



# NF-kB (human p50/p65) Combo Transcription Factor Assay Kit

Catalog Number KA1340

96 assays

Version: 03

Intended for research use only

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## Introduction

### Background

The NF- $\kappa$ B/Rel family of transcription factors is comprised of several structurally-related proteins that form homodimers and heterodimers and include p50/p105, p52/p100, RelA (p65), c-Rel/NF- $\kappa$ B.<sup>1</sup> Members of this family are responsible for regulating over 150 target genes, including the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules. Because of this, NF- $\kappa$ B has often been called a 'central mediator of the human immune response.'<sup>1,2</sup> Acting as dimers, these transcription factors bind to DNA sequences collectively called  $\kappa$ B sites, thereby regulating expression of target genes. In most cells, Rel/ NF- $\kappa$ B transcription complexes are present in an inactive form in the cytoplasm, bound to an inhibitor I $\kappa$ B. Certain stimuli result in the phosphorylation, ubiquitination, and subsequent degradation of I $\kappa$ B proteins, thereby enabling translocation of NF- $\kappa$ B into the nucleus.<sup>3</sup> The most common Rel/NF- $\kappa$ B dimer in mammals contains p50-RelA (p50/ p65) heterodimers and is specifically called NF- $\kappa$ B. One of the target genes activated by NF- $\kappa$ B is that encoding I $\kappa$ B. This feedback mechanism allows newly-synthesized I $\kappa$ B to enter the nucleus, remove NF- $\kappa$ B from DNA, and transport it back to the cytoplasm thereby restoring its inactive state. The importance of Rel/NF- $\kappa$ B transcription factors in human inflammation and certain diseases makes them attractive targets for potential therapeutics.<sup>4-6</sup>

### Principle of the Assay

NF- $\kappa$ B (human p50/p65) Combo Transcription Factor Assay Kit is a non-radioactive, sensitive method for detecting specific transcription factor DNA binding activity in nuclear extracts. A 96-well enzyme-linked immunosorbent assay (ELISA) replaces the cumbersome radioactive electrophoretic mobility shift assay (EMSA). A specific double stranded DNA (dsDNA) sequence containing the NF- $\kappa$ B response element is immobilized onto the wells of a 96-well plate. NF- $\kappa$ B contained in a nuclear extract binds specifically to the NF- $\kappa$ B response element. NF- $\kappa$ B p50 and p65 are detected by addition of a specific primary antibody directed against NF- $\kappa$ B p50 or p65. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. NF- $\kappa$ B (human p50/p65) Combo Transcription Factor Assay Kit detects human NF- $\kappa$ B p50 and human, mouse, and rat p65.

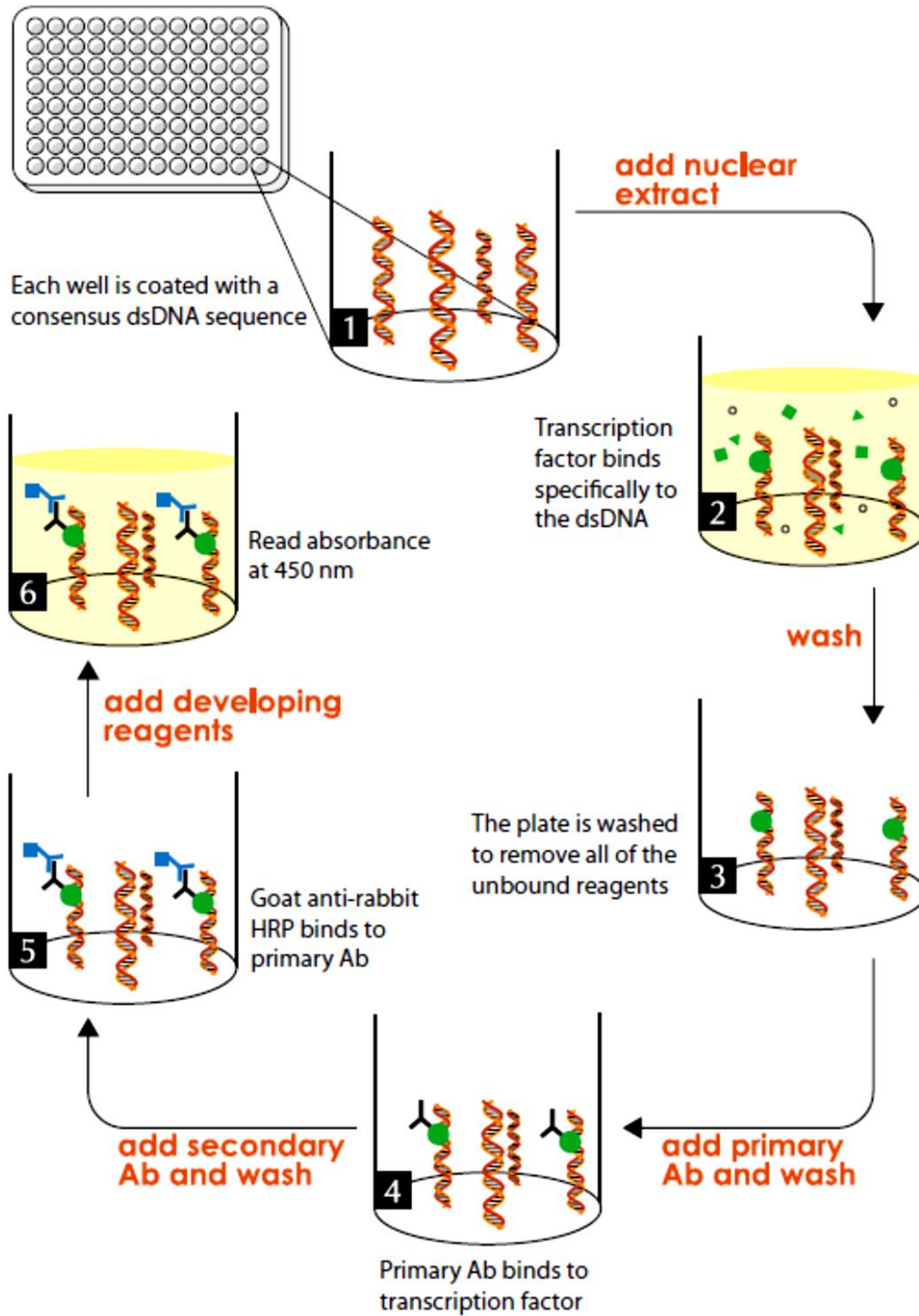


Figure 1. Schematic of the Transcription Factor Binding Assay

## General Information

### Materials Supplied

List of component

| Item  | Quantity/Sizes |
|---|----------------|
| Transcription Factor Binding Assay Buffer (4X)                | 3 mL           |
| Transcription Factor Reagent A                                | 120 µL         |
| Transcription Factor NF-κB (human p50) Combo Positive Control | 75 µL          |
| Transcription Factor NF-κB (human p65) Combo Positive Control | 75 µL          |
| Transcription Factor Antibody Binding Buffer (10X)            | 3 mL           |
| Transcription Factor NF-κB (human p50) Combo Primary Antibody | 60 µL          |
| Transcription Factor NF-κB (p65) Combo Primary Antibody       | 60 µL          |
| Wash Buffer Concentrate (400X)                                | 5 mL           |
| Polysorbate 20  | 3 mL           |
| Transcription Factor NF-κB Competitor dsDNA                   | 120 µL         |
| Transcription Factor Goat Anti-Rabbit HRP Conjugate           | 120 µL         |
| Transcription Factor NF-κB 96-Well Strip Plate                | 1 plate        |
| 96-Well Cover Sheet   | 1 cover        |
| Transcription Factor Developing Solution                      | 12 mL          |
| Transcription Factor Stop Solution                            | 12 mL          |

### Storage Instruction

This kit will perform as specified if stored as directed and used before the expiration date indicated on the outside of the box.

| Item  | Storage |
|---|---------|
| Transcription Factor Binding Assay Buffer (4X)                | 4°C     |
| Transcription Factor Reagent A                                | -20°C   |
| Transcription Factor NF-κB (human p50) Combo Positive Control | -80°C   |
| Transcription Factor NF-κB (human p65) Combo Positive Control | -80°C   |
| Transcription Factor Antibody Binding Buffer (10X)            | 4°C     |
| Transcription Factor NF-κB (human p50) Combo Primary Antibody | -20°C   |
| Transcription Factor NF-κB (p65) Combo Primary Antibody       | -20°C   |
| Wash Buffer Concentrate (400X)                                | RT      |
| Polysorbate 20  | RT      |
| Transcription Factor NF-κB Competitor dsDNA                   | -20°C   |
| Transcription Factor Goat Anti-Rabbit HRP Conjugate           | -20°C   |

|   |     |
|---|-----|
| Transcription Factor NF- $\kappa$ B 96-Well Strip Plate | 4°C |
| 96-Well Cover Sheet                                     | RT  |
| Transcription Factor Developing Solution                | 4°C |
| Transcription Factor Stop Solution                      | RT  |

### **Materials Required but Not Supplied**

- ✓ A plate reader capable of measuring absorbance at 450 nm
- ✓ Adjustable pipettes and a repeat pipettor
- ✓ A source of UltraPure water; glass Milli-Q or HPLC-grade water is acceptable
- ✓ 300 mM dithiothreitol (DTT)

*NOTE: The components in each kit lot have been quality assured and warranted in this specific combination only; please do not mix them with components from other lots.*

### **Precautions for Use**

Warning: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

- General Information
  - ✓ It is not necessary to use all the wells on the plate at one time; however a Positive Control should be run every time.
  - ✓ For each plate or set of strips, it is recommended that two Blk, two Non-Specific Binding (NSB), and two PC wells be included.
- Pipetting Hints
  - ✓ Use different tips to pipette each reagent.
  - ✓ Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
  - ✓ Do not expose the pipette tip to the reagent(s) already in the well.

## Assay Protocol

### Reagent Preparation

- ✓ Transcription Factor Antibody Binding Buffer - One vial contains 3 mL of a 10X stock of Transcription Factor Antibody Binding Buffer (ABB) to be used for diluting the primary and secondary antibodies. To prepare 1X ABB, dilute 1:10 by adding 27 mL of UltraPure water. Store at 4°C for up to six months.
- ✓ Wash Buffer Concentrate (400X) - One vial contains 5 mL of 400X Wash Buffer. Dilute the contents of the vial to a total volume of 2 liters with UltraPure water and add 1 mL of Polysorbate 20. *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a pipette. A positive displacement device such as a syringe should be used to deliver small quantities accurately.* A smaller volume of Wash Buffer Concentrate can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 mL/liter of Wash Buffer). Store at 4°C for up to two months.
- ✓ Transcription Factor Binding Assay Buffer (4X) - One vial contains 3 mL of a 4X stock of Transcription Factor Binding Assay Buffer (TFB). Prepare Complete Transcription Factor Binding Buffer (CTFB) immediately prior to use in 1.5 mL centrifuge tubes or 15 mL conical tubes as outlined in Table 1. This buffer is now referred to as CTFB. *It is recommended that the CTFB be used the same day it is prepared.*

| Component                                    | Volume/Well | Volume/Strip | Volume/96-well plate |
|--|-------------|--------------|----------------------|
| UltraPure water                              | 73 µL       | 584 µL       | 7,008 µL             |
| 4X Transcription Factor Binding Assay Buffer | 25 µL       | 200 µL       | 2,400 µL             |
| Reagent A                                    | 1 µL        | 8 µL         | 96 µL                |
| 300 mM DTT                                   | 1 µL        | 8 µL         | 96 µL                |
| Total Required                               | 100 µL      | 800 µL       | 9,600 µL             |

Table 1. Preparation of Complete Transcription Factor Binding Assay Buffer

- ✓ Transcription Factor NF-κB (human p50) Positive Control - One vial contains 75 µL of clarified cell lysate. This lysate is provided as a positive control for NF-κB p50 activation; it is not intended for plate to plate comparisons. The Positive Control provided is sufficient for 7 reactions and will provide a strong signal (>0.5 AU at 450 nm) when used at 10 µL/well. When using this Positive Control, a decrease in signal may occur with repeated freeze/thaw cycles. It is recommended that the Positive Control be aliquoted at 25 µL per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw cycles.
- ✓ Transcription Factor NF-κB (human p65) Positive Control - One vial contains 75 µL of clarified cell lysate. This lysate is provided as a positive control for NF-κB p65 activation; it is not intended for plate to plate comparisons. The Positive Control provided is sufficient for 7 reactions and will provide a strong signal (>0.5 AU at 450 nm) when used at 10 µL/well. When using this Positive Control, a decrease in signal may occur with repeated freeze/thaw cycles. It is recommended that the Positive Control be aliquoted at 25 µL per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw cycles.

### **Sample Preparation**

- ✓ Nuclear Extraction PBS (10X)  
1.71 M NaCl, 33.53 mM KCl, 126.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 22.04 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.4
- ✓ Nuclear Extraction PBS (1X)  
Dilute 100 mL of 10X stock with 900 mL distilled H<sub>2</sub>O
- ✓ Nuclear Extraction Phosphatase Inhibitor Cocktail (50X)  
0.05 M NaF  
0.05 M β-glycerophosphate  
0.05 M Na<sub>3</sub>VO<sub>4</sub>  
Store at -80°C
- ✓ Nuclear Extraction PBS/Phosphatase Inhibitor Solution (1X)  
Add 200 μL of 50X Phosphatase Inhibitor Solution to 10 mL of 1X Nuclear Extraction PBS, mix well, and keep on ice. Make fresh daily.
- ✓ Nuclear Extraction Protease Inhibitor Cocktail (100X)  
10 mM AEBSF  
0.5 mM Bestatin  
0.2 mM Leupeptin Hemisulfate Salt  
0.15 mM E-64  
0.1 mM Pepstatin A  
0.008 mM Aprotinin from Bovine Lung  
Made in DMSO, store at -20°C
- ✓ Nuclear Extraction Hypotonic Buffer (10X)  
100 mM HEPES, pH 7.5, containing 40 mM NaF, 100 μM Na<sub>2</sub>MoO<sub>4</sub>, and 1 mM EDTA, Store at 4°C.
- ✓ Complete Extraction Hypotonic Buffer (1X)  
Prepare as outlined in Table 2. The phosphatase and protease inhibitors lose activity shortly after dilution; therefore any unused 1X Complete Extraction Hypotonic Buffer should be discarded.

| Reagent                      | 150 mm plate~1.5 x 10 <sup>7</sup> cells |
|------------------------------|--|
| Hypotonic Buffer (10X)       | 100 μL                                   |
| Phosphatase Inhibitors (50X) | 20 μL                                    |
| Protease Inhibitors (100X)   | 10 μL                                    |
| Distilled Water              | 870 μL                                   |
| Total Volume                 | 1,000 μL                                 |

Table 2. Preparation of Complete Extraction Hypotonic Buffer

- ✓ Nonidet P-40 Assay Reagent (10%)  
Nonidet P-40 or suitable substitute at a concentration of 10% (v/v) in H<sub>2</sub>O Store at room temperature.

- ✓ Nuclear Extraction Buffer (2X)  
20 mM HEPES, pH 7.9, containing, 0.2 mM EDTA, 3 mM MgCl<sub>2</sub>, 840 mM NaCl, and 20% glycerol (v/v), Store at 4°C.
- ✓ Complete Nuclear Extraction Buffer (1X)  
Prepare as outlined in Table 3. Some of the phosphatase and protease inhibitors lose activity shortly after dilution; therefore any remaining 1X Extraction Buffer should be discarded.

| Reagent                        | 150 mm plate~1.5 x 10 <sup>7</sup> cells |
|--------------------------------|--|
| Nuclear Extraction Buffer (2X) | 75 µL                                    |
| Protease Inhibitors (100X)     | 1.5 µL                                   |
| Phosphatase Inhibitors (50X)   | 3.0 µL                                   |
| DTT (10 mM)                    | 15 µL                                    |
| Distilled Water                | 55.5 µL                                  |
| Total Volume                   | 150 µL                                   |

Table 3. Preparation of Complete Nuclear Extraction Buffer

- Purification of Cellular Nuclear Extracts  
The procedure below can be used for a 15 mL cell suspension grown in a T75 flask or adherent cells (100 mm dish 80-90% confluent) where 10<sup>7</sup> cells yields approximately 50 µg of nuclear protein.
  1. Collect ~10<sup>7</sup> cells in pre-chilled 15 mL tubes.
  2. Centrifuge suspended cells at 300 x g for five minutes at 4°C.
  3. Discard the supernatant. Resuspend the cell pellet in 5 mL of ice-cold 1x Nuclear Extraction PBS/Phosphatase Inhibitor Solution and centrifuge at 300 x g for five minutes at 4°C. Repeat one time.
  4. Discard the supernatant. Add 500 µL ice-cold Hypotonic Buffer. Mix gently by pipetting and transfer resuspended pellet to pre-chilled 1.5 mL microcentrifuge tube.
  5. Incubate cells on ice for 15 minutes allowing cells to swell.
  6. Add 100 µL of 10% Nonidet P-40 (or suitable substitute). Mix gently by pipetting.
  7. Centrifuge for 30 seconds (pulse spin) at 4°C in a microcentrifuge. Transfer the supernatant which contains the cytosolic fraction to a new tube and store at -80°C.
  8. Resuspend the pellet in 100 µL ice-cold Complete Nuclear Extraction Buffer (1X) (with protease and phosphatase inhibitors). Vortex 15 seconds at the highest setting and then gently rock the tube on ice for 15 minutes using a shaking platform. Vortex sample for 30 seconds at the highest setting and gently rock for an additional 15 minutes.
  9. Centrifuge at 14,000 x g for 10 minutes at 4°C. The supernatant contains the nuclear fraction. Aliquot to clean chilled tubes, flash freeze and store at -80°C. Avoid freeze/ thaw cycles. The extracts are ready to use in the assay.
  10. Keep a small aliquot of the nuclear extract to quantitate the protein concentration.

## Assay Procedure

- Performing the Assay

Binding of active NF- $\kappa$ B to the consensus sequence:

- Equilibrate the plate and buffers to room temperature prior to opening. Remove the plate from the foil and select the number of strips needed. The 96-well plate supplied with this kit is ready to use.

*NOTE: If you are not using all of the strips at once, place the unused strips back in the plate packet and store at 2-4°C. Be sure that the packet is sealed with the desiccant inside.*

- Prepare the CTFB as outlined in Table 1.

- Add appropriate amount of reagent(s) listed below to the designated wells as follows:

Blk - add 100  $\mu$ L of CTFB to designated wells.

NSB - add 100  $\mu$ L of CTFB to designated wells. Do not add samples or Positive Control to these wells.

C1 - Add 80  $\mu$ L of CTFB prior to adding 10  $\mu$ L of Transcription Factor NF- $\kappa$ B Specific Competitor dsDNA to designated wells. Add 10  $\mu$ L of control cell lysate, or unknown sample.

*NOTE: Competitor dsDNA must be added prior to adding the positive control or nuclear extracts.*

S1-S44 - Add 90  $\mu$ L of CTFB followed by 10  $\mu$ L of Nuclear Extract to designated wells.

PC - Add 90  $\mu$ L of CTFB followed by 10  $\mu$ L of Positive Control to appropriate wells.

- Use the cover provided to seal the plate. Incubate overnight at 4°C or one hour at room temperature without agitation (incubation for one hour will result in a less sensitive assay).
- Empty the wells and wash five times with 200  $\mu$ L of 1X Wash Buffer. After each wash empty the wells in the sink. After the final wash (wash #5), tap the plate on a paper towel to remove any residual Wash Buffer.

- Addition of Transcription Factor NF- $\kappa$ B Combo Primary Antibody

- Dilute the Transcription Factor NF- $\kappa$ B Combo Primary Antibody 1:100 in 1X ABB as outlined in Table 4 below. Add 100  $\mu$ L of diluted NF- $\kappa$ B (human p50 or p65) to each well except the Blk wells.

| Component                       | Volume/Well | Volume/Strip | Volume/96-well plate |
|---------------------------------|-------------|--------------|----------------------|
| 1X ABB                          | 99 $\mu$ L  | 792 $\mu$ L  | 9,504 $\mu$ L        |
| NF- $\kappa$ B Primary Antibody | 1 $\mu$ L   | 8 $\mu$ L    | 96 $\mu$ L           |
| Total required                  | 100 $\mu$ L | 800 $\mu$ L  | 9,600 $\mu$ L        |

Table 4. Dilution of Primary Antibody

- Use the adhesive cover provided to seal the plate.
- Incubate the plate for one hour at room temperature without agitation.
- Empty the wells and wash each well five times with 200  $\mu$ L of 1X Wash Buffer. After each wash, empty the contents of the plate into the sink. After the final wash (wash #5), tap the plate three to five times on a paper towel to remove any residual Wash Buffer.

- Addition of the Transcription Factor Goat Anti-Rabbit HRP Conjugate
1. Dilute the Transcription Factor Goat Anti-Rabbit HRP Conjugate 1:100 in 1X ABB as outlined in Table 5 below. Add 100  $\mu$ L of diluted HRP Conjugated to each well except the Blk wells.

| Component                      | Volume/Well | Volume/Strip | Volume/96-well plate |
|--------------------------------|-------------|--------------|----------------------|
| 1X ABB                         | 99 $\mu$ L  | 792 $\mu$ L  | 9,504 $\mu$ L        |
| Goat Anti-Rabbit HRP Conjugate | 1 $\mu$ L   | 8 $\mu$ L    | 96 $\mu$ L           |
| Total required                 | 100 $\mu$ L | 800 $\mu$ L  | 9,600 $\mu$ L        |

Table 5. Dilution of HRP Conjugate

2. Use the adhesive cover provided to seal the plate.
3. Incubate for one hour at room temperature without agitation.
4. Empty the wells and wash five times with 200  $\mu$ L of 1X Wash Buffer. After each wash, empty the contents of the plate into the sink. After the final wash (wash #5), tap the plate three to five times on a paper towel to remove any residual Wash Buffer.

- Develop and Read the Plate

1. To each well being used add 100  $\mu$ L of Transcription Factor Developing Solution which has been equilibrated to room temperature.
2. Incubate the plate for 15 to 45 minutes at room temperature with gentle agitation protected from light. Allow the wells to turn medium to dark blue prior to adding Transcription Factor Stop Solution. (This reaction can be monitored by taking absorbance measurements at 655 nm prior to stopping the reactions; An OD<sub>655</sub> of 0.4-0.5 yields an OD<sub>450</sub> of approximately 1). Monitor development of sample wells to ensure adequate color development prior to stopping the reaction. *NOTE: Do not overdevelop; however Positive Control wells may need to overdevelop to allow adequate color development in sample wells.*
3. Add 100  $\mu$ L of Stop Solution per well being used. The solution within the wells will change from blue to yellow after adding the Stop Solution.
4. Read absorbance at 450 nm within five minutes of adding the Stop Solution. Blank the plate reader according to the manufacturer's requirements using the blank wells.

- Summary

*NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when initially performing the assay.*

1. Prepare CTFB as described in the Pre-Assay Preparation section, Table 3.
2. Add 90  $\mu$ L CTFB per sample well (80  $\mu$ L if adding Competitor dsDNA), 100  $\mu$ L to Blk and NSB wells).
3. Add 10  $\mu$ L of Competitor dsDNA (optional) to appropriate wells.
4. Add 10  $\mu$ L of Positive Control to appropriate wells.
5. Add 10  $\mu$ L of Sample containing NF- $\kappa$ B to appropriate wells.
6. Incubate overnight at 4°C without agitation.
7. Wash each well five times with 200  $\mu$ L of 1X Wash Buffer.

8. Add 100  $\mu$ L of diluted NF- $\kappa$ B Antibody per well (except Blk wells).
9. Incubate one hour at room temperature without agitation.
10. Wash each well five times with 200  $\mu$ L of 1X Wash Buffer.
11. Add 100  $\mu$ L of diluted Goat Anti-Rabbit Secondary antibody (except Blk wells).
12. Incubate one hour at room temperature without agitation.
13. Wash each well five times with 200  $\mu$ L of 1X Wash Buffer.
14. Add 100  $\mu$ L of Developing Solution per well.
15. Incubate 15 to 45 minutes with gentle agitation.
16. Add 100  $\mu$ L of Stop Solution per well.
17. Measure the absorbance at 450 nm.

| Steps            | Reagent   | Blk         | NSB         | PC          | C1          | S1-S44      |
|------------------|---|-------------|-------------|-------------|-------------|-------------|
| 1. Add reagents  | CTFB  | 100 $\mu$ L | 100 $\mu$ L | 90 $\mu$ L  | 80 $\mu$ L  | 90 $\mu$ L  |
|                  | Competitive dsDNA   |             |             |             | 10 $\mu$ L  |             |
|                  | Positive Control  |             |             | 10 $\mu$ L  | 10 $\mu$ L  |             |
|                  | Samples   |             |             |             |             | 10 $\mu$ L  |
| 2. Incubate      | Cover plate and incubate overnight at 4°C without agitation             |             |             |             |             |             |
| 3. Wash          | Wash all wells five times   |             |             |             |             |             |
| 4. Add reagents  | Primary Antibody  |             | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L |
| 5. Incubate      | Cover plate and incubate one hour at room temperature without agitation |             |             |             |             |             |
| 6. Wash          | Wash all wells five times   |             |             |             |             |             |
| 7. Add reagents  | Goat Anti-Rabbit HRP Conjugate  |             | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L |
| 8. Incubate      | Cover plate and incubate one hour at room temperature without agitation |             |             |             |             |             |
| 9. Wash          | Wash all wells five times   |             |             |             |             |             |
| 10. Add reagents | Developer Solution  | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L |
| 11. Incubate     | Monitor development in wells  |             |             |             |             |             |
| 12. Add reagents | Stop Solution   | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L | 100 $\mu$ L |
| 13. Read         | Read plate at wavelength of 450 nm                                      |             |             |             |             |             |

Table 6. Quick Protocol Guide

## Data Analysis

### Performance Characteristics

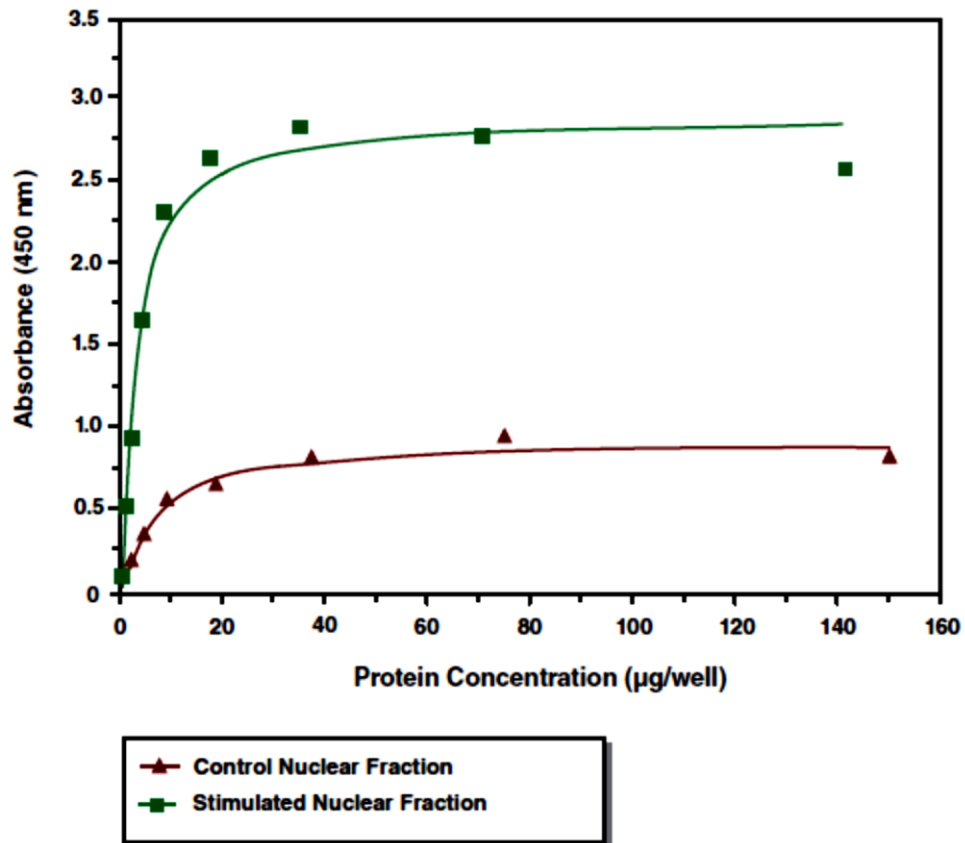


Figure 1. Assay of NF- $\kappa$ B (human p50) from the nuclear fraction of TNF- $\alpha$  activated HeLa cells

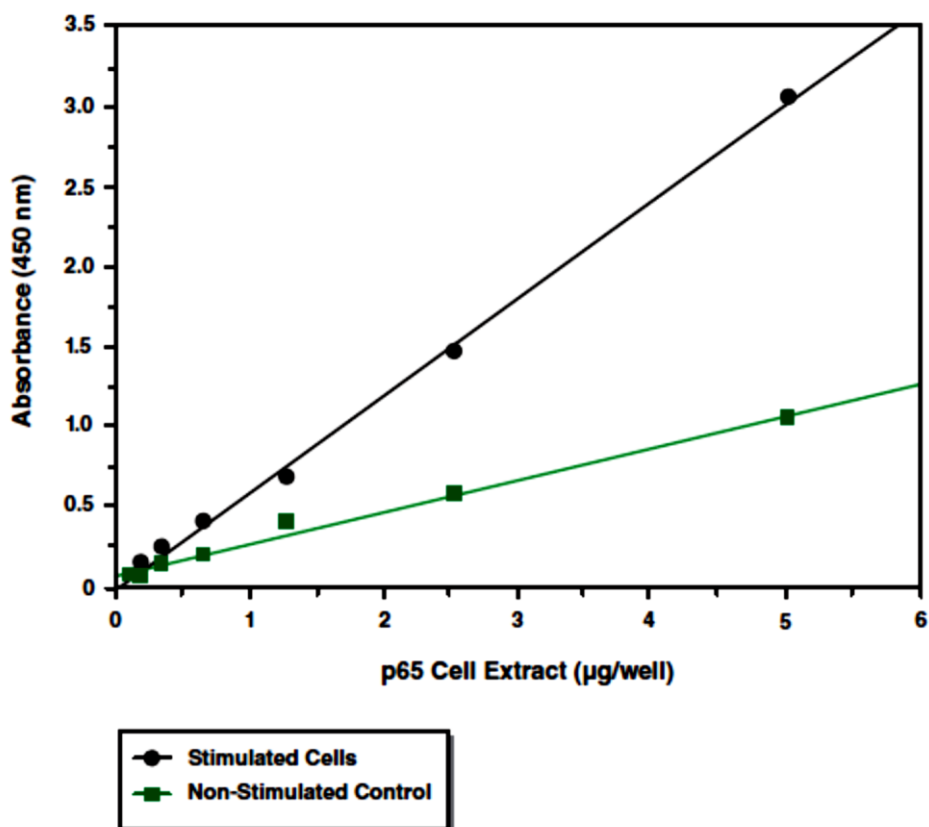


Figure 2. Assay of cell lysates isolated from stimulated (20 ng/mL TNF- $\alpha$  for 30 min.) and non-stimulated HeLa cells demonstrating NF- $\kappa$ B (p65) activity

- Interferences

The following reagents were tested for interference in the assay.

| Reagent                            | Will Interfere (Yes or No) |
|------------------------------------|----------------------------|
| EGTA ( $\leq 1$ mM)                | No                         |
| EDTA ( $\leq 0.5$ mM)              | No                         |
| ZnCl (any concentration)           | Yes                        |
| DTT (between 1 and 5 mM)           | No                         |
| Dimethylsulfoxide ( $\leq 1.5\%$ ) | No                         |

## Resources

### Troubleshooting

| Problem                                    | Possible Causes   | Recommended Solutions   |
|--|---|---|
| No signal or weak signal in control wells. | <p>A. Omission of key reagent.</p> <p>B. Plate reader settings not correct.</p> <p>C. Reagent/reagents expired.</p> <p>D. Salt concentrations affected binding between DNA and protein.</p> <p>E. Developing reagent used cold.</p> <p>F. Developing reagent not added at correct volume.</p> | <p>A. Check that all reagents have been added and in the correct order. Perform the assay using the Positive Control.</p> <p>B. Check wavelength setting on plate reader and change to 450 nm.</p> <p>C. Check expiration date on reagents.</p> <p>D. Reduce the amount of nuclear extract used in the assay, or reduce the amount of salt in the nuclear extracts (alternatively can perform buffer exchange).</p> <p>E. Pre-warm the Developing Solution to room temperature prior to use.</p> <p>F. Check pipettes to ensure correct amount of developing solution was added to wells.</p> |
| High signal in all wells.                  | <p>A. Incorrect dilution of antibody (too high).</p> <p>B. Improper/inadequate washing of wells.</p> <p>C. Overdeveloping.</p>  | <p>A. Check antibody dilutions and use amounts outlined in instructions.</p> <p>B. Follow the protocol for washing wells using the correct number of times and volumes.</p> <p>C. Decrease the incubation time when using the developing reagent.</p>   |
| High background (NSB).                     | Incorrect dilution of antibody (too high).  | Check antibody dilutions and use amounts outlined in the instructions.  |
| Weak signal in sample wells.               | <p>A. Sample concentration is too low.</p> <p>B. Incorrect dilution of antibody.</p> <p>C. Salt concentrations affecting binding between DNA and protein.</p>   | <p>A. Increase the amount of nuclear extract used. Loss of signal can occur with multiple freeze/thaw cycles of the sample. Prepare fresh nuclear extracts and aliquot as outlined in product insert.</p> <p>B. Check antibody dilutions and use amounts outlined in the instructions.</p> <p>C. Reduce the amount of nuclear extract used in the assay or reduce the amount of salt in the nuclear extracts (alternatively can perform buffer exchange).</p>   |

## **References**

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**Plate Layout**

|   |    |    |     |     |     |     |     |     |     |     |     |     |
|---|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|   | 1  | 2  | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
| A | S1 | S1 | S9  | S9  | S17 | S17 | S25 | S25 | S33 | S33 | S41 | S41 |
| B | S2 | S2 | S10 | S10 | S18 | S18 | S26 | S26 | S34 | S34 | S42 | S42 |
| C | S3 | S3 | S11 | S11 | S19 | S19 | S27 | S27 | S35 | S35 | S43 | S43 |
| D | S4 | S4 | S12 | S12 | S20 | S20 | S28 | S28 | S36 | S36 | S44 | S44 |
| E | S5 | S5 | S13 | S13 | S21 | S21 | S29 | S29 | S37 | S37 | NSB | NSB |
| F | S6 | S6 | S14 | S14 | S22 | S22 | S30 | S30 | S38 | S38 | PC  | PC  |
| G | S7 | S7 | S15 | S15 | S23 | S23 | S31 | S31 | S39 | S39 | Blk | Blk |
| H | S8 | S8 | S16 | S16 | S24 | S24 | S32 | S32 | S40 | S40 | C1  | C1  |

S1-S44=Sample Wells; NSB=Non-specific Binding Wells; PC=Positive Control Wells; Blk=Blank Wells; C1=Competitor dsDNA Wells