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# Ultrasensitive fluorescence detection of transcription factors based on kisscomplex formation and the T7 RNA Polymerase amplification method

Kai Zhang*, Ke Wang, Xue Zhu, Minhao Xie*

*Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi, Jiangsu 214063, China*

* Corresponding author. Fax: +86-510-85508775; Tel: +86-510-85508775

E-mail addresses: zhangkai@jsinm.org (K. Zhang), xieminhao@jsinm.org (M, Xie)

## 1. Experimental section

### *1.1 Materials and chemicals*

T7 RNA polymerase and NTPs were purchased from Thermo Fisher Scientific Co., Ltd. (Waltham, MA, USA). Nuclease inhibitor RNasin, and diethypyrocarbonate (DEPC) were ordered from Promega. Oligonucleotides were purchased from Genscript Biotechnology Co., Ltd (Nanjing, China) and listed in Table S1. The purified recombinant Microphthalmia-associated transcription factor (MITF) was purchased from Promega (Madison, WI). The NF-κB p65 was purchased from Abcam (Cambridge, UK). Other chemicals were all of analytical grade. All solutions were prepared with Milli-Q (Branstead) purified double distilled water having specific resistance of > 18 MΩ cm. All oligonucleotide samples were prepared with phosphate buffer (2.7 mM KCl, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 8.1 mM $Na_2HPO_4$, 1 unit $\mu L^{-1}$ RNasin, 0.1% DEPC). Two buffer solutions were prepared using pH 7.4 phosphate buffered saline (PBS) (2.7 mM KCl, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 8.1 mM $Na_2HPO_4$, 1 unit $\mu L^{-1}$ RNasin, 0.1% DEPC) for the assay. Then, 20 μL of reaction mixture (containing 60 units of T7 RNA Polymerase Plus, 1.25 μM NTP, 1× T7 buffer) was added. The tips and tubes are RNase-free and do not require pretreatment to inactivate RNases.

### *1.2 MITF assay and NF-κB p65 assay.*

To immobilize DNA1 on the 96 well microtiter plate, 200 μL DNA1 (1 μM) in phosphate buffer (2.7 mM KCl, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 8.1 mM $Na_2HPO_4$, 1 unit $\mu L^{-1}$ RNasin and 0.1% DEPC) was added in each well (with an active ester surface) by incubation at room temperature for 12 h overnight. After rinsing each well twice with 300 μL of washing buffer (2.7 mM KCl, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 8.1 mM $Na_2HPO_4$, 1 unit $\mu L^{-1}$ RNasin, 0.1% DEPC, and 0.05 w/v % Tween-20), the plate was coated with 300 μL blocking buffer (2.7 mM KCl, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 8.1 mM $Na_2HPO_4$, 1 unit $\mu L^{-1}$ RNasin, 0.1% DEPC, 0.05 w/v% Tween-20 and 0.1 w/w % bovine serum albumin (BSA)).

Next, the MITF (200 μL) with different original concentrations (0, 0.5, 1, 2, 5, 10, 20, 35, 50, 100, 150, 200, 300, 500, and 1000 pM) was added to each well and incubated at 37 °C for 30 min in the presence of protein binding buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg $mL^{-1}$ yeast tRNA, 10% glycerol, 0.25 mM DTT, 1 unit $\mu L^{-1}$ RNasin and 0.1% DEPC. For the detection of MITF in the nuclear extracts (200 μL), the sample was added into the well at 37 °C for 30 min in the binding buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.25 mM DTT, 2 mM sodium phosphate (pH 7.0), 20 ng $\mu L^{-1}$ HaeIII-cut *Escherichia coli* (*E. coli*) DNA, 25 ng $\mu L^{-1}$ yeast tRNA, 1 unit $\mu L^{-1}$ RNasin and 0.1% DEPC.

After rinsing each well twice with 300 μL of washing buffer, the plate was coated with 300 μL DNA2/T7 promoter primer duplex (1 μM) in 20 mM HEPES buffer (140 mM KOAc, 20 mM NaOAc, 10 mM $MgCl_2$, 1 unit $\mu L^{-1}$ RNasin and 0.1% DEPC, pH 7.4) at 10 °C for 10 min to form the kisscomplex as pervious report.[1] After rinsing each well twice with 300 μL of washing buffer,

200 μL of reaction mixture (containing 60 units of T7 RNA Polymerase Plus, 1.25 μM NTP, 1× T7 buffer) was added to each well. RNA amplification was performed at 37 °C for 3h. The RNA intercalating dye RiboGreen was added to the reaction mixture (200 μL, 1:200 diluted in the TE buffer supplied by the manufacturer) and the plate was read (excitation wavelength 485 nm and emission wavelength 534 nm) in a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices,USA).

The procedure of assay NF-κB p65 was the same as which for the detection of MITF except the adding of DNA3 and the target NF-κB p65 instead of DNA1 and target MITF, respectively.

*1.3 Cell Culture*

A549 cells were cultured in RPMI-1640 (GIBCO) supplemented with 10% fetal calf serum, penicillin (100 μg $mL^{-1}$), and streptomycin (100 μg $mL^{-1}$) at 37 °C in a humidified atmosphere containing 5% $CO_2$ (in water-jacketed $CO_2$ incubator, Thermo 3111, Billups-Rothenberg, Del Mar, CA). Cell number was determined with a Petroff-Hausser cell counter (USA).

*1.4 Obtain Cell Nuclear Extract*

Cells were collected in the exponential phase of growth, and $5 \times 10^7$ cells were collect and washed twice with ice-cold PBS (0.1 M, pH 7.4), then incubated with 20 ng $mL^{-1}$ TNF-α (PeproTech, Rocky Hill, NJ) for 30 min. The nuclear extracts were collected using a nuclear extract kit (ActiveMotif, Carlsbad, CA) according to the manufacturer's instructions. Then the extracts were diluted to 200 μL with different concentrations and storage at -80 °C for future use.

**Table S1.** Oligonucleotides sequence used in this method. The colors of the sequences are the same as given in scheme 1. The underline bases of DNA1 can combine with underline base of DNA1 for the formation of kisscomplex. The double underline bases are the amplification sequence. The italic part of DNA2 can hybridize with T7 promoter primer.

| note | sequence (5' to 3') |
|---|---|
| DNA1 | $NH_2$-AAAAAAAACACGTGCUGGGGCGCACGTCTCTTCACGCCCCAG |
| DNA2 | GTGATGGTCGTCTGCTGGT*CCCTATAGTGAGTCGTATTA*TTTTTUGCUCGGCCCCGCGAGCA |
| DNA3 | $NH_2$-AAAAAAAAGGGACTTTCCCUGGGGCGGGAAAGTCCCTCTTCACGCCCCAG |
| T7 promoter primer | *TAATACGACTCACTATAGGG* |

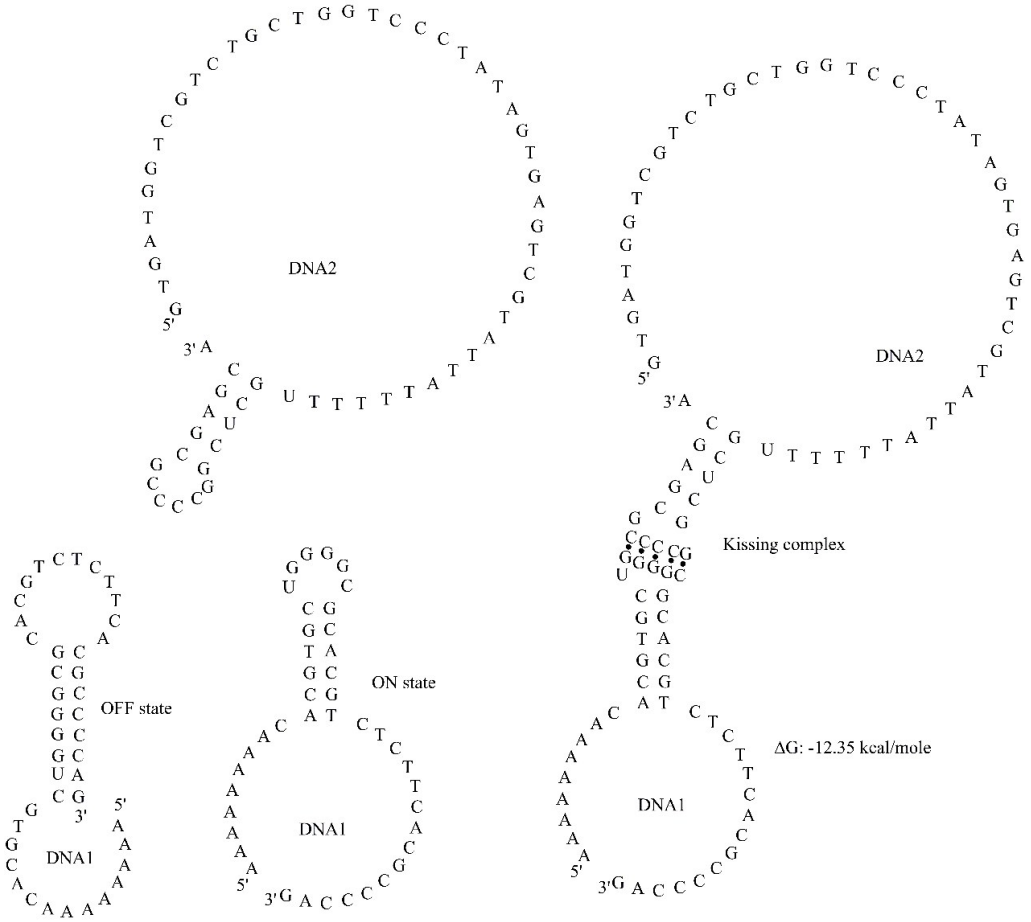


**Fig. S1.** The structures of DNA1 (OFF state and ON state), DNA2 and kissing complex. The formation of kissing complex has a ΔG of -12.35 kcal/mole (calculated by OligoAnalyzer 3.1).

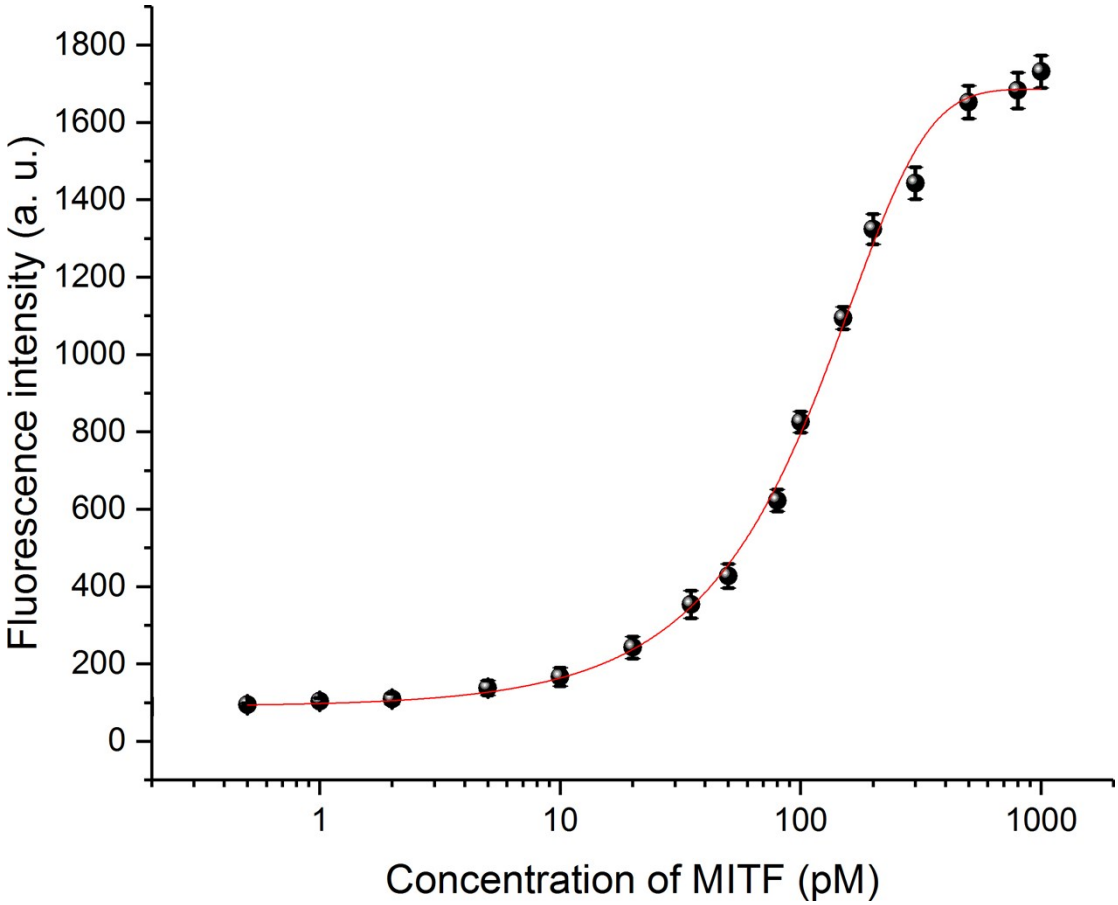


**Fig. S2.** The relationship between the fluorescence emission intensity increase and the logarithms of the concentrations of MITF.

**Table S2**. Comparison of the analytical performance for the assay of transcription factor by the as-proposed method and other reported in the literature.

| **strategy** | **detection modes** | **detection limit** | **reference** |
|---|---|---|---|
| DNAzyme-based assay | absorbance | 10 nM | 2 |
| Electrochemical assay | square wave voltammetry | 4 ± 2 nM | 3 |
| Microcantilever arrays | vertical-cavity surface-emitting laser | 100 nM | 4 |
| Nicking Enzyme based amplification | fluorescence | 2.2 pM | 5 |
| DSN based amplification | fluorescence | 1.1 pM | 6 |

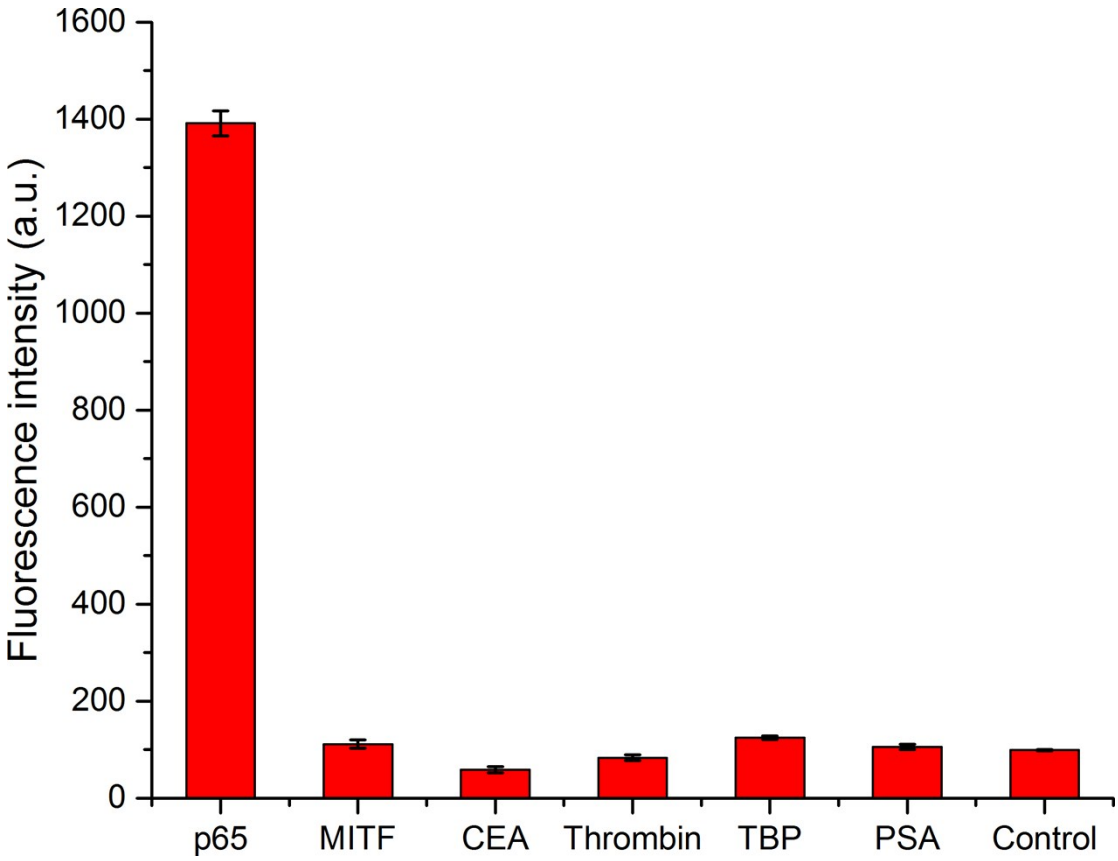


**Fig. S3** Fluorescence intensity of the KPFA test of NF-κB p65 (p65) and other proteins, showing the specificity of the assay. The concentration of the proteins was all 200 pM.


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