ELISA Kit: NF-κB (p65) TRANSCRIPTION FACTOR ASSAY KIT

Catalog # KAA065

I. Overview

Rockland's NF-κB (p65) Transcription Factor Assay is a non-radioactive, sensitive method for detecting specific transcription factor DNA binding activity in nuclear extracts and whole cell lysates. 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-κB response element is immobilized onto the bottom of wells of a 96 well plate (see Figure 1). NF-κB contained in a nuclear extract specifically binds to the NF-κB response element. NF-κB (p65) is detected by addition of a specific primary antibody directed against NF-κB (p65). A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. Rockland's NF-κB (p65) Transcription Factor Assay detects human NF-κB (p65). It will not cross-react with NF-κB (p50).

Please read the entire product insert prior to use.

II. Kit Principle

The NF-κB/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-κB [1]. 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-κB has often been called a 'central mediator of the human immune response' [2]. Acting as dimers, these transcription factors bind to DNA sequences, collectively called κB, sites thereby regulating expression of target genes. In most cells, Rel/ NF-κB transcription complexes are present in an inactive form in the cytoplasm, bound to an inhibitor IκB. Certain stimuli result in the phosphorylation, ubiquitination, and subsequent degradation of IκB proteins thereby enabling translocation of NF-κB into the nucleus [3]. The most common Rel/NF-κB dimer in mammals contains p50-RelA (p50/p65) heterodimers and is specifically called NF-κB. One of the target genes activated by NF-κB is that encoding IκBα. This feedback mechanism allows newly-synthesized IκBα to enter the nucleus, remove NF-κB from DNA and transport it back to the cytoplasm thereby restoring its inactive state. The importance of Rel/NF-κB transcription factors in human inflammation and certain diseases makes them attractive targets for potential therapeutics [4-6].

III. Intended Use

Use Rockland's NF-κB (p65) Transcription Factor Assay for detection of transcription factor in lysates or cell extracts by ELISA. If you require additional assistance please call or e-mail our technical service representatives at 800-656-7625 or tech@rockland-inc.com.

IV. Storage and Stability

This kit will perform as specified if stored at temperatures outlined in Table 1 and used before the expiration date indicated on the Certificate of Analysis.

V. Number of Assays

Each kit contains one (1) plate capable of detecting transcription factor in 44 sample wells run in duplicate when the recommended plate configuration is used (see Figure 3).

VI. Kit Components and Component Layout

A. Kit Components

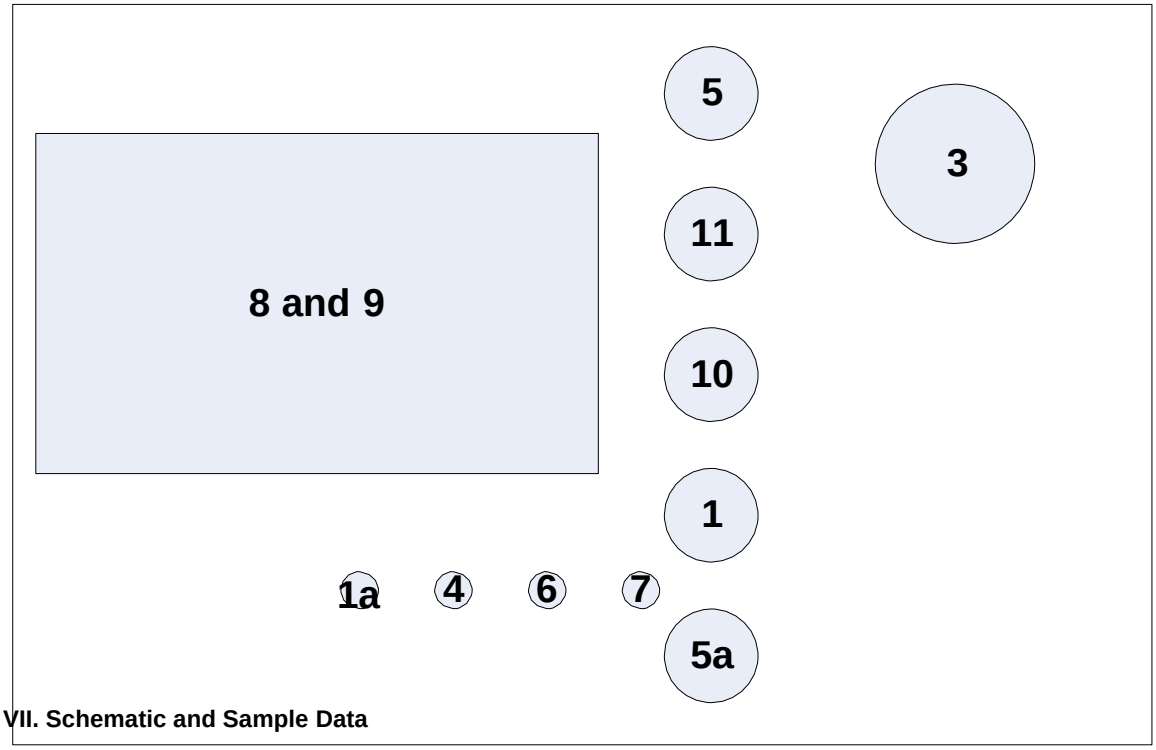
Please store all kit components as outlined in Table 1 below. For long-term storage, the positive control should be thawed on ice, aliquoted at 20 µL/vial and stored at -80°C. After use we recommend each kit component be stored according to the temperature listed below. If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 656-7625 or (610) 369-1008. We cannot accept any returns without prior authorization.

Table 1.

Number	Item	Storage	Quantity
1	Transcription Factor Binding Assay Buffer (4X)	4 °C	1 vial
1a	Transcription Factor Reagent A	-20 °C	1 vial
2	Transcription Factor NF-κB (p65) Positive Control	-80 °C†	1 vial
3	Transcription Factor Antibody Binding Buffer (10X)	4 °C	1 vial
4	Transcription Factor NF-κB (p65) Primary Antibody	-20 °C	1 vial
5	Wash Buffer Concentrate (400X)	4 °C	1 vial
5a	Tween 20	Room temperature	1 vial
6	Transcription Factor NF-κB Specific Competitor dsDNA	-20 °C	1 vial
7	HRP Goat Anti-Rabbit Secondary Antibody Conjugate	-20 °C	1 vial
8	Transcription Factor NF-κB 96 Well Plate	4 °C	1 plate
9	Plate Cover		1 cover
10	Transcription Factor Developing Solution	4 °C	1 vial
11	Transcription Factor Stop Solution	4 °C	1 vial

† Transcription Factor NF-κB (p65) Positive Control is packed separately at -80 °C.

B. Component Layout



VII. Schematic and Sample Data

Figure 1. Schematic of the Transcription Factor Binding Assay

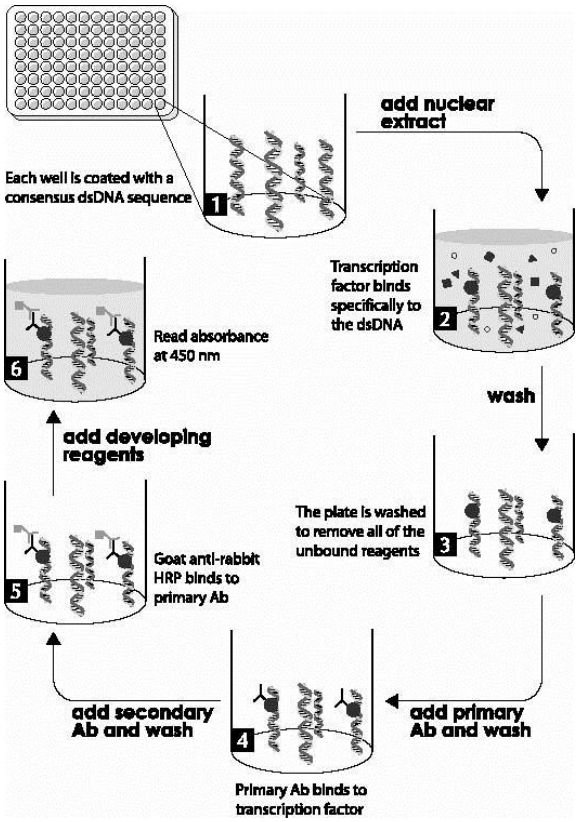
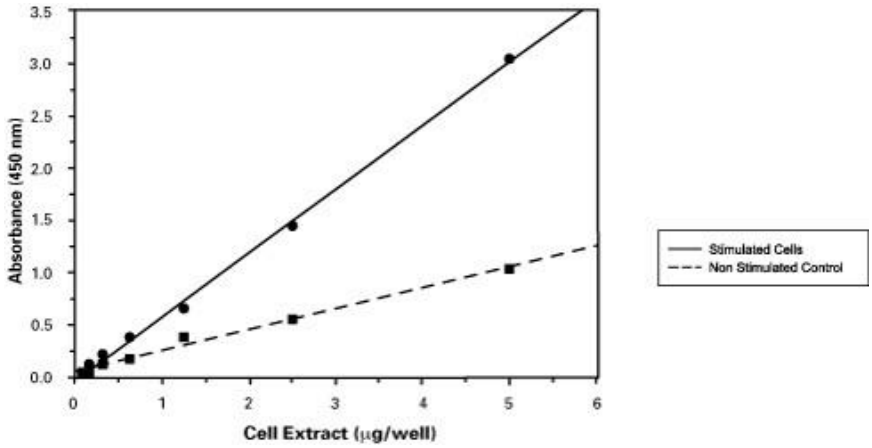


Figure 2. Assay of cell lysates isolated from stimulated (20 ng/mL TNFα for 30 min.) and non-stimulated HeLa cells demonstrating NF-κB (p65) activity.



VIII. Pre-Assay Preparation

A. Required Buffers not included in Kit (i.e. provided by the User)

1. **10X PBS (or p/n MB-008)**
   Dissolve 80 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 800 ml distilled H_20.
   Adjust pH to 7.4 with HCl
   Adjust volume to 1 L with H_2O

2. **1X PBS**
   100 ml of 10X stock into 900 ml distilled H_20

3. **Phosphatase Inhibitor Solution (50X)**
   1 M NaF
   0.05 M β-glycerophosphate
   0.05 M Na_3VO_4
   Store at -80°C

4. **PBS/Phosphatase Inhibitor Solution**
   Add 250 µL of 50X Phosphatase Inhibitor Solution to 10 mL of 1X PBS, mix well and keep on ice. Make fresh daily.

5. **Hypotonic Buffer (pH 7.5)**
   20 mM HEPES (pH 7.5)
   5 mM NaF
   10 µM Na_2MoO_4
   0.1 mM EDTA
   Store at 4°C.

6. **Extraction Buffer***
   10 mM HEPES (pH 7.9)
   0.1 mM EDTA
   1.5 mM $MgCl_2$
   420 mM NaCl
   0.5 mM DTT
   0.5 mM PMSF
   1 µg/ml Pepstatin A
   1 µg/ml Leupeptin
   10 µg/ml Aprotinin
   20 mM NaF
   1 mM ß-glycerophosphate
   10 mM Na_3VO_4
   25% glycerol (v/v)

*This buffer cannot be stored and must be made fresh on the day of use.

B. Purification of Cellular Nuclear Extract

This procedure can be used for a 15 mL cell suspension grown in a T75 flask or adherent cells (100 mm dish 80-90% confluent) where 10^7 cells yields approximately 50 µg of nuclear protein.

1. Collect 10^7 cells in pre-chilled 15 mL tubes.
2. Centrifuge suspended cells at 300 x g for 5 minutes at 4°C.
3. Discard the supernatant. Resuspend cell pellet in 5 ml of ice-cold PBS/Phosphatase Inhibitor Solution and centrifuge at 300 x g for 5 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 50 µL of 10% Nonidet P-40. Mix gently by pipetting.
7. Centrifuge for 30 seconds (pulse spin) at 4°C in a microcentrifuge. Transfer the supernatant containing the cytosolic fraction to a new tube and store at -80°C.

8. Resuspend the pellet in 50 µL ice-cold extraction buffer (with protease inhibitors). Vortex 15 seconds at highest setting then gently rock the tube on ice for 15 minutes using a shaking platform. Vortex sample for 30 seconds at 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 assay.
10. Keep small aliquot of nuclear extract to quantify the protein concentration.

C. Preparation of Buffers provided in this kit (see page 2).

1. Antibody Binding Buffer

Kit component **#3** contains 3 mL of a 10X stock of Antibody Binding Buffer (ABB) to be used for diluting the primary and secondary antibodies. For preparing 1X ABB dilute to 1X by adding 27 mL of UltraPure water. Store at 4°C for up to two months.

2. Wash Buffer

Kit component **#5** 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 Tween 20 (vial **#5a**). *[NOTE: Tween 20 is a viscous liquid and cannot be measured by a pipet. 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 Tween 20 (0.5 mL/liter of Wash Buffer). Store at 4°C for up to two months.

3. NF-κB (p65) Positive Control

Kit component **#2** contains 150 µL of TNFα-stimulated HeLa cell extract (stored at -80°C). This lysate is provided as a positive control for NF-κB (p65) activation; it is not intended for plate-to-plate comparisons. The cell lysate provided is sufficient for 15 reactions and will provide a strong signal (>0.5 AU at 450 nm) when used at 10 µL/well. When using this control, a decrease in signal may occur with repeated freeze/thaw cycles. It is recommended that the clarified cell lysate be aliquoted at 20 µL per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw.

4. Transcription Factor Binding Assay Buffer (4X)

Kit component **#1** contains 3 mL of a 4X stock of Transcription Factor Binding Assay Buffer (TFB). Prepare TFB immediately prior to use in 1.5 mL centrifuge tubes or 15 mL conical tubes as outlined in Table 2. This buffer is now referred to as Complete Transcription Factor Binding Assay Buffer (CTFB). *[NOTE: It is recommended that the CTFB be used the same day it is prepared.]*

Component	Volume Required per well	Volume Required per strip	Volume Required per 96 well plate
UltraPure water (p/n MB-010-0100)	73 µL	584 µL	7,008 µL
4X Transcription Factor Binding Assay Buffer	25 µL	200 µL	2,400 µL
Reagent A (kit component **#1a**)	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 2. Preparation of Complete Transcription Factor Binding Assay Buffer

IX. Materials Required but Not Supplied

Nearly all components required for this ELISA are provided for your convenience in Rockland's NF-κB (p65) Transcription Factor Assay kit. However, some addition materials needed are:

1. A plate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipettes and a repeat pipettor.
3. A source of UltraPure water. Deionized or HPLC-grade water is acceptable.
4. 300 mM dithiothreitol (DTT).
5. Buffers for preparation of nuclear extracts.
6. Nuclear extracts.

[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.]

X. Transcription Factor Binding Assay Method

A. Plate Configuration

There is no fixed specific pattern for using the wells on the plate. A typical layout of NF-κB (p65) positive control (PC), competitive dsDNA (C1) and unknown samples of nuclear extracts (U1 through U44) run in duplicate is given below. We suggest you record the contents of each well on the template sheet provided (see page 11).

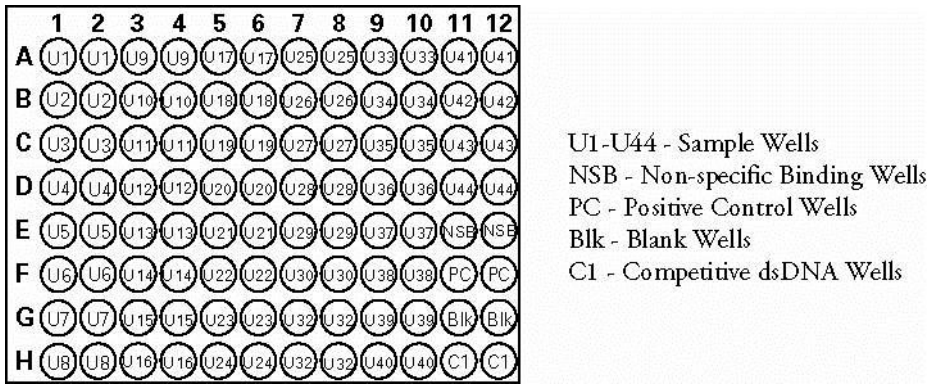


Figure 3. Sample Plate Format

B. Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps to maintain more precise times of incubation.
- Before pipetting each reagent, equilibrate the pipet tip in that reagent (i.e., fill the tip and expel the contents; repeat several times).
- Do not expose the pipet tip to the reagent(s) already in the well.
- 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 blanks (Blk), two non-specific binding (NSB), and two positive control wells be included.

C. Protocol

1. Binding of active NF-κB (p65) to the consensus sequence:

a) 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 2-4ºC. Be sure that the packet is sealed with the desiccant inside.]

b) Prepare the complete transcription factor buffer (CTFB) as outlined in Table 2 (page 5).

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

- Blank wells (Blk) - add 100 µL of CTFB to designated wells.
- Non-specific Binding wells (NSB) - add 100 µL of CTFB to designated wells. Do not add NF-κB (p65) to these wells.
- Competitor wells (C1) - Add 80 µL of CTFB prior to adding 10 µL of competitor dsDNA to designated wells. Add 10 µL of control cell lysates or unknown sample.

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

- Sample wells (U1-U44) - Add 90 µL of CTFB prior to adding 10 µL of Nuclear extract to designated wells. A protocol for isolation of nuclear extracts is given on page 4-5.
- Positive Control wells (PC) - Add 90 µL of CTFB prior to adding 10 µL of positive control to appropriate wells.

d) Use the cover provided (kit component **#9**) to seal the plate. Incubate overnight at 4°C or 1 h at room temperature without agitation (incubation for one hour will result in a less sensitive assay).

e) Empty the wells and wash 5 times with 200 µL of 1X wash buffer. After each wash empty the wells in the sink. After the final wash (i.e. 5th wash), tap the plate on a paper towel to remove any residual wash buffer.

2. Addition of Anti-NF-κB (p65) Primary Antibody

a) Dilute the Anti-NF-κB (p65) antibody 1:100 in 1X antibody binding buffer (ABB) as outlined in Table 3 below. Add 100 µL of diluted Anti-NF-κB (p65) antibody to each well except the Blank (Blk) wells.

Component	Volume Required per well	Volume Required per strip	Volume Required per 96 well plate
1X ABB	99 µL	792 µL	9,504 µL
Anti-NF-κB (p65) Primary Antibody	1 µL	8 µL	96 µL
Total required	**100 µL**	**800 µL**	**9,600 µL**

Table 3. Dilution of Primary Antibody

b) Use the adhesive cover provided to seal the plate.

c) Incubate the plate for 1 h at room temperature without agitation.

d) Empty the wells and wash each well 5 times with 200 µL of 1X wash buffer. After each wash, empty the contents of the plate into the sink. After the final wash (i.e. 5th wash), tap the plate 3 to 5 times on a paper towel to remove any residual wash buffer.

3. Addition of the HRP Goat anti-Rabbit conjugated Secondary Antibody

a) Dilute the HRP-conjugated secondary antibody (kit component **#7**) 1:100 in 1X Antibody binding buffer (ABB) as outlined in Table 4 below. Add 100 µL antibody to each well except the Blank (Blk) wells.

Component	Volume Required per well	Volume Required per strip	Volume Required per 96 well plate
1X ABB	99 µL	792 µL	9,504 µL
HRP Goat anti-Rabbit (p/n 611-103-122)	1 µL	8 µL	96 µL
Total required	**100 µL**	**800 µL**	**9,600 µL**

Table 4. Dilution of Secondary Antibody

b) Use the adhesive cover provided to seal the plate.

c) Incubate for 1 hour at room temperature without agitation.

d) Empty the wells and wash 5 times with 200 µL 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 3 to 5 times on a paper towel to remove any residual wash buffer.

4. Develop and Read the Plate

a) To each well being used add 100 µL of developing solution (kit component **#10**) which has been equilibrated to room temperature.

b) 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 stop solution. Taking absorbance measurements at 655 nm prior to stopping the reactions can monitor this reaction. An OD_{655} of 0.4 to 0.5 yields an OD_{450} of approximately 1.0. Monitor development of sample wells to ensure adequate color development prior to stopping the reaction.

[NOTE: Do not overdevelop the plate. However positive control wells (only) may need to overdevelop to allow adequate color development in sample wells.]

c) Add 100 µL of stop solution (kit component **#11**) per well being used. The solution within the wells will change from blue to yellow after adding the stop solution.

d) Read absorbance at 450 nm within 5 minutes of adding the stop solution. Blank the plate reader according to the manufacturer's requirements using the blank wells.

XI. Assay Procedure Summary

[NOTE: This procedure is provided as a quick reference for experienced users. Follow the

detailed procedure when initially performing the assay.]

1. Prepare Complete Transcription Factor Binding Buffer (CTFB) as described in the Pre-Assay Preparation section, Table 2.
2. Add 90 µL CTFB per well (80 µL if adding Competitive dsDNA, 100 µL to Blank (Blk) and non-specific binding (NSB) wells).
3. Add 10 µL of Competitive dsDNA (optional) to appropriate wells.
4. Add 10 µL of Positive Control to appropriate wells.
5. Add 10 µL of Sample containing NF-κB (p65) wells.
6. Incubate overnight at 4°C without agitation.
7. Wash each well five times with 200 µL of 1X Wash Buffer.
8. Add 100 µL of diluted Anti-NF-κB (p65) antibody per well (except Blank wells).
9. Incubate 1 h at room temperature without agitation.
10. Wash each well five times with 200 µL of 1X Wash Buffer.
11. Add 100 µL of diluted Goat anti-Rabbit HRP conjugated secondary antibody (except Blank wells).
12. Incubate 1 h at room temperature without agitation.
13. Wash each well five times with 200 µL of 1X Wash Buffer.
14. Add 100 µL of Developing Solution per well.
15. Incubate 15 to 45 min with gentle agitation.
16. Add 100 µL of Stop Solution per well.
17. Measure the absorbance at 450 nm.

XII. Quick Protocol Guide (Steps 1-17 listed above)

Steps	Reagent	Blk	NSB	PC	C1	U1-U44
(1-5) add reagents	CTFB	100 µL	100 µL	90 µL	80 µL	90 µL
	Competitive dsDNA				10 µL	
	Positive control			10 µL	10 µL	
	Samples					10 µL
(6) incubate	Cover plate and incubate overnight at 4°C without agitation					
(7) wash	Wash all wells five times					
(8) add reagent	Primary antibody		100 µL	100 µL	100 µL	100 µL
(9) incubate	Cover plate and incubate 1 h at room temperature without agitation					
(10) wash	Wash all wells five times					
(11) add reagent	Gt anti-Rabbit HRP		100 µL	100 µL	100 µL	100 µL
(12) incubate	Cover plate and incubate 1h at room temperature without agitation					
(13) wash	Wash all wells five times					
(14) add reagent	Developer	100 µL	100 µL	100 µL	100 µL	100 µL
(15) incubate	Monitor development in wells					
(16) add reagent	Stop solution	100 µL	100 µL	100 µL	100 µL	100 µL
(17) read plate	Read plate at wavelength of 450 nm					

Table 5. Quick Protocol Guide

XIII. Interferences

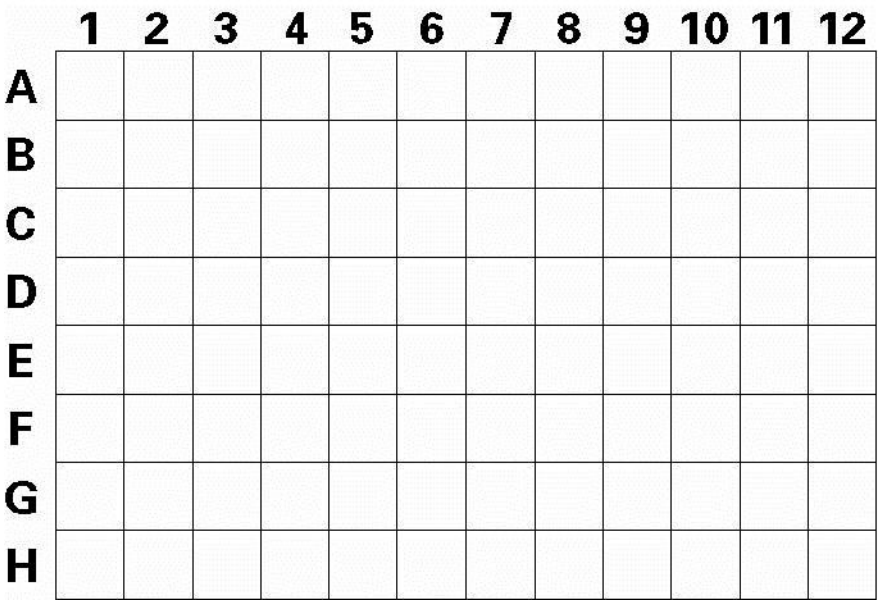
The following reagents were tested for interference in the assay.

Reagent	Will Interfere (Yes or No)
EGTA (≤1 mM)	No
EDTA (≤0.5 mM)	No
ZnCl (any concentration)	Yes
DTT (between 1 and 5 mM)	No
Dimethylsulfoxide (≤1.5%)	No

XIV. Troubleshooting Guide

Problem:	No signal or weak signal in all wells.
Cause:	Omission of key reagent. *-or-* Plate reader settings not correct. *-or-* Reagent/reagents expired.
Solution:	Check that all reagents have been added and in the correct order. Perform the assay using the positive control. *-or-* Check wavelength setting on plate reader and change to 450 nm. *-or-* Check expiration date on reagents.
Problem:	No signal or weak signal in all wells.
Cause:	Salt concentrations affected binding between DNA and protein. *-or-* Developing reagent used cold. *-or-* Developing reagent not added at correct volume.
Solution:	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). *-or-* Prewarm the developing solution to room temperature prior to use. *-or-* Check pipettes to ensure correct amount of developing solution was added to wells.
Problem:	High signal in all wells.
Cause:	Incorrect dilution of antibody (too high). *-or-* Improper/inadequate washing of wells. *-or-* Overdeveloping.
Solution:	Check antibody dilutions and use amounts outlined in instructions. *-or-* Follow the protocol for washing wells using the correct number of times and volumes. *-or-* Decrease the incubation time when using the developing reagent.
Problem:	High background (NSB).
Cause:	Incorrect dilution of antibody (too high).
Solution:	Check antibody dilutions and use amounts outlined in the instructions.
Problem:	Weak signal in sample wells.
Cause:	Sample concentration is too low. *-or-* Incorrect dilution of antibody. *-or-* Salt concentrations affecting binding between DNA and protein.
Solution:	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. *-or-* Check antibody dilutions and use amounts outlined in the instructions. *-or-* 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).

XV. Plate Template



XVI. References

1. Gilmore, T.D. The Rel/NF-κB signal transduction pathway: Introduction. *Oncogene* **18**, 6842-6844 (1999).

2. Pahl, H.L. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* **18**, 6853-6866 (1999).

3. Karin, M. The beginning of the end: IκB kinase (IKK) and NF-κB activation. *J. Biol. Chem.* **274(39)**, 27339-27342 (1999).

4. Gilroy, D.W., Lawrence, T., Perretti, M., *et al.* Inflammatory resolution: New opportunities for drug discovery. *Nature Reviews* **3**, 401-416 (2004).

5. Maeda, S., Hsu, L.-C., Liu, H., *et al.* Nod2 mutation in Crohn's disease potentiates NF-κB activity and IL-1β processing. *Science* **307**, 734-738 (2005).

6. Arkan, M.C., Hevener, A.L., Greten, F.R., *et al.* IKK-β links inflammation to obesity-induced insulin resistance. *Nature Med.* **11(2)**, 191-198 (2005).

XVII. Disclaimer

XVIII. Additional Kits and Antibodies

Additional Kits	Code	Size	Price
NF-κB (p50) Transcription Factor Assay Kit	KAA064	1 each	www.rockland-inc.com
NF-κB (p65) Transcription Factor Assay Kit	KAA065	1 each	www.rockland-inc.com

Additional Antibodies	Code	Additional Antibodies	Code
Anti-NFκB (p50) (NFKB1) [Rabbit]	100-4164	Anti-NFκB cRel [Rabbit]	100-4166
Anti-NFκB (p65) (Rel A) [Rabbit]	100-4165	Anti-NFκB (p105) [Rabbit]	100-4184
Anti-NFκB (p65) (Rel A) N-TERMINAL SPECIFIC [Rabbit]	100-4165N	Anti-NFκB (p52) [Rabbit]	100-4185
Anti-NFκB (p65) NLS specific [Rabbit]	600-401-271	Anti-IκB alpha (Hu, Ms, Rt Specific) C-terminal [Rabbit]	100-4167C
Anti-NFκB (p65) (Rel A) phospho specific pS276 [Rabbit]	100-401-264	Anti-IκB beta (Hu, Ms, Rt Specific) [Rabbit]	100-4186
Anti-NFκB (p65) (Rel A) phospho specific pS529 [Rabbit]	100-401-266	Anti-IKK alpha [Rabbit]	100-401-219
Anti-IKKe phospho specific pT501 [Rabbit]	600-401-267	Anti-IKK beta [Rabbit]	100-401-220