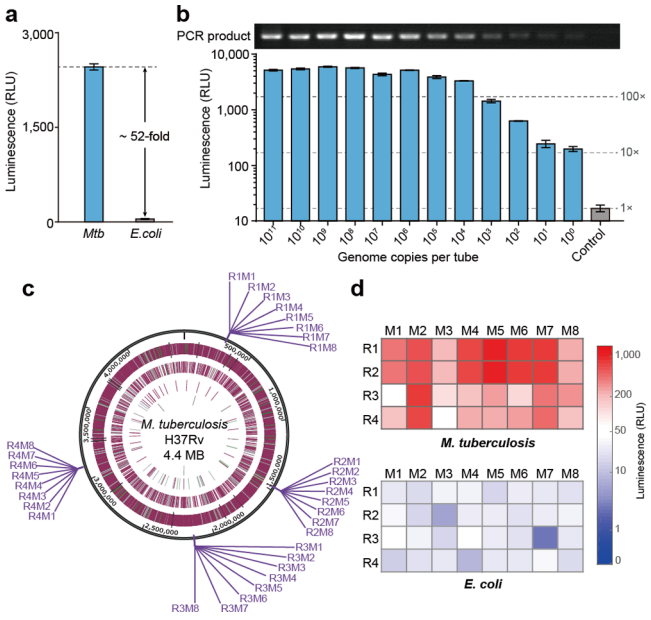


Figure 3. PC reporter in the detection of *Mycobacterium tuberculosis* (*Mtb*)H37Rv genome. (a,b) Specificity and sensitivity of the PC reporter targeting the *Mtb* 16S rRNA gene. In (a), equimolar *Mtb* H37Rv and *E. coli* MG1655 DNA was PCR amplified and used as the experiment and the control group, respectively. In (b), numbers of *Mtb* H37Rv genome in the crude sample pre-PCR are indicated on the *x*-axis. The control group contained no DNA. Gel bands of the PCR products of *Mtb* H37Rv DNA are shown on top of each bar. In both (a) and (b), error bars show standard error of three experimental replicates. (c,d) Multitarget array detection of the *Mtb* H37Rv genome. In each of the four regions (R1-R4) across the *Mtb* H37Rv genome, eight marker sequences (M1-M8) were selected and the target specificities programmed to PC reporters in a 4 × 8 detection array. *E. coli* genomic DNA was used in the control plates. Color shows luminescent intensities in one representative plate of three replicates in the experiment (top) and the control (bottom) groups.

sequence, as well as fragmented DNA, may present problems for existing NA detection methods that target only a tiny region in the entire genome of interest. As a proof of principle, we designed an *Mtb* genome detection array. A pool of 3791 candidate target sequences specific to *Mtb* against the human oral metagenome, which is representative of the genetic composition of patients' sputum samples, and satisfying the PAM-out orientation with 19−23 bp distance between the two 23 bp segments were mapped out along the *Mtb* genome by an automated algorithm. From this pool, we selected 32 target sequences in four regions distributed across the *Mtb* genome, each of ∼3 kb long, and synthesized the complementary sgRNAs (Figure 3c). These sgRNAs were used to program the PC reporter in each well simultaneously. Homogeneous solutions containing *Mtb* genomic DNA or the nonspecific *E. coli* genomic DNA were applied to this customized reporter array followed by a statistical analysis on the luminescence matrices. Relative luminescence levels varied between wells as a result of differential performances of each individual target sequence (Figure 3d). Nevertheless, with the array design utilizing much more sequence information than a traditional single probe test, the *Mtb* genome was detected with a *p*-value of 8×10^{-23} (by Wilcoxon ranked sum test). A reporter array for *B. subtilis* was designed likewise and detailed in Online Methods (also see Supplemental Figure S3).

The PC reporter system we developed here uses dCas9 as an adapter between the bioluminescent reporter and the probing sequences. In itself, a pair of dCas9 proteins is able to encode in total 44 bp of target specificity with an additional spacing constraint, and makes easy the attachment of enzymatic reporters that produce highly sensitive reaction output with minimal background signals. Even more profoundly, the novel modular design of the PC reporter makes it recyclable, and its target specificity readily reprogrammable to virtually any sequence of our choosing. For this reason, we are confident that it will provide an alternative solution to NA detection that is both more powerful and more affordable than available probe-based approaches. In comparison with the single-strand DNA in molecular beacon reporter, the single gRNA in paired dCas9 system is more fragile and easily degraded by the ubiquitous RNase. We believe that some chemical modifications on the sgRNA might improve the stability of an active dCas9-sgRNA complex in the future study.

Currently in clinical practice, pathogen DNA detection still relies on PCR. The new generation of isothermal PCR technologies has obviated the need for thermal cyclers and thus presents a major step toward point-of-care diagnostics.[11] However, for these methods sensitivity comes at the cost of specificity,[12] and the dsDNA dyes or chelants used as reporters could not distinguish nonspecific amplification products such as those from primer dimerization.[13] We have demonstrated how our PC reporter utilized extra sequence information to reliably detect the *M. tuberculosis* genome. The added statistical power in our array design could prove especially valuable in real world situations where either pathogen DNA is present at very low concentrations or nonspecific DNA is ample to confound test results. Luciferase was used in this study as the reporter; however, for field applications, both electrochemical signals and ELISA colorimetric readout might be more desirable for the development of automatic readers and test strips, respectively.

Perhaps the most exciting application of the PC reporter is the fast prototyping of arrays and miniature systems to access DNA sequence content in a sample at significantly dropped costs compared to direct sequencing. Such platforms could be useful for multipathogen detection, multigene detection in metagenomic samples, or even as "DNA readers" which will require an additional step of short reads assembly. The experiments in this study were performed in homogeneous solutions; however, application for *in vivo* bioimagining is highly conceivable, given the universality of CRISPR-Cas across many different model and nonmodel organisms.

METHODS

Cloning and Protein Purification. The codon-optimized DNA sequences of FKBP, FRB, firefly luciferase and dCas9 were ordered from Generay Biotech (Shanghai) (see Supplemental Figure S5). *Bsa*I restriction sites were added at both ends of each protein coding sequence. NFluc-FRB, FKBP-CFluc, NFluc-dCas9, CFluc-dCas9, dCas9-NFluc, and dCas9-CFluc were constructed in pET21a using Golden Gate Assembly. The linker between luciferase fragments and dCas9/FKBP/FRB was GGGGSGGGGS. Each fusion protein contained a 6 × His-tag on its N or C terminus. The *E. coli* BL21 (DE3) strain was used for protein expression, and protein purification followed standard protocol.[18] The purified proteins were stored in 50% glycerol at −80 °C.

The backbone plasmid for sgRNA expression was constructed based on pSB1C3 (iGEM registry) by Gibson assembly (Supplemental Figure S6), and the protospacers were inserted into the backbone plasmid by Golden gate method. The target sequences in Figures 1b,c and 2a,b were designed in the annealed

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primers and cloned into pSB1C3 vector by Gibson assembly (Supplemental Figure S7).

sgRNA Production and *In Vitro* Measurement of Luminescence. Linear DNA fragments containing the "T7 promoter-sgRNA-T7 terminator" cassette were first PCR amplified from sgRNA expression plasmids and then subjected to cell-free transcription using HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs) to obtain sgRNA products, followed by purification. The sgRNA products were stored at −20 °C.

To obtain protein:sgRNA complexes, NFluc-dCas9 and CFluc-dCas9 were, respectively, incubated with their corresponding sgRNA using the following method: 3 μL sgRNA (diluted to 1000 nM) and 2 μL fusion protein (diluted to 0.25 μM) were mixed in 8.5 μL HEPES buffer (20 mM HEPES, 150 mM KCl, pH 7.5) and incubated at 25 °C for 10 min. Three μL DNA substrate, diluted to specified concentrations, was then added into the reaction mixture containing two protein:sgRNA complexes (2 × 13.5 μL = 27 μL), and further incubated at 37 °C for 30 min (30 μL in total). The total solution was transferred into a black flat-bottom 96-well plate (prewarmed at 37 °C), followed by the addition of 100 μL Luciferase Assay Reagent (Promega E4050, prewarmed at 37 °C). Once the preparation is complete, luminescence was promptly measured using microplate reader (Thermo Scientific Varioskan Flash).

To verify the functional complementation of NFluc and CFluc (Supplemental Figure S1), 2 μL NFluc-FRB and FKBP-CFluc (both diluted to 0.25 μM) were combined into 16 μL HEPES buffer. 40 nM rapamycin (Sigma-Aldrich, dissolved in DMSO) was added immediately. After incubation at 37 °C for 10 min, luminescence was measured as above.

Search for gRNA Candidates Throughout the *M. tuberculosis* Genome. We first enumerated PAM sites (5′-CCN$(N)_{20}$ or $(N)_{20}$NGG-3′) in the *Mycobacterium tuberculosis* H37Rv genome which was isolated by Prof. Cuihua Liu from a tuberculosis patient. A Python 3.4.3 build-in regular expression was used to search these candidates on both forward- and reverse-strands. We evaluated the specificity of each target *in silico* against Human Oral Meta-Genome (HOMG, http://www.hmpdacc.org/HMRGD/) by blastn (BLAST-2.2.31+, NCBI, ref 19) and the biophyton-1.65 module. Parameters for BLAST are listed in Supplemental Table S1. Candidates whose PAM-proximal 12 bp sequence appeared in the HOMG collection were subsequently excluded. An algorithm of CRISPR Design tool (ref 20 and http://crispr.mit.edu/about) was used to score the off-target effect for the remaining target candidates. The candidates were then ranked with an index scored from 100% to 0% (Supplemental Figure S4), and the ones with a score lower than 45% were further removed. Finally, according to our experimental data (Figure 2b), target sequences satisfying the spacing constraint, *i.e.*, 19−23 bp between half sites, were paired as a marker in the *Mtb* genome. In total, 3751 markers were selected throughout the *Mtb* genome. Four *Mtb* genomic regions, each containing eight markers, were selected for the array test (Figure 3c, Supplemental Table S2). As a positive control, a genomic detection array was designed for *B. subtilis*, using markers selected by the same method from nine regions in the *B. subtilis* genome with each containing seven or eight markers (70 of 2583 candidates used in final array) (Supplemental Table S3).

Single-Target Assay and Target-Array Assay of *M. tuberculosis*. Isolated genome of *M. tuberculosis* H37Rv was purified by Dr. Cuihua Liu. A pair of target sequences in the 16S rRNA gene was used to demonstrate the reliability of the PC reporter in Figure 3a,b. In Figure 3b, the product of 35-cycle PCR amplification was added to the PC reporter detection buffer without further purification. For the array-based high-throughput assay, 32 markers in *E. coli* and *Mtb* genomes were tested on 96-well microplates, respectively. The array assay of *B. subtilis* genome detection (Supplemental Figure S3) was performed in the same way. The *p*-values of the array assays were calculated by Wilcoxon ranked sum test.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00215.

Supplementary figures; synthetic gene sequences; markers selected throughout *M. tuberculosis* H37Rv genome and *B. subtilis* genome. (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors have filed a provisional patent application related to this work.

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