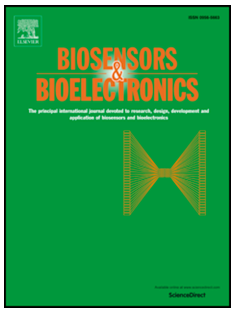

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A CRISPR-derived biosensor for the sensitive detection of transcription factors based on the target-induced inhibition of Cas12a activation

Bingzhi Li[1], Anqi Xia[1], Shilin Zhang[1], Tiying Suo[1], Yujie Ma[2], He Huang[1, *], Xing Zhang[1, *], Yue Chen[2, *], Xuemin Zhou[3, *]

[1] School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Nanjing 210023, China

[2] School of Nursing, Nanjing Medical University, Nanjing 211166, China

[3] School of Pharmacy, Nanjing Medical University, Nanjing 211166, China

[*] **Corresponding Authors:**

He Huang (huangh@njnu.edu.cn);

Xing Zhang (zhangxing@njnu.edu.cn);

Yue Chen (chenyue1005@njmu.edu.cn)

Xuemin Zhou (xueminzhou001_001@hotmail.com)

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Abstract

Transcription factors (TFs) are the key proteins for the decision of cell fates, and they have been recognized as potent markers for diagnostic and treatment of diseases. Herein, we report on a highly sensitive biosensor for the detection of TFs based on the CRISPR/Cas12a system. This biosensor was accomplished based on the competitive binding of the Cas12a-crRNA and TFs towards a dsDNA referred to as activator. Without TFs, the activator can be recognized by Cas12a-crRNA and cause the activation of the DNase activity of Cas12a. When TFs were added, the TFs can bind with the activator because the activator was designed to contain the specific binding sites of target TFs. We find that this binding can inhibit the association between Cas12a-crRNA and the activator, which hinders the activation of Cas12a. As a proof-of-concept, the rapid detection of five kinds of TFs was presented, and the detection was extended to the analysis of TFs expression in xenograft solid tumors from mice. This investigation is the first attempt to apply CRISPR technology in the sensing of TFs, and it discloses that the blocking of activator can be applied as a new sensing mechanism for the development of CRISPR-based biosensor.

Keywords: Transcription factors; CRISPR; Cas12a; Biosensors; DNA binding proteins

As medical research paves into the post-genomic era, accumulating research interest has been attracted to the revealing of the functions of biomolecules (Tsutakawa et al. 2020; Suo et al. 2020). Among all these biomolecules, transcription factors (TFs) are of critical importance because of their pivotal roles in the regulation of gene expression (Li et al. 2017; Ma et al. 2019). They collect the information from upstream pathways, then associates with the DNA-binding domains in specific genes to trigger the initiation of transcription (Darnell 2002; Napetschnig and Wu 2013). Different from the other signaling molecules, the TFs have much tighter connections with gene expression, and their dysregulations are highly related to cancers, autoimmune diseases, and virus infections (Ben-Neriah and Karin 2011; Karin and Greten 2005; Rasaiyaah et al. 2013). Investigations have revealed that the monitoring of TFs values in suspicious lesions and blood samples could help the early diagnostic of multiple diseases including cancer (Ding et al. 2013; Darnell 2002; Pazos et al. 2016; Vitiello et al. 2012), however, the lab test of TFs still heavily relies on conventional and lengthy methods such as ELISA, Western blot, and electrophoretic mobility shift assay (EMSA). With the involvement of signal amplification and functional nanoprobes, unconventional TFs assays are emerging (Chen et al. 2020; Li et al. 2017; Lin et al. 2019; Zhang et al. 2017). Nevertheless, most of those improved methods required multiple digestions, ligations, and amplification processes, which compromised the simplicity to reach high sensitivity. Along with the development of TFs-based treatment and diagnostic, there is a foreseeable demand for facile monitoring of critical TFs, thus it is meaningful to explore TFs assays meet the

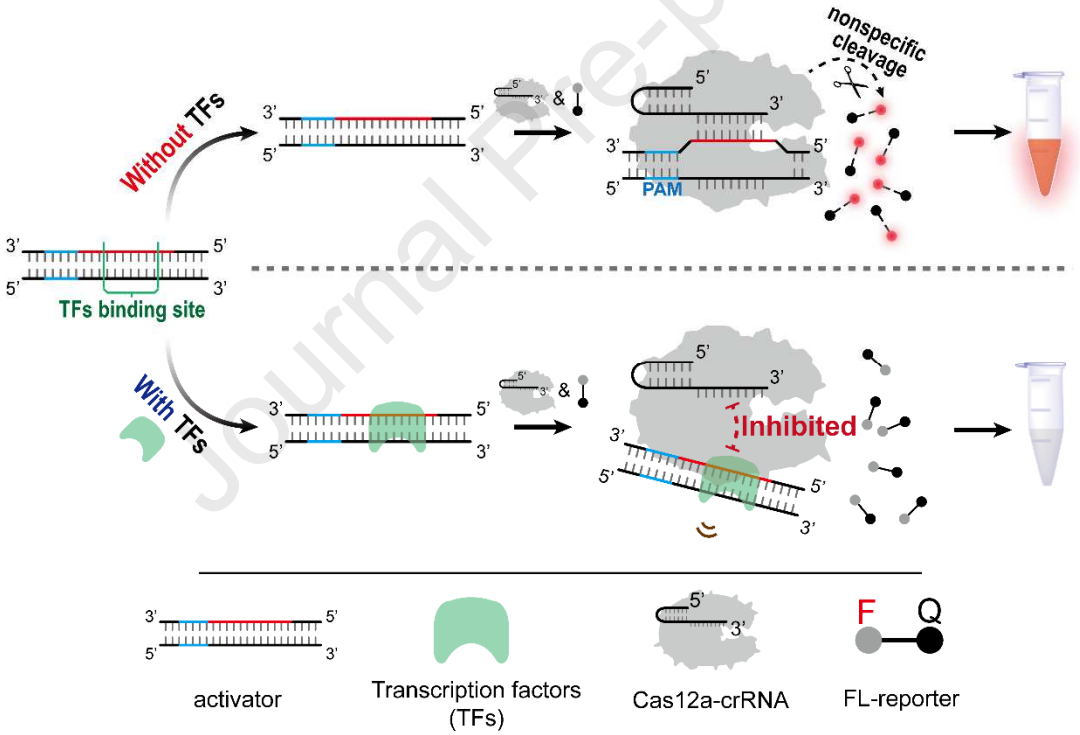
requirements for point-of-care test (POCT) settings.

The recent emergence of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) enzymes have provided powerful tools for genome editing (Wang et al. 2013), bioengineering (English et al. 2019), and diagnostics (Gootenberg et al. 2017). Highly sensitive biosensors for nucleic acids have been developed based on the tunable nuclease activity of Cas9 (Liu et al. 2016; Zhou et al. 2018), Cas13 (Chen et al. 2019; Myhrvold et al. 2018), and Cas12a (Chen et al. 2018; Li et al. 2018). The Cas-crRNA complex, formed by the binding between the Cas enzymes and a single-stranded guide CRISPR RNA (crRNA), can recognize nucleic acids through base pairing and make a response by initiating enzymatic activities. For example, Cas12a-crRNA can recognize both ssDNA and dsDNA, then generating nonspecific DNase activity to cleave nearby ssDNA (Kellner et al. 2019; Li et al. 2018). Benefit from specific recognition, efficient cleavage, and portability for potential POCT applications, Cas enzymes are competent tools in the detection of nucleic acids (Chang et al. 2019; Gootenberg et al. 2018; Xu et al. 2020), however, CRISPR has rarely been applied in the sensing of non-nucleic-acid biomarkers. Though recent research has proposed some strategies to applying Cas enzymes in the detection of non-nucleic-acid targets (Shen et al. 2020; Xiong et al. 2019), their underlying mechanism is to transduce the signal of targets recognition to the generation of Cas-recognizable nucleic acid substrates. The transduction typically involved the enzyme-assisted reactions and nucleic acid reaction network (Chen et al.

2019; Shen et al. 2020), with the need for multiple steps of temperature control, purifications, and centrifugation. During these procedures, the loss and distortion of the target signal may occur, resulting in decreased sensitivity and accuracy. Therefore, it is highly desirable for developing more direct mechanisms to enrich the mechanisms of CRISPR-based sensing.

According to the best of our knowledge, there has been no research regarding the CRISPR-based sensing of TFs published. Although Tan and Zhang's group has reported on a sensing system composed of allosteric TFs and Cas12a (Liang et al. 2019), the TFs were served as sensing component and the purpose of their research was to detect small molecules. In this research, we attempted to extend the application of the CRISPR/Cas system to the sensing of TFs. Both Cas12a-crRNA and TFs can recognize specific dsDNA domain, and we are curious about whether this common point could be utilized as a sensing mechanism. Thus inspired, here we report on a target-induced inhibition strategy for the direct detection of TFs based on CRISPR/Cas. As shown in Scheme 1, a double-stranded DNA (dsDNA) containing protospacer adjacent motif (PAM), TFs binding site (TBS), and crRNA-recognizable-sequence (denoted in red) was used as the activator of Cas12a-crRNA (referred to as "activator"). When there is no target TFs in the sample, the activator will associate with the Cas12a-crRNA by hybridizing with crRNA. This association will unleash the nonspecific DNase activity of Cas12a, which causes cleavage of collateral ssDNA (Chen et al. 2018). The cleavage indiscriminately shreds

the FL-reporter (a 5 nt ssDNA modified with 5'-Cy3 and 3'-BHQ2) to separate the fluorophore (Cy3) and quencher (BHQ2), generating fluorescence enhancement. Differently, when TFs are added, they can stably bind to the TBS region and form a stable protein-DNA complex. The complex exhibits a considerable size effect which prevented the Cas12a-crRNA from approaching the activator. As a result, the activation of the Cas12a will be inhibited, so the FL-reporter remains intact. In this profile, the TFs control the activity of Cas12a through the activator, then probed by the FL-reporter.



Scheme 1. The schematic illustration of the Cas12a-based biosensor for TFs detection.

We used the p50 subunit of nuclear factor kappa-B (NF-κB) to demonstrate the principle of this biosensor, and the specific TBS of NF-κB p50 was adopted from previous reports (Ma et al. 2011; Vallée-Bélisle et al. 2011; Wang 2005). Increased

concentrations of NF-κB p50 were incubated with the activator, then added with the mixture of Cas12a-crRNA and ssDNA reporter (a 15 nt ssDNA instead of FL-reporter), followed by native-PAGE analysis. As shown in Figure 1A, Lane 1 indicated the location of the ssDNA reporter, and it can be found that the band intensity of the ssDNA reporter increased with the intensified concentrations of NF-κB p50 from Lane 2 to Lane 5. In Lane 6, no significant band can be observed, indicating that the ssDNA reporter was all cleaved. These results reveal that the addition of NF-κB p50 inhibited the activation of the DNase activity of Cas12a in the system. Furthermore, an activator(TBS-) having PAM and crRNA recognizable sequence, but lack of the TBS of NF-κB p50, was designed. This activator(TBS-) were incubated with different concentrations of NF-κB p50, then added with Cas12a-crRNA and ssDNA reporter. We found that all ssDNA was cleaved in this set of tests, regardless of how much NF-κB p50 was added (Figure S1). This result indicated that the activator with the ability to bind NF-κB p50 mediated the inhibition effect of NF-κB p50 towards Cas12a-crRNA. The results of gel analyses together demonstrated that the association of the activator and the Cas12a-crRNA can be impeded by the binding between NF-κB p50 and the activator.

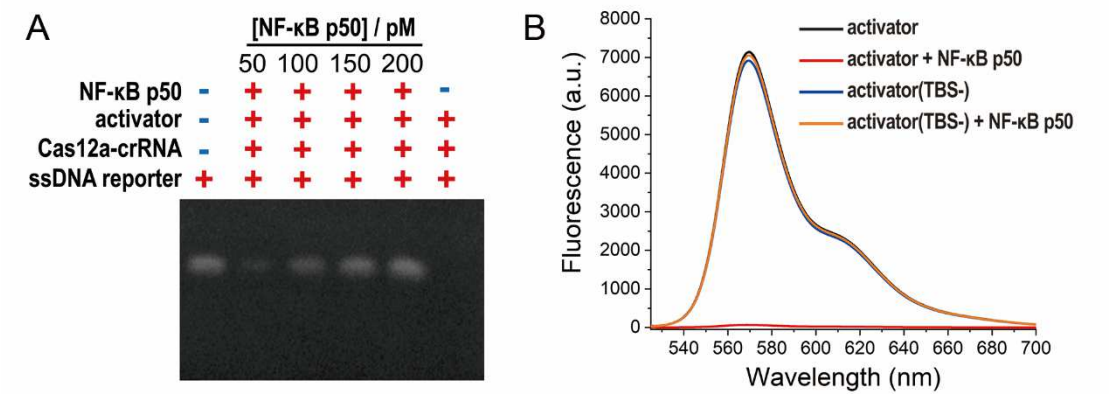


Figure 1. The verification of this sensing mechanism. (A) Native-PAGE verification of the cleavage of the ssDNA reporter under different concentrations of NF-κB p50. The concentrations of activator, Cas12a-crRNA, and ssDNA reporter were 0.2 nM, 0.2 nM, and 1 μM respectively, and the cleavage of ssDNA reported was taken for 1 h. (B) Fluorescence verification of this biosensor in various conditions. The concentrations of activator, activator(TBS-), and NF-κB p50 were all 0.2 nM if they were added, and 1 nM of FL-reporter was used throughout the fluorescent test. The separated spectra can be found in Figure S2.

The feasibility of this biosensor was further tested by the fluorescent spectra in different conditions (Figure 1B). When the activator, Cas12a-crRNA, and FL-reporter were mixed, a strong emission can be observed. However, when the activator was incubated with NF-κB p50 first, only negligible fluorescence is recorded. This change indicated that the addition of NF-κB p50 inhibited the cleavage of the FL-reporter. To further demonstrate that the inhibited cleavage is derived from preventing the association between activator and Cas12a-crRNA, the activator(TBS-) was used. As shown in the blue and orange curves, when activator(TBS-) was added to the reaction,

the samples are both highly emitted regardless of the addition of NF-κB p50. The two tests with activator(TBS-) indicated that the DNase activity of Cas12a will be unleashed if the activator cannot bind with NF-κB p50. Taken the above results together, the binding between NF-κB p50 and the activator can inhibit the activation of Cas12a, which reinforces the realization of this biosensor. The results from fluorescent spectra and the above native-PAGE analysis are in accordance with each other, further revealed the feasibility of this biosensor.

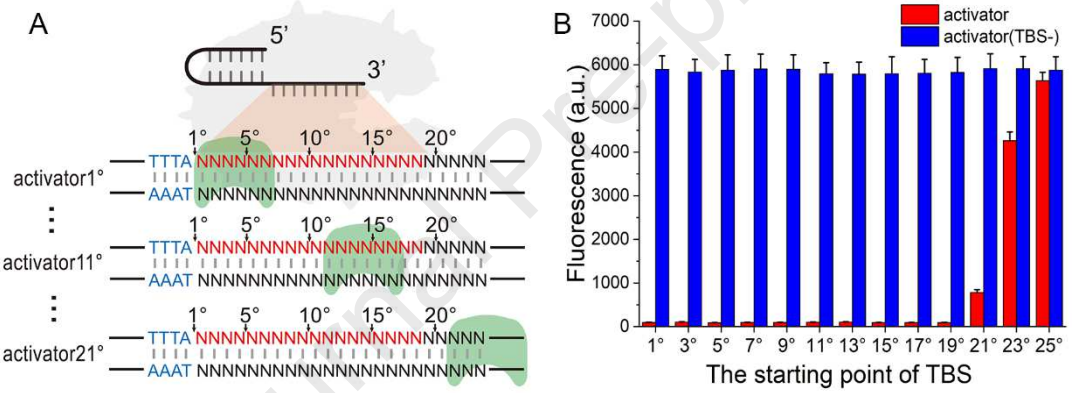


Figure 2. The relationship between the starting point of TBS in the activator and the inhibitory effect towards the activation of Cas12a. (A) The design rule of activators with different TBS starting points. The TBS-negative version of activators is designed by replacing the TBS sequence with the random sequence with the same formula of nucleotides. (B) The fluorescence signal of samples added with different kinds of activators. All the conditions, including the concentrations of NF-κB p50 (0.2 nM), Cas12a-crRNA (0.2 nM), activator (0.1 nM), and FL-reporter (1 nM), are identical in this set of tests, except for different kinds of activators are used. The cleavage was conducted for 2 h in this test to testify the inhibitory effect. Error bars indicate the

standard deviation of three replicate determinations.

In this work, the activator is “locked” by TFs to lose the ability to triggering Cas12a, thus the location of TBS in the activator played essential roles in the sensing profile. As shown in Figure 2A, the starting points of TBS are designed at the various position downstream of the PAM. To minimize the influence of sequence affinity, the TBS is inserted to the activator as a whole in different positions to form a new activator. According to the inserting point of TBS, the corresponding activators are referred to as activator1° to activator25° (complete sequence in Table S1). Besides, a set of activators with the TBS replaced by random sequences were designed, and they are named as activator1°(TBS-) to activator25°(TBS-). Figure 2B indicates the fluorescent signal of the samples with various activators, and it can be found that a significant difference can be observed in the TBS-positive and -negative samples from activator1° to activator19°. However, starting from activator21° (e.g., the TBS inserting point is 20 bp away from PAM), the difference decreased. These results indicated that the triggering ability of activator towards Cas12a-crRNA was significantly inhibited when the binding position of NF-κB p50 is in the range from position 1° to 19°. As the binding position went beyond this range, the inhibitory effects decreased sharply. It has been established that the recognition between Cas12a-crRNA and activator is sequence-sensitive, which is capable of distinguishing single nucleotide polymorphism (Myhrvold et al. 2018; Zhang et al. 2018). The binding of TFs can effectively impede the association between Cas12a-crRNA and the

activator, even when the NF-κB p50 is bounded 4 bp downstream of the hybridization region. The location-insensitivity implies that the binding between NF-κB p50 and the activator causes a considerable change in the chemical environment of the activator.

A time-interval of 20 min was set for the fully binding of NF-κB p50 to the activator, then the kinetics of the activation of Cas12a and subsequent collateral cleavage at different temperatures was recorded. As shown in Figure S3, the reaction accelerates as the temperature increased from 15 °C to 45 °C, which can be ascribed to the change of enzymatic activity. Interestingly, when the temperature is higher than 40 °C, larger proportions of fluorescence enhancement occurred. The Cas12a is active from 16 to 48 °C according to the manufacturer's protocol, while the human-derived NF-κB p50 quickly loses its activity when the temperature is higher than 40 °C. Therefore, the increased fluorescence enhancement after 40 °C can be ascribed to the inactivation of NF-κB p50, which makes some activator released from the complex and associated with Cas12a-crRNA. The reaction temperature was set at 25 °C to unify with the above procedure, and a 20 min of time-interval was adopted.

The analytical procedures are shown in Figure 3A, and it can be found that the total workflow took 40 min with two steps of sample addition. Figure 3B shows that the fluorescence of samples declined as the concentrations of NF-κB p50 intensified. An excellent linear relationship from 5 pM to 5 nM can be established, and the limit of detection (LOD) of 2.7 pM was obtained following the 3σ/slope rule (methodology

detailed in Supplementary Material). It can be found that the error bars showed relatively high deviation, which could derive from the instrumental instability and operating errors. Figure S4 shows that the deviation in the measuring a blank sample for ten times was not high, which demonstrated that the instrumental instability was not the main reason for high deviations. Due to low concentrations of FL-reporter was applied in the sensing, the high signal gain was adopted, which may amplify the operating errors and cause high deviations. The change of analytical conditions such as the ratio and concentrations of sensing components may result in different signal response and linear range, thus the sensor can be further customized based on specific sensing purposes. As an assay without the participation of nucleic acids amplification and complicated circuit reactions, the obtained low picomolar sensitivity is quite advantageous compared with the efforts in recent reports (detailed in Table S2). We assumed that the acquisition of excellent sensitivity is related to the high turnover of Cas12a (Dai et al. 2019; Wang et al. 2019). When compared with conventional assays including ELISA, EMSA, and ChIP, this biosensor can realize homogeneous quantification without the need for washing, purification, and gel electrophoresis, thus significantly reduced the operational time from 0.5-2 days to ~40 min. Besides, this biosensor is free of expensive and unstable antibody, which significantly increases the convenience and portability of this method.

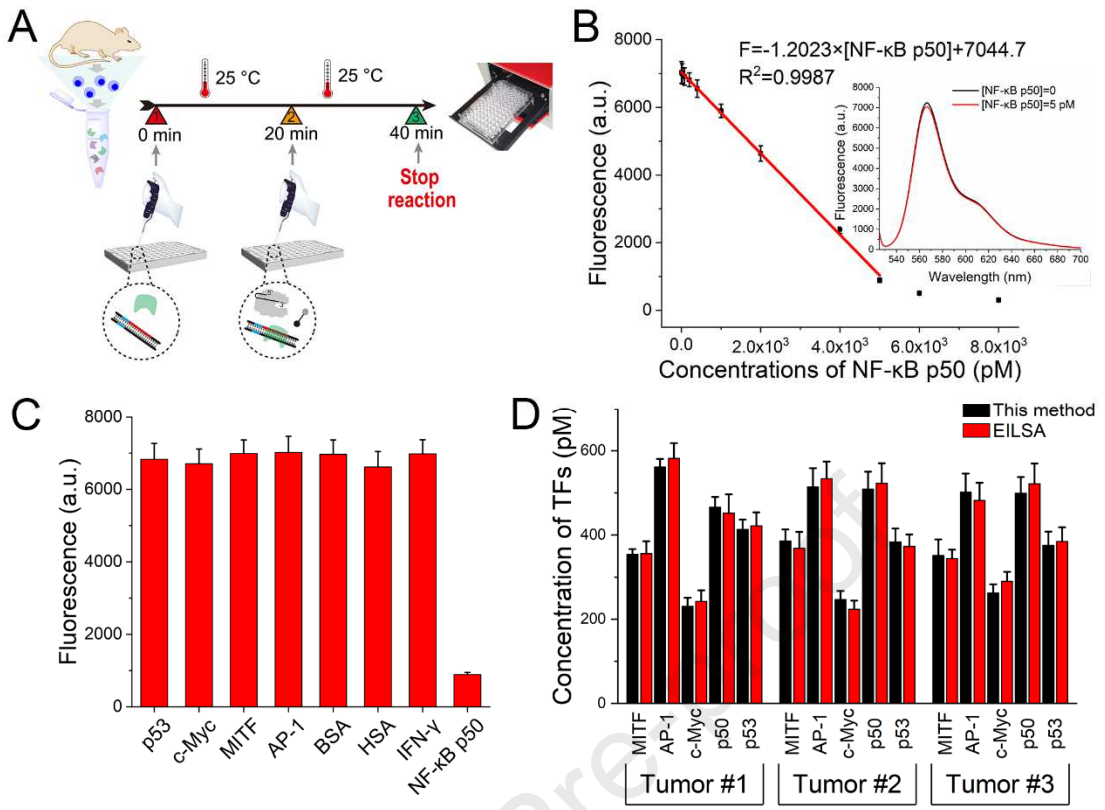


Figure 3. Performance of the Cas12a-based biosensor in the detection of TFs. (A) The workflow for the detection of TFs from biological samples. (B) The relationship between the fluorescence of the samples and the concentration of NF-κB p50. Inset is the fluorescent spectra of the biosensor without the addition of NF-κB p50 and with the addition of 5 pM of NF-κB p50 (λ_{ex}=510 nm). (C) The selectivity of this method towards NF-κB p50. The concentration of all the interferent is 50 nM, while that of NF-κB p50 is 5 nM. (D) The detection of five kinds of TFs in three solid tumors obtained from xenograft mice. Black columns represent the results from this biosensor, and the red columns represent the results from commercial ELISA kits. Error bars indicate the standard deviation of three replicate determinations.

The biosensor was added with other kinds of TFs (p53, c-Myc, MITF, and AP-1) and

biomolecules (BSA, HSA, IFN-γ). As shown in Figure 3C, only the sample with NF-κB p50 was weakly emitted, even the concentration of all the other biomolecules is 10-fold higher than NF-κB p50. These results indicated that only NF-κB p50 can inhibit the activation of Cas12a by forming a complex with the activator. The biosensor was also challenged by a group of analogs of the activator. The analogs were created by deleting the PAM or replacing some nucleotides in the TBS, and it was found that only the right activator with PAM and fully matched TBS can be used for probing the concentrations of NF-κB p50 (Figure S5). This excellent specificity can be ascribed to the affinity between NF-κB p50 and its specific TBS (Lu et al. 2017; Lin et al. 2019), as well as the recognition specificity between Cas12a-crRNA and its activator.

The real sample-based recovery tests were further conducted. The concentrations of NF-κB p50 in from xenograft solid tumors were measured using the calibration curve presented in Figure 3B, followed by calculating the recovery after spiking. Recovery results ranged from 96.2-108.0% was obtained (Table S3), which demonstrated that this linear relationship can be applied in the real sample analysis with acceptable errors. Additionally, intra- and inter-day precision were <5.8% and 3.5% as shown in Table S4 and Table S5, respectively. All the test results meet the criteria of bioanalysis (Xu et al. 2017; Zhou et al. 2018), indicating that the specificity, accuracy, and precision of this biosensor could make practical analysis possible.

Encouraged by the realization of NF-κB p50 detection, we further investigated the versatility of this biosensor in detecting other kinds of TFs. The activators were modularly re-designed by embedding the highly specific TBS of p53 (Heyduk and Heyduk 2002), c-Myc (Halazonetis and Kandil 1991), MITF (Zhang et al. 2017), AP-1 (Yin et al. 2014), and Nanog (Ma et al. 2016). As shown in Figure S6, the fluorescence of the biosensor has a strong relationship with the concentrations of target TF, and the LOD of 3.6 pM, 2.3 pM, 4.2 pM, 4.5 pM, and 6.0 pM were obtained for the quantification of p53, c-Myc, MITF, AP-1, and Nanog, respectively. Besides, when the concentration of TFs was set at the lowest concentration of the linear range, a significant decrease in fluorescence can be observed (B, D, F, H, and J of Figure S6). Surprisingly, this biosensor exhibited certain similarities in the signal response and analytical performance when detecting the selected TFs. Because the adopted binding sites were reported to possess a strong affinity towards the corresponding TFs, we assumed that this phenomenon may be derived from the efficient inhibition of the activators. Whereas, it should also be noticed that the recognition between Cas12a-crRNA and the activator, as well as the enzymatic activity of Cas12a, could both influence the performance of this biosensor. The particular explanation of the interesting phenomenon remains to be elucidated, however, our results indicated that this method can be repurposed for the detection of different kinds of TFs *via* reengineering the activators.

The detection of NF-κB p50, p53, c-Myc, MITF, and AP-1 in solid tumors was

conducted by both the Cas12a-based biosensor and commercial ELISA kits. The results were shown in Figure 3D, and it can be found that the two methods are generally in agreement with each other, which reinforced the feasibility of the current biosensor. Though similar results were obtained, this biosensor required only 1/9 of detection time (~40 min *vs.* ~6 h) and 1/10 of the detection sample. Additionally, and cost of this biosensor is ~$0.5 per test, which was similar to SHERLOCK (Gootenberg et al. 2017), however, the cost of ELISA kits is nearly $ 7 per test (a 96T kit typically cost $700). Therefore, this biosensor has the potential to revolutionize the quantification of TFs by integrating improved performance and reduced cost.

In summary, we have developed a biosensor based on CRISPR/Cas12a for the detection of five kinds of TFs. The binding between target TFs and the dsDNA activator impeded the approaching and association between Cas12a-crRNA and the activator, thus inhibited the activation of Cas12a. This process converted the binding between TFs and activator into the regulation of the DNase activity of Cas12a, which enabled the specific quantification of multiple kinds of TFs with well-defined binding sites, and yielded LOD at the low picomolar level. We were surprised to find that this biosensor exhibited similar analytical performance in the detection of the selected TFs, while the underlying mechanism remains unclear. Furthermore, because the sensing profile is based on the binding between TFs and their binding sites, its application scope is currently limited to those TFs with well-defined TBS; however, with the development of methodologies for the discovering of TFs binding sites, the

application scope would be expanded accordingly. Additionally, it will also be a meaningful work to apply this biosensor in the monitoring of weakly expressed yet critical TFs based on comprehensive verifications. This investigation suggested a new sensing mechanism by using sensing target to intervene the association between Cas12a-crRNA and the activator, which could promote the application of CRISPR/Cas-based system in the detection of non-nucleic-acid targets. Taken together, the present study not only provided a powerful analytical tool for TFs-based healthcare and research but also broaden the utilization of CRISPR/Cas in analytical fields. Future works will be focused on exploring the key factors that determine the performance of this biosensor (such as binding affinity), as well as the application of this biosensor in the detection of weakly expressed TFs.

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

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1. It is found that TFs can compete and inhibit the dsDNA-induced activation of Cas12a.

2. CRISPR technology is first applied in the biosensing of transcription factors.

3. The inhibition-based sensing mechanism for CRISPR is proposed.

4. Rapid sensing of multiple TFs with picomolar sensitivity is realized.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: