Background

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 phosphorylattion, 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]

About This Assay

NF-κB (human p50) Transcription Factor Assay Kit 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, binds specifically to the NF-κB response element. NF-κB (p50) is detected by addition of specific primary antibody directed against NF-κB (p50). A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. The NF-κB (human p50) Transcription Factor Assay Kit detects human NF-κB (p50). It will not cross-react with NF-κB (p65).

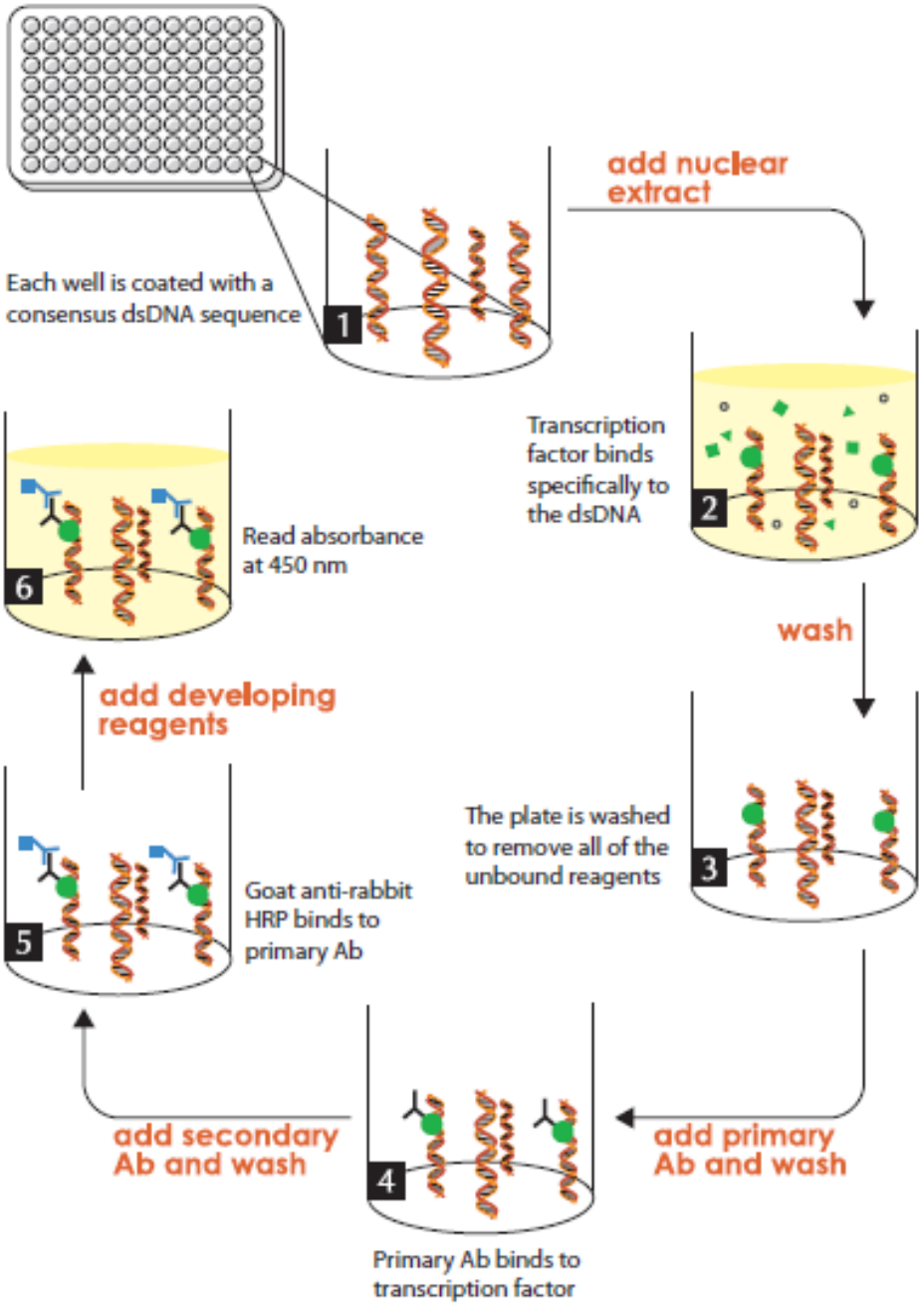


Figure 1. Schematic of the Transcription Factor Binding Assay

Material Supplied

Item	Quantity	Storage
Transcription Factor Binding Assay Buffer (4X)	1 vial	4℃
Transcription Factor Reagent A	1 vial	-20℃
Transcription Factor NF-κB (p50) Positive Control	1 vial	-80℃
Transcription Factor Antibody Binding Buffer (10X)	1 vial	4℃
Transcription Factor NF-κB (human p50) Primary Antibody	1 vial	-20℃
Wash Buffer Concentrate (400X)	1 vial	4℃
Tween 20	1 vial	RT
Transcription Factor NF-κB Specific Competitor dsDNA	1 vial	-20℃
Transcription Factor Goat Anti-Rabbit HRP Conjugate	1 vial	-20℃
Transcription Factor NF-κB 96-Well Strip Plate	1 plate	4℃
96-Well Cover Sheet	1 cover	RT
Transcription Factor Developing Solution	1 vial	4℃
Transcription Factor Stop Solution	1 vial	4℃

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

Materials Needed 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 are 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.

Storage and Stability

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

Sample Buffer Preparation

All buffers and reagents below required for preparation of Nuclear Extracts:

- ✓ PBS (10X) – 1.37 M NaCl, 0.027 M KCl, 0.1 M Na_2HPO_4, 0.017 M KH_2PO_4, pH 7.4
- ✓ PBS (1X) - Dilute 100 ml of 10X stock with 900 ml distilled H_2O
- ✓ Nuclear Extraction Phosphatase Inhibitor Cocktail (50X) - 0.05 M β-glycerophosphate and 1M NaF, 0.05 M Na_3OV_4, Store at -80℃
- ✓ PBS/Phosphatase Inhibitor Solution - Add 200 µl of 50X Phosphatase Inhibitor Solution to 10 ml of 1X 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 -80℃
- ✓ Nuclear Extraction Hypotonic Buffer (10X) -100 mM HEPES, pH 7.5, containing 40 mM NaF, 100 µM Na_2MoO_4, and 1 mM EDTA, Store at 4℃.
- ✓ Complete Extraction Hypotonic Buffer (1X) - Prepare as outlined in Table 1. 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 10^7 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 1. 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_2O Store at room temperature
- ✓ Nuclear Extraction Buffer (2X) - 20 mM HEPES, pH 7.9, containing, 0.2 mM EDTA, 3 mM $MgCl_2$, 840 mM NaCl, and 20% glycerol (v/v), Store at 4℃
- ✓ Complete Nuclear Extraction Buffer (1X) - Prepare as outlined in Table 2. 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 10^7 cells
Nuclear Extraction Buffer (2X)	50 µl
Protease Inhibitors (100X)	1.0 µl
Phosphatase Inhibitors (50X)	2.0 µl
DTT (10 mM)	5 µl
Distilled Water	42 µl
Total Volume	100 µl

Table 2. Preparation of Complete Nuclear Extraction Buffer

Purification of Cellular Nuclear Extracts

The procedure described 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 10^7 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 five minutes at 4℃.
3. Discard the supernatant. Resuspend cell pellet in 5 ml of ice-cold PBS/Phosphatase Inhibitor Solution and centrifuge at 300 x g for five minutes at 4℃. Repeat one time.
4. Discard the supernatant. Add 500 µl ice-cold 1X Hypotonic buffer. Mix gently by pipetting and transfer resuspended pellet to a 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℃ in a microcentrifuge. Transfer the supernatant which contains the cytosolic fraction to a new tube and store at -80℃.
8. Resuspend the pellet in 50 µl ice-cold Complete Nuclear Extraction Buffer (1X) (with protease and phosphatase 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℃. The supernatant contains the nuclear fraction. Aliquot to clean chilled tubes, flash freeze and store at -80℃. 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.

Reagent Preparation

1. Transcription Factor Antibody Binding Buffer (10X) - 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℃ for up to six months.
2. 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 Tween 20. *NOTE: Tween 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 Tween 20 (0.5 ml/liter of Wash Buffer). Store at 4℃ for up to two months.
3. 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 Assay Buffer (CTFB) immediately prior to use in 1.5 ml centrifuge tubes or 15 ml conical tubes as outlined in Table 3, 12. This buffer is now referred to as CTFB. *It is recommended that the CTFB be used the same day it is prepared.*

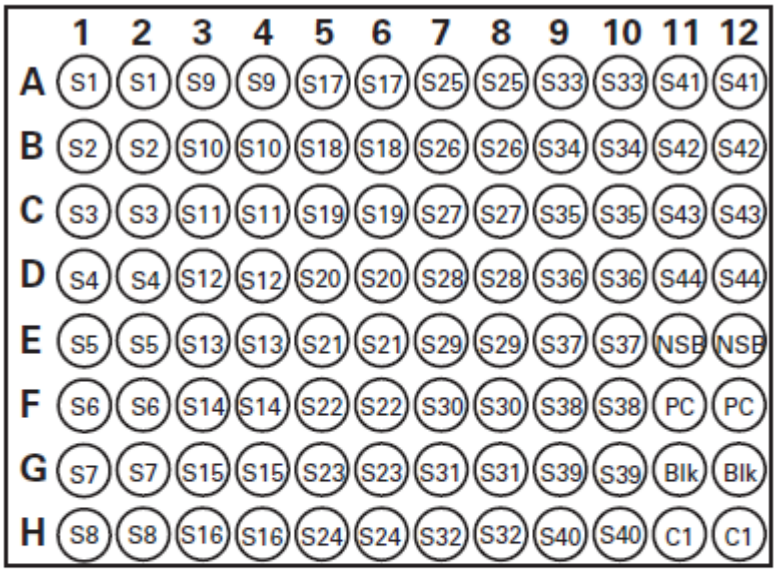
Component	Volume/Well	Volume/Strip	Volume/96-well plate
UltraPure Water	73 µl	584 µl	7,008 µl
4X Transcription Factor Binding Assay Buffer	25 µl	200 µl	2,400 µl
Reagent A	1 µl	8 µl	96 µl
300 mM DTT	1 µl	8 µl	96 µl
Total Required	100 µl	800 µl	9,600 µl

Table 3. Preparation of Complete Transcription Factor Binding Assay Buffer

4. Transcription Factor NF-κB (p50) Positive Control - One vial contains 150 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 15 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 20 µl per vial and stored at -80℃ to avoid loss in signal from repeated freeze/thaw cycles.

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of NF-κB (p50) Positive Control (PC), Competitor dsDNA (C1), and samples of nuclear extracts (S1-S44) to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided.



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

Figure 2. Sample plate format

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.

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.

Performing the Assay

Binding of active NF-κB (p50) to the consensus sequence

1. 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℃. Be sure that the packet is sealed with the desiccant inside.
2. Prepare the CTFB as outlined in Table 3.
3. Add appropriate amount of reagent(s) listed below to the designated wells as follows: **Blk -** add 100 µl of CTFB to designated wells.
   NSB - add 100 µl of CTFB to designated wells. Do not add samples or Positive Control to these wells.
   C1 - Add 80 µl of CTFB prior to adding 10 µl of Transcription Factor NF-**κ**B Specific Competitor dsDNA to designated wells. Add 10 µl 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 µl of CTFB followed by 10 µl of Nuclear Extract to designated wells.
   PC - Add 90 µl of CTFB followed by 10 µl of Positive Control to appropriate wells.
4. Use the cover provided to seal the plate. Incubate overnight at 4℃ or one hour at room temperature without agitation (incubation for one hour will result in a less sensitive assay).
5. Empty the wells and wash five times with 200 µl 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-κB (human p50) Primary Antibody

1. Dilute the Transcription Factor NF-κB (human p50) Primary Antibody 1:100 in 1X ABB as outlined in Table 4 below. Add 100 µl of diluted NF-κB (human p50) Primary Antibody to each well except the Blk wells.

Component	Volume/Well	Volume/Strip	Volume/96-well plate
1X ABB	99 µl	792 µl	9,504 µl
NF-κB (human p50) Primary Antibody	1 µl	8 µl	96 µl
Total required	100 µl	800 µl	9,600 µl

Table 4. Dilution of Primary Antibody

2. Use the adhesive cover sheet provided to seal the plate.
3. Incubate the plate for one hour at room temperature without agitation.
4. Empty the wells and wash each well five 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 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 µl of diluted secondary antibody to each well except the Blk wells.

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

Table 5. Dilution of Secondary Antibody

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 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 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 µl 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_{655} of 0.4-0.5 yields an OD_{450} 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 µl 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.

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 CTFB as described in the Pre-Assay Preparation section, Table 3.
2. Add 90 µl CTFB per sample well (80 µl if adding Competitor dsDNA), 100 µl to Blk and NSB wells).
3. Add 10 µl of Competitor dsDNA (optional) to appropriate wells.
4. Add 10 µl of Positive Control to appropriate wells.
5. Add 10 µl of Sample containing NF-κB (human p50) to appropriate wells.
6. Incubate overnight at 4℃ without agitation.
7. Wash each well five times with 200 µl of 1X Wash Buffer.
8. Add 100 µl of diluted NF-κB (human p50) Primary Antibody per well (except Blk wells).
9. Incubate one hour 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 Conjugate (except Blk wells).
12. Incubate one hour 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 minutes with gentle agitation.
16. Add 100 µl 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 µl	100 µl	90 µl	80 µl	90 µl
	Competitor dsDNA				10 µl	
	Positive Control			10 µl	10 µl	
	Samples					10 µl
2. Incubate	Cover plate and incubate overnight at 4℃ without agitation					
3. Wash	Wash all wells five times					
4. Add reagents	Primary Antibody		100 µl	100 µl	100 µl	100 µl
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 µl	100 µl	100 µl	100 µl
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 µl	100 µl	100 µl	100 µl	100 µl
11. Incubate	Monitor development in wells					
12. Add reagents	Stop Solution	100 µl	100 µl	100 µl	100 µl	100 µl
13. Read	Read plate at wavelength of 450 nm					

Table 6. Quick Protocol Guide

Performance Characteristics

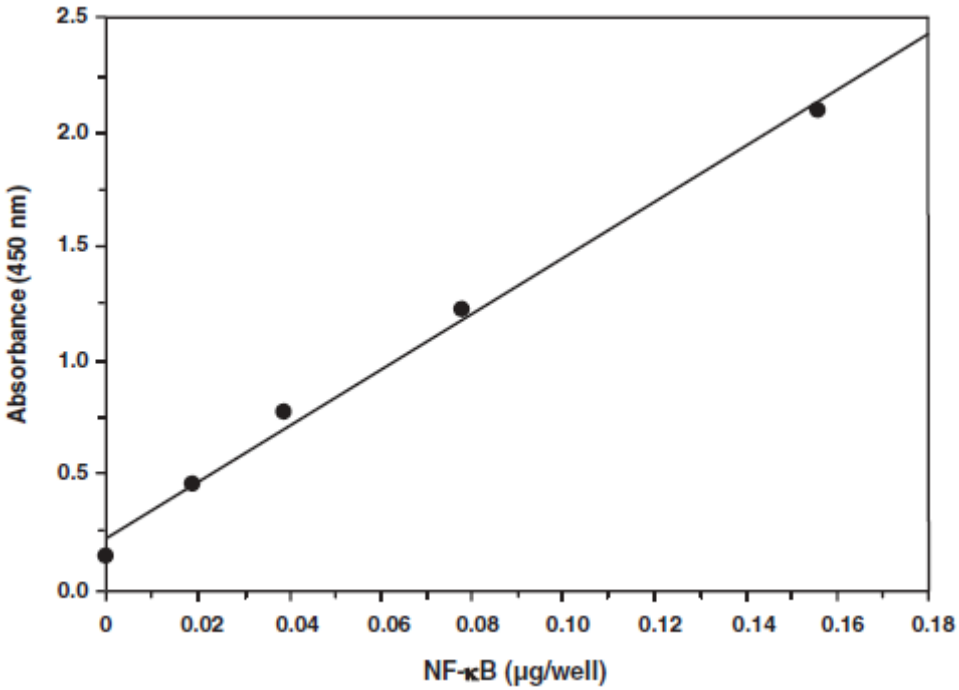


Figure 3. Sensitivity of the NF-κB assay using purified human recombinant p50

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

Troubleshooting

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

Problem (cont.)	Possible Causes (cont.)	Recommended Solutions (cont.)
Weak signal in sample wells	A. Sample concentration is too low B. Incorrect dilution of antibody C. Salt concentrations affecting binding between DNA and protein	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 B. Check antibody dilutions and use amounts outlined in the instructions 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)

References

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