Electronic Supplementary Information

Development of Bidirectional Isothermal Amplification Strategy for Sensitive Detection of Transcription Factors in Cancer Cells

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EXPERIMENTAL SECTION

Materials and reagents. All oligonucleotides (Table 1) were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The purified recombinant NF-κB p50 (rhNF-κB p50) and NF-κB p65 (p65) were purchased from Active Motif (Carlsbad, CA, U.S.A.). The Cellular Jun (c-Jun) protein was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Bovine serum albumin (BSA) was bought from Sigma Aldrich Company (St. Louis, MO, U.S.A.). The Exonuclease III (Exo III),

Klenow Fragment polymerase (3'→5' exo-), the Nb.BtsI nicking endonuclease and Endonuclease IV (Endo IV) were bought from New England Biolabs (Beijing, China). Oridonin was obtained from Shanghai Macklin Biochemical Company (Shanghai, China). Deoxynucleotide solution mixture (dNTPs) was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). SYBR Gold was obtained from Life Technologies (Carlsbad, CA, U.S.A.). Human cervical carcinoma cells line (HeLa cells) was obtained from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All other chemicals were of analytical grade and used without further purification. Ultrapure water was prepared by a Millipore filtration system (Millipore, Milford, MA, U.S.A.).

Table S1. Sequences of the oligonucleotides [α]

note	sequence (5'-3')
p50-s	TCG CAT CTA AGG CAC GCA GTG↓ AGT CGC ATC TAA GGC ACC* C*G A GTT T**GG GAC TTT CC**G TGC
p50-antis	TCG CAT CTA AGG CAC GCA GTG↓ AGT CGC ATC TAA GGC AC**G GAA AGT CCC** AAA CT* C* GG
signal probe	FAM-TCG CAT CXA AGG CAC-BHQ

[α] In p50-s probe and p50-antis probe, the asterisks indicate the phosphorothioate modification. The boldface bases represent the binding region of NF-κB p50. The arrows in the probes indicate the nicking positions of Nb.BtsI. In signal probe, the X denotes the tetrahydrofuran abasic site mimic.

Protein-DNA interaction and exonuclease digestion. The 10 μM p50-s and 10 μM p50-antis were incubated in the annealing buffer containing 100 mM NaCl and 10 mM Tris-HCl (pH 7.5) at

95 °C for 5 min and then slowly cooled to room temperature. The 1 μL of purified recombinant NF-κB p50 at various concentrations and 500 nM TF-binding probes were incubated at 37 °C for 30 min in 10 μL of binding buffer (10 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg/mL yeast tRNA, 10% glycerol, 0.25 mM DTT). Then 10 U of Exo III and 1 μL of 10 × NEB buffer 1 were added and incubated for another 10 min at 37 °C, followed by heating at 80 °C for 10 min to inactive Exo III.

Amplification reaction and fluorescence measurement. The Exo III-digested products were added into 50 μL of reaction solution containing 500 nM dNTPs, 2.5 U of Klenow Fragment polymerase, 5 U of Nt.BstI, 300 nM signal probe, 5 U of Endo IV, 5 μL of 10 × NEBuffer 2, and 5 μL of 10 × CutSmart, followed by incubation at 37 °C for 60 min. The fluorescence spectra were measured by using a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan) at an excitation wavelength of 490 nm, and the fluorescence intensity at 521 nm was recorded for data analysis.

Real-time PCR assay. The Exo III-digested products were added into 50 μL of reaction solution containing 500 nM dNTPs, 2.5 U of Klenow Fragment polymerase, 5 U of Nt.BstI, 300 nM signal probe, 5 U of Endo IV, 5 μL of 10 × NEBuffer 2, and 5 μL of 10 × CutSmart. The polymerization experiments were performed on Bio-Rad CFX 96 Real-Time PCR instrument (Bio-Rad) at 37 °C for 100 min. The fluorescence signals were detected at intervals of 30 s.

Gel electrophoresis. The Exo III-digested products were added into 50 μL of reaction solution containing 500 nM dNTPs, 2.5 U of Klenow Fragment polymerase, 5 U of Nt.BstI, 5 μL of 10 × NEBuffer 2, and 5 μL of 10 × CutSmart, followed by incubation at 37 °C for 60 min. The products

of bidirectional EXPAR were mixed with the fluorescent indicator SYBR Gold, and then the mixture was resolved on a 12% nondenaturating polyacrylamide gel electrophoresis (PAGE) in 1 × Tris-borate-EDTA (TBE) buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 110 V constant voltage for 45 min at room temperature. The image of gel electrophoresis was visualized by a ChemiDoc MP Imaging system (Hercules, CA, U.S.A.).

Inhibition assay. For transcription factor inhibition assay, oridonin at various concentrations was preincubated with 0.25 mg/mL nuclear extracts, and 500 nM TF-binding probes at 37 °C for 30 min in 10 μL of binding buffer. The subsequent reactions followed the above steps, and fluorescence intensity was measured as described above.

Cell culture and preparation of cell extracts. Human cervical carcinoma cells line (HeLa cells) were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, U.S.A.), supplemented with 10% fetal bovine serum (FBS, Invitrogen, U.S.A.) and 1% penicillin-streptomycin at 37 ºC in a humidified 5% CO_2 incubator. After HeLa cells were incubated with 20 ng/mL TNF-α (Invitrogen, USA) for 40 min, the nuclear extracts were prepared using the nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of total proteins was measured using Bradford-based assay.

EMSA assay. The TNF-α-treated nuclear extracts at various concentrations and 500 nM TF-binding probes were incubated at 37 °C for 30 min in 10 μL of binding buffer (10 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg/mL yeast tRNA, 10% glycerol, 0.25 mM DTT). The glycerol was added into the samples, then the mixture was loaded on a 10% nondenaturating polyacrylamide gel electrophoresis (PAGE) in 1× Tris-borate-EDTA (TBE) buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 110 V constant voltage for 60 min at room temperature. The gel was stained by SYBR Gold, and scanned by a ChemiDoc MP Imaging system (Hercules, CA, U.S.A.).

SUPPLEMENTARY RESULTS

1. Optimization of experimental conditions. To achieve the best analytical performance, three experimental parameters including the concentration of signal probe, the amount of Endo IV, and reaction time were optimized. As shown in Fig. S1A, the F/F_0 value enhances with the increasing concentration of signal probes from 100 to 300 nM, and then gradually decreases beyond 300 nM (F and F_0 are the fluorescence intensity in the presence and absence of p50 protein, respectively). Thus, the optimal concentration of signal probes is 300 nM. The effect of Endo IV amount was further investigated under the fixed concentration of signal probe (300 nM). As shown in Fig. S1B, the F/F_0 value enhances with the increasing amount of Endo IV and reaches a plateau at the amount of 5 U (F and F_0 are the fluorescence intensity in the presence and absence of p50 protein, respectively). Thus, 5 U of Endo IV is used in the subsequent research. We optimized the reaction time as well, As shown in Fig. S1C, the fluorescence intensity enhances with reaction time from 0 to 60 min, and reaches a plateau at 60 min. Thus, the reaction time of 60 min is used in the subsequent research.

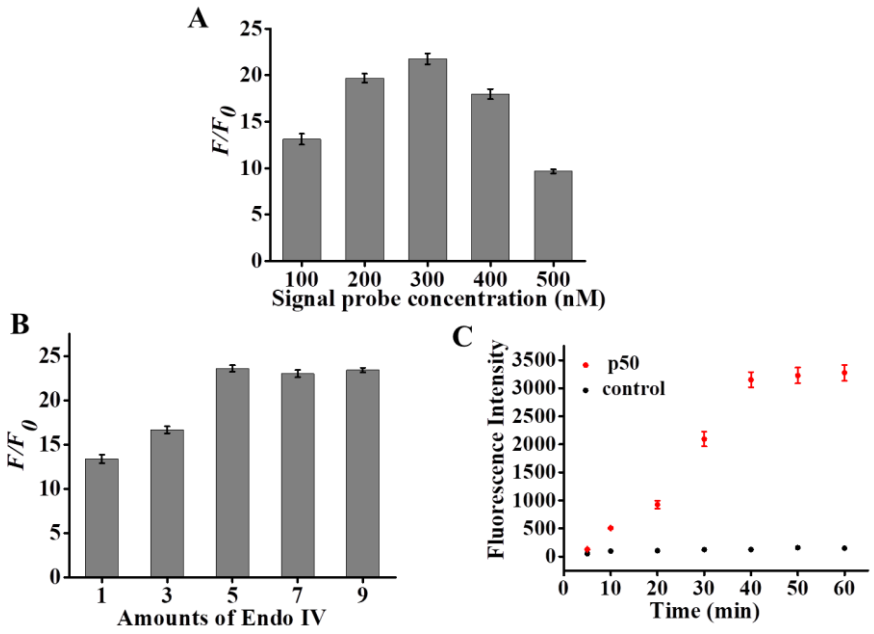

Fig. S1. (A) Variance of F/F_0 value in response to different concentrations of signal probes at a fixed amount of Endo IV (5 U). (B) Variance of F/F_0 value in response to different amounts of Endo IV at a fixed concentration of signal probe (300 nM). (C) Variance of fluorescence intensity with reaction time. The concentration of pure NF-κB p50 is 8×10^{-7} M, the concentration of TF binding probe is 500 nM. Error bars show the standard deviation of three independent experiments.

2. Detection specificity. The specificity of the proposed method is mainly dependent on the specific binding interaction between TF and TF-binding probes. To evaluate the detection specificity, we chose bovine serum albumin (BSA), cellular Jun (c-Jun), and NF-κB p65 (p65) as the model interferent. BSA is an irrelevant protein. The c-Jun is a member of basic zipper (bZIP) family of dimeric transcriptional activators, and it can recognize and bind the enhancer heptamer motif 5'-TGACGTCA-3'.[1] Like NF-κB p50, NF-κB p65 belongs to NF-κB family, but it has a different sequence recognition site.[2] As shown in Fig. S2, distinct fluorescence signal is observed

in the presence of NF-κB p50, while no significant fluorescence signal is observed in the presence of BSA (blue column, Fig. S2), c-Jun protein (green column, Fig. S2), and p65 (yellow column, Fig. S2), respectively. Although NF-κB p65 is a member of NF-κB family,[2] no distinct fluorescence signal is obtained due to the different binding site between the NF-κB p65 and NF-κB p50. Only the NF-κB p50 can efficiently bind to the specific TF-binding probe to initiate the bidirectional EXPAR amplification. These results demonstrate the good specificity of the proposed method towards NF-κB p50.

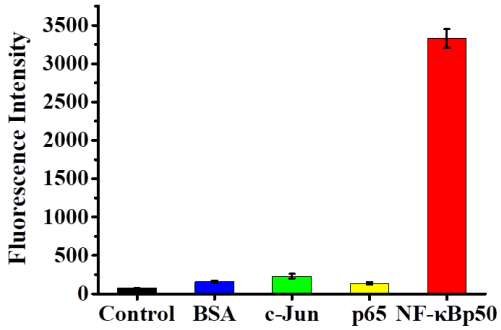

Fig. S2. Measurement of the fluorescence intensity in response to only 500 nM TF binding probe (control, black column), 500 nM TF binding probe + 1 mg/mL BSA (blue column), 500 nM TF binding probe + 8×10^{-7} M c-Jun (green column), 500 nM TF binding probe + 8×10^{-7} M p65 (yellow column), 500 nM TF binding probe + 8×10^{-7} M NF-κB p50 (red column). Error bars show the standard deviation of three independent experiments.

3. Screening of TF inhibitors

NF-κB exists in the cytoplasm in an inactive state and it is activated by a large variety of stimuli including pro-inflammatory cytokine, oxidative stress and hyperglycaemia.[3] The NF-κB can regulate the inducible expression of genes that are involved in tumor promotion, angiogenesis, and metastasis,[4] and thus NF-κB has become a major target for drug development and cancer therapy. To investigate the capability of the proposed method for NF-κB p50 inhibition assay, we used oridonin as the model NF-κB inhibitor. Oridonin directly interferes with the DNA-binding activity of NF-κB towards its response DNA sequence.[5] As shown in Fig. S3, oridonin shows a significant inhibitory effect upon the DNA-binding activity of NF-κB p50 in a concentration dependent manner (Fig. S3A) with a half-maximal inhibitory concentration (IC_{50}) of 28.9 μM (Fig. S3B). Moreover, we used the real-time PCR to detect the DNA-binding activity of NF-κB in the presence of oridonin. The oridonin exhibits significant inhibitory effect upon the DNA-binding activity of NF-κB p50 (ESI†, Fig. S4). These results demonstrate that the proposed method can be used to screen the TF inhibitors.

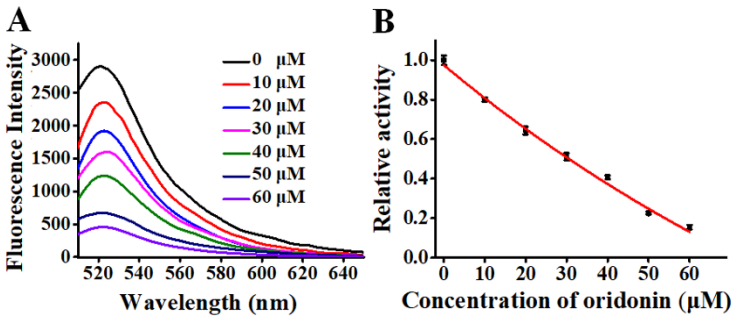

Fig. S3. (A) Measurement of fluorescence intensity in response to different concentrations of inhibitor oridonin. (B) Inhibition effect of oridonin upon the DNA-binding activity of NF-κB p50. The 500 nM TF-binding probes and 0.25 mg/mL nuclear extracts were used in the experiments. Error bars show the standard deviation of three independent experiments.

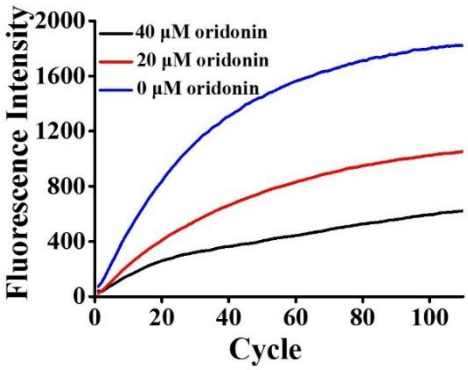

Fig. S4. Real-time fluorescence curves in response to 40 μM oridonin (black line), 20 μM oridonin (red line), and the control group without oridonin (blue line), respectively. The concentration of TF-binding probe is 500 nM.

4. EMSA analysis

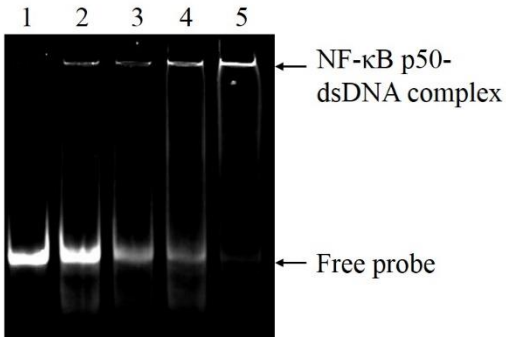

Fig. S5. Detection of endogenous NF-κB activity by EMSA in HeLa cell nuclear extracts. Lane 1, in the absence of nuclear extracts; Lane 2, 1 mg/mL TNF-α-treated nuclear extracts; Lane 3, 2 mg/mL TNF-α-treated nuclear extracts; Lane 4, 4 mg/mL TNF-α-treated nuclear extracts; Lane 5, 5 mg/mL TNF-α-treated nuclear extracts. The 500 nM TF-binding probes and 20 ng/mL TNF-a were used in the experiments.

5. Fluorescence measurement

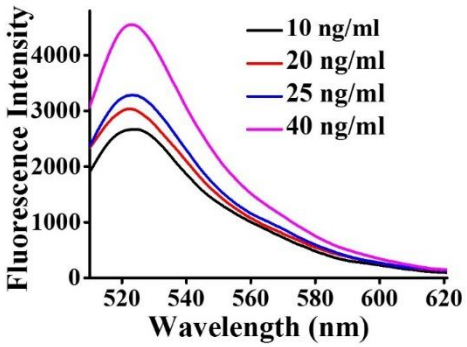

Fig. S6. Fluorescence emission spectra in response to the same amounts of total nuclear extract (0.25mg/ml) treated by different concentrations of TNF-a. The concentration of TF-binding probe is 500 nM.

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