A simplified probe preparation for ELISA-based NF-κB activity assay

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Abstract

Nuclear factor-κB (NF-κB) is critically involved in the transcriptional regulation of many genes and multiple biological and pathobiological processes. To efficiently monitor and to rapidly screen NF-κB transcriptional activity, an ELISA-based assay has been increasingly and successfully employed as a new method in a variety of cell lines and experimental models since its first demonstration and recent development. In the ELISA-based assay, NF-κB is captured by a double-stranded DNA probe pre-linked on multi-well plates. Typically, the DNA probe contains the double-stranded consensus binding sequence for active NF-κB and another double-stranded sequence linking the consensus binding sequence with the plate (linker sequence). Since nuclear factor has no binding activity with single-stranded DNA, we modified the probe construction as containing the double-stranded consensus binding sequence and a single-stranded-linker sequence. Our results show that this kind of probe is highly sensitive and specific for NF-κB activity assay, whereas the preparation of this kind of probe is much more convenient. A single-stranded-linker sequence may largely decrease nonspecific protein binding and thus increase the sensitivity of this assay. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Several traditional techniques have been successfully established to reveal the critical role of nuclear factor-κB (NF-κB) in a variety of cellular responses or biological processes [1–3] such as our previous study on Ca^{2+} oscillation frequency-regulated NF-κB transcriptional activation [2]. These include electrophoretic mobility shift assay (EMSA), also called gel retardation [4]; reporter gene assays like chloramphenicol acetyltransferase (CAT) [2], luciferase [5] and green fluorescent protein [6]; cytoplasmic/nuclear distribution of NF-κB and/or its inhibitory protein, I-κB revealed by Western blot or immunofluorescent staining [7]; as well as the specific recognition by antibodies of the nuclear localization sequence (NLS) of NF-κB [8]. Unfortunately, all these methods have major disadvantages, such as being highly time-consuming, requiring radioactive labeling, constituting an indirect assay, or producing non-quantitative measurements.

An ELISA-based nuclear factor activity assay was originally described by Gubler and Abarzua [9] and has been recently modified by Renard et al. [10]. In this kind of assay, the nuclear factor of interest is captured by a double-stranded oligonucleotidic probe containing the consensus binding sequence and then detected by a primary antibody, followed by a secondary antibody conjugated to horseradish peroxidase. This method is convenient, sensitive and quantitative. In fact, this kind of assay has been increasingly and extensively employed in a variety of experimental research projects [11–18].

A key step in this kind of assay is the probe design and preparation. As illustrated in Fig. 1, the regular probe usually contains a double-stranded consensus binding sequence and a double-stranded-linker sequence. The linker sequence is often biotin-labeled to allow it to be fixed on an avidin-coated well. In the probe design, the consensus binding sequence serves as a binding site for specific capture of NFκB. The linker sequence between avidin and the consensus binding sequence is also necessary, presumably providing sufficient space for the binding of NFκB to the consensus binding sequence. However, the preparation of the probe is still dependent on PCR and subsequent purification of the PCR products [10] with the potential problems of contamination or lack of specificity.

In the present study, we propose a new design of the probe based on a structure using the double-stranded consensus binding sequence with a single-stranded-linker sequence. The probe preparation becomes much more convenient by eliminating the process of PCR and its subsequent product purification. In addition, this kind of probe should theoretically increase the sensitivity of the assay since a protein like NFκB has no binding activity with single-stranded DNA. Our results show that this new probe is highly sensitive, specific and reliable in detecting NFκB transcriptional activity.

![Fig. 1. Schematic illustration of probe design for ELISA-based transcription factor activity assay (upper: classical; lower: modified).](image)
2. Materials and methods

2.1. Cell culture and preparation of cell extracts

The cell line ECV304, a human umbilical vein endothelial cell line, was purchased from the China Center for Type Culture Collection (Wuhan, Hubei 430072). The cells were cultured and maintained in M199 medium (HyClone, Logan, UT 84321) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin). To activate NF-κB, cells cultured at 70–80% confluence in 50 cm² flask were stimulated with 5 ng/ml IL-1β (Calbiochem-Novabiochem Corporation, La Jolla, CA92039) for 30 min at 37 °C. After stimulation, cells were rinsed twice with cold PBS, digested with 0.25% trypsin and centrifuged for 10 min at 1000 rpm. The pellet was then resuspended in 100 μl lysis buffer (20 mM HEPES pH 7.5, 0.35 M NaCl, 20% glycerol, 1% NP-40, 1 mM MgCl₂·6H₂O, 0.5 mM EDTA, 0.1 mM EGTA) containing a protease inhibitor cocktail (Calbiochem, San Diego, CA 92121). After incubating on ice for 10 min, the lysate was centrifuged for 20 min at 14000 rpm. The supernatant constituted the total protein extract. After quantification with BCA reagent (Pierce, Rockford, IL 61105), the cell extract was kept frozen at −80 °C until NF-κB activity measurement.

2.2. Construction and preparation of the probes

Seven single-stranded oligonucleotide chains were synthesized by Shanghai Sangon Biotechnology Corporation (Shanghai, Shanghai 200031). The sequences are as follows:

No. 1: Wild type chain 1: 5’-AGTTGAGGGACTTTCCCAGGC-C-(C)34-C-3’, the 3’ end is biotinylated. This sequence contains 1 copy of the consensus binding sequence of NF-κB [10].

No. 2: Wild type chain 2: 5’-AGTTGAGGGACTTTCCCAGGCAGTTGAG-GGGACTTTCCAGGC-C-(C)12-C-3’, the 3’ end is biotinylated. This sequence contains 2 copies of the consensus binding sequence of NF-κB [10].

No. 3: Complementary chain of wild type chain: 5’-GCCTGGGAAGTCCCCTCAACT-3’

No. 4: Mutated chain: 5’-AGTTGAGCTCACTTTCCCAGGC-C-(C)34-C-3’, the 3’ end is biotinylated.

No. 5: Complementary chain of mutated chain: 5’-GCCTGGGAAGTGAGCTCAACT-3’

No. 6: Complementary chain of wild type chain with poly-G: 5’-G-(G)34-G-GCCTGGGAAG-TGCCCTCAACT-3’

No. 7: Complementary chain of mutated chain with poly-G: 5’-G-(G)34-G-GCCTGGGAAG-TGAGCTCAACT-3’

The oligonucleotides No. 1 and No. 3, No. 2 and No. 3, and No. 4 and No. 5 were mixed in ratios of 1:1, 1:2, and 1:1, respectively, denatured at 94 °C for 10 min, allowed to anneal at room temperature overnight and then stored at −80 °C. The corresponding double-stranded probes are marked as DS1WSSL for double-stranded wild type probe with single-stranded-linker, DS2WSSL for double-stranded wild type probe with single-stranded-linker and with two copies of the consensus binding sequence of NF-κB, and DS1MSSL for double-stranded mutated type probe with single-stranded-linker, respectively. The corresponding single-stranded probes are marked as SS1W for single-stranded wild type probe, SS2W for single-stranded wild
type probe with two copies of the consensus binding sequence of NF-κB and SS1M for single-stranded mutated type probe, respectively.

The double-stranded wild type probe with double-stranded-linker (DS1WDSL) and the double-stranded mutated type probe with double-stranded-linker (DS1MDSL) were also constructed by mixing equal amounts of oligoneucleotide No. 1 with No. 6 or oligoneucleotide No. 4 with No. 7, respectively, through the same procedures for the double-stranded wild or mutated type probes with single-stranded-linker (DS1WSSL or DS1MSSL) preparation.

2.3. Binding of the double-stranded oligonucleotidic probe on multi-well plates

The 3′ end biotinylated probe was linked to streptavidin-coated 96-well plates (Roche Diagnostics Corporation, Indianapolis, IN 46256) by the following procedure: 2 pmol of probe per well was incubated for 1 h at 37 °C in 50 μl phosphate-buffered saline (PBS). Plates were then washed twice with PBS containing 0.1% Tween-20 to remove the probe in excess and once with PBS alone.

2.4. Binding of NF-κB to the double-stranded probe

This assay was performed with whole-cell lysate from IL-1β stimulated cells or unstimulated control. 20 μl of cell extract containing varying amounts of protein were mixed with 30 μl of binding buffer (4 mM HEPES pH 7.5, 100 mM KCl, 8% glycerol, 5 mM DTT, 0.2% BSA, 40 μg/ml salmon sperm DNA) in the above microwells coated with the probes. After a 1-h incubation at room temperature with mild agitation (200 rpm), the microwells were washed three times with PBS containing 0.1% Tween-20.

2.5. Binding of anti-NFκB antibodies to the NFκB–DNA complex

100 μl mouse anti-NFκB p65 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA 95060, 200 μg/ml), diluted 1000 times in 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl and 1% non-fat dried milk, were incubated in each well for 1 h at room temperature. The microwells were then washed three times each with 200 μl PBS containing 0.1% Tween-20.

2.6. Binding of peroxidase-conjugated anti-mouse IgG to the anti-NFκB antibodies

Peroxidase-conjugated goat anti-mouse IgG (Santa Cruz), diluted 1000 times in 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl and 1% non-fat dried milk, were incubated in each well at room temperature for 1 h. The microwells were then washed four times each with 200 μl PBS containing 0.1% Tween-20.

2.7. Colorimetric detection

100 μl tetramethylbenzidine (Shanghai Lizhu-Dongfeng Biotechnology Corporation, Shanghai, Shanghai 200031) was incubated in each microwell at room temperature for 10 min before adding 100 μl of stopping solution (2 M H2SO4). Optical density was then read at 450 nm under a microplate reader (TECAN, Austria), using a 655-nm reference wavelength. Backgrounds are determined in lysis buffer, instead of cell lysate-incubated microwells, and
3. Results

3.1. Comparison of detection sensitivity between wild type and mutated type double-stranded probe

To test the sensitivity of this new probe in detecting NFκB DNA-binding activity, the DNA probe-coated microwells were incubated with increasing amounts of cell lysates (0.5, 1, 2, 5, 10, 25 and 50 µg of proteins per well), prepared either from unstimulated cells (control) or from IL-1β-stimulated cells. As shown in Fig. 2, when wild type probe (DS1WSSL) was coated, a significant increase of NFκB activity was detected in IL-1β-stimulated cells in a concentration-dependent manner. The NFκB activity can even be revealed in a small amount of lysate with protein level as low as 0.5 µg/well, under which the probe detects ~4-fold increase in NFκB activity in IL-1β-stimulated cells versus unstimulated control (0.187 ± 0.009 vs. 0.048 ± 0.003, n = 3 for each). It is noted that there is also a somewhat dose-dependent increase in NFκB activity in unstimulated cells, especially for lysates with 10, 25 and 50 µg of proteins per well; this probably reflects basal NFκB activity in these cell lysates. The NF-kB DNA-binding activity in unstimulated cells does not represent nonspecific activity since the mutated type probe does not detect any increase in NFκB activity in the same samples (Fig. 2).

Also shown in Fig. 2, when mutated type probe (DS1MSSL) was used, the basic NFκB activity is very low and no significant increase of NFκB activity was detected either in IL-1β-stimulated cells or in unstimulated control. This result indicates that the activities detected by the wild type probe are specific for NFκB, whereas the low activity detected by mutated type probe probably presents nonspecific binding.

Fig. 2. A comparison of NFκB detection between a double-stranded wild type probe (DS1WSSL) and a mutated type probe (DS1MSSL). To test the sensitivity of detection for NFκB activity, the microwells coated with probe DS1WSSL or probe DS1MSSL were incubated with increasing amounts of cell lysates (0.5, 1, 2, 5, 10, 25 and 50 µg of proteins per well), prepared either from unstimulated cells (control) or from IL-1β-stimulated cells. The NFκB activity was determined by a monoclonal antibody against NFκB followed by a peroxidase-conjugated secondary antibody as described in detail in Materials and methods. Data represent means ± SD of three separate experiments (it is noted that the black triangle curve is behind the white triangle).
Taken together, these experiments show that this new kind of probe, containing a double-stranded NFκB binding consensus sequence and a single-stranded-linker sequence, can detect NFκB activity in a very sensitive and specific manner.

Regression analysis further reveals an excellent linear relationship between the NFκB activity (OD value in Fig. 2) and the protein level (microgram/well in Fig. 2), ranging from 0.5 to 25 μg/well, OD value = 0.260 + (0.0721 * protein level-μg), R = 0.99, p < 0.001 for regression.

Fig. 3. A comparison of NFκB detection between a double-stranded wild type probe with one copy of consensus binding sequence (DS1WSSL) and a double-stranded wild type probe with two copies of consensus binding sequence (DS2WSSL). To compare the double-stranded wild type probe with one copy of consensus binding sequence (DS1WSSL) and double-stranded wild type probe with two copies of consensus binding sequence (DS2WSSL) in detecting NFκB activity, microwells coated with probe DS1WSSL or probe DS2WSSL were incubated with increasing amounts of cell lysates (0.5, 1, 2, 5, 10, 25 and 50 μg of proteins per well), prepared either from unstimulated cells (control) or from IL-1β-stimulated cells. The NFκB activity was determined as described in detail in Materials and methods. Data represent means ± SD of three separate experiments.

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Fig. 4. A comparison of NFκB detection between single-stranded probes and double-stranded probes. To compare the double-stranded probes (DS1WSSL, DS2WSSL and DS1MSSL) and single-stranded probes (SS1W, SS2W and SS1M) in detecting NFκB activity, the microwells were coated with different probes and incubated with increasing amounts of cell lysates containing a total of 5 μg protein per well, prepared either from unstimulated cells (control, black) or from IL-1β-stimulated cells (IL-1β, blank). NFκB activity was determined as described above and also in detail in Materials and methods. Data represent means ± SD of three separate experiments.
3.2. Comparison of detection results between DS1WSSL and DS2WSSL

To reveal any difference between probes with one or with two copies of the consensus binding sequence, microwells containing the DNA probe were also incubated with increasing amounts of cell lysates (0.5, 1, 2, 5, 10, 25 and 50 μg of proteins/well) prepared either from unstimulated cells (control) or from IL-1β-stimulated cells. No significant difference was detected between cell lysates probed by DS1WSSL and DS2WSSL at any concentration (Fig. 3).

3.3. Comparison of detection results between single-stranded probe and double-stranded probe

To determine whether a single-stranded probe can detect any NFκB activity, experiments were performed on single-stranded probe-coated microwells and the assay was conducted in cell
lysate containing a total protein of 5 μg per well. Single-stranded probe, either wild type or mutated type, failed to detect any NFκB activities in unstimulated cells. In the cells stimulated by IL-1β, wild type single-stranded probe detected a very weak NFκB activity, whereas mutated type, single-stranded probe did not detect any signal (shown in Fig. 4). By contrast, double-stranded probe effectively detects NFκB activity in IL-1β-stimulated cells.

3.4. Comparison of detection results between double-stranded probes with single-stranded-linker and double-stranded-linker

The ELISA-based analysis was also performed to reveal any difference in nonspecific binding between the double-stranded-linker probe and the single-stranded-linker probe, assuming that the nonspecific binding is detected by mutated probes. As shown in Fig. 5a, the activity detected by mutated probe with double-stranded-linker (DS1MDSL) was significantly higher than that by mutated probe with single-stranded-linker (DS1MSSL) both in unstimulated and IL-1β-stimulated cell lysate (0.067 ± 0.005 and 0.069 ± 0.006 for DS1MDSL with unstimulated or IL-1β-stimulated cell lysate, respectively, p<0.05 vs. 0.041 ± 0.003 and 0.042 ± 0.002 for DS1MSSL with unstimulated or IL-1β-stimulated cell lysate, respectively, N=3 for each, one-way ANOVA). These results indicate that the single-stranded-linker probe decreases the nonspecific binding of other protein with the consensus motif as compared to the double-stranded-linker probe.

It is noted that the wild type probes with either single-stranded-linker or double-stranded-linker detect the similar NFκB activities in both unstimulated control and IL-1β stimulated cells (shown in Fig. 5b).

4. Discussion

In this study, we define a successful modification of the probe design for an ELISA-based NFκB assay. The modified, new type of probe described in this study contains a double-stranded consensus binding sequence and a single-stranded-linker sequence, rather than a double-stranded-linker sequence in the classical type of probe reported in a previous study [10]. We show that this new probe design remains highly sensitive and is more specific than the traditional probe design in detecting NFκB activity in agonist-stimulated cell lysate.

The major advantage of this modification of probe design is the convenience in probe preparation. The probe was designed as a kind of “double–single-stranded” one and could be easily obtained by denaturing and annealing of two fragments of oligonucleotide in equimolar amounts since the two fragments of oligonucleotides are different in length. After annealing, a probe with double-stranded NFκB consensus binding sequence and single-stranded-linker sequence was formed. The process of probe generation is simplified by eliminating PCR and its product purification. Consequently, the employment of this new kind of probe in detecting NFκB activity would be expected to save time, cost and effort.

In the experiments of NFκB assay using multiple microplates in this study, we generally used the protocol of Renard et al., as described before [10]. Regression analysis reveals an excellent linear relationship between the NFκB activity and the protein level, in the range of 0.5–25 μg/well. This is wider than the linear range of 2–10 μg of protein suggested by the study with the classical probe [10]. The explanation for this improved range is still under investigation. One possible explanation may lie on the fact that proteins do not bind or interact with single-stranded DNA sequence in general. Our probe design of single-stranded, instead of double-stranded-
linker, sequence may decrease the nonspecific binding or interaction of protein with DNA probe especially at high protein concentrations.

We found that the use of a single-stranded-linker significantly reduced nonspecific background binding. Assuming that nonspecific activity is detected by double-stranded mutated probes, the experimental results in Fig. 5a showed that the binding activity detected by double-stranded mutated probe with single-stranded-linker (DS1MDSL) was significantly lower than that by double-stranded mutated probe with double-stranded-linker (DS1MDSL) both in unstimulated and IL-1β-stimulated cell lysates. These results strongly support the hypothesis that the single-stranded-linker design decreases the nonspecific binding or interaction of protein with DNA probe.

In this study, we also investigated whether tandem-binding sequences improved the performances of the ELISA-based assay. The experiment data showed no any difference between the probe with one copy of the binding sequence and that with two copies of the binding sequence (Fig. 3) in detecting NFκB activity in endothelial cells. It seems that repeated binding sequence is unnecessary in detecting NFκB activity in this type of cells. This indicates that the amount of NFκB in IL-1β-activated endothelial cells is sufficient to be detected by a single binding site. Whether tandem-binding sequences improve the assay performance using extracts from other cell types remains to be determined.

Additional experiments in this study showed that single-stranded probe is not able to detect any NFκB DNA binding activity. This is consistent with the fact that single-stranded DNA does not interact with protein and proves that probe with single strand is not an appropriate design.

The single-stranded-linker sequence in this study was arbitrarily chosen to be poly “C”. Two different lengths of 34 “C” and 12 “C” were tested and found to be not different. The design of the single-stranded-linker seems very flexible. It is anticipated that a single-stranded-linker with a random sequence of any other kind and a length between 12 and 34 bases will work similarly.

5. Simplified description of the method and its (future) applications

As a conclusion, the probe for ELISA-based NF-κB activity assay can be easily obtained by denaturing and annealing of two fragments of oligonucleotide, a double-stranded consensus binding sequence and a single-stranded-linker sequence. This novel strategy for this kind of probe design and preparation may be generally applicable to other nuclear factors.

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