

**NFκB p65**  
**Transcription Factor Assay**  
**Chemiluminescent**

**Cat. No. SGT620**

**FOR RESEARCH USE ONLY**  
**Not for use in diagnostic procedures**

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## Application

The transcription factor NF $\kappa$ B (Nuclear Factor kappa B) is involved in the expression and regulation of a number of important cellular and physiological processes such as growth, development, apoptosis, immune and inflammatory response, and activation of various viral promoters including human immunodeficiency virus long terminal repeats<sup>1,2</sup>. NF $\kappa$ B represents a group of structurally related and evolutionarily conserved proteins related to the proto-oncogene c-Rel with five members in mammals that include Rel (cRel), RelA (p65), RelB, NF $\kappa$ B1 (p50 and its precursor p105), and NF $\kappa$ B2 (p52 and its precursor p100)<sup>1,2</sup>. NF $\kappa$ B/Rel proteins exist as homo- or heterodimers to form transcriptionally competent or repressive complexes. Although most NF $\kappa$ B dimers are activators of transcription, the p50/50 and p52/52 homodimers can repress the transcription of their target genes<sup>2,3</sup>. The p50/p65 heterodimer of NF $\kappa$ B is the most abundant in cells<sup>3</sup>.

A critical component in NF $\kappa$ B regulation is the I $\kappa$ B Kinase (IKK) complex<sup>3</sup>. In a majority of unstimulated cells, the NF $\kappa$ B transcription factors exist in their inactive form and are retained in the cytoplasm by the bound inhibitory I $\kappa$ B proteins<sup>4,5</sup>. Upon stimulation by multiple inducers including viruses or cytokines, such as TNF $\alpha$ , IL-1, or PMA, I $\kappa$ B $\alpha$  is rapidly phosphorylated and degraded, resulting in the release of the NF $\kappa$ B complex, most commonly the p105/p65 heterodimer. The p105 subunit is cleaved into its active p50 form. This cleavage exposes the NLS sequence on the p50 subunit. The p50/p65 heteroduplex then translocates to the nucleus where it activates gene transcription. NF $\kappa$ B induces the transcription of its own inhibitor, I $\kappa$ B $\alpha$ , causing an autoregulatory mechanism of NF $\kappa$ B activity and generating the inactive form of NF $\kappa$ B. The newly formed nuclear NF $\kappa$ B-I $\kappa$ B $\alpha$  complexes are then exported out to the cytoplasm, thereby reestablishing the cytoplasmic pool of inactive NF $\kappa$ B complexes primed for another round of activation to take place<sup>2</sup>. The wide variety of genes regulated by NF $\kappa$ B includes those encoding cytokines, chemokines, adhesion molecules, acute phase proteins, and inducible effector enzymes<sup>3</sup>.

The CHEMICON<sup>®</sup> Non-Radioactive Chemiluminescent NFκB p65 Transcription Factor Assay provides a fast, ultra-sensitive method to detect specific transcription factor DNA binding activity in very low quantities of nuclear extracts with an incredibly large dynamic range. This assay combines the principle of the electrophoretic mobility shift assay (EMSA) with the 96-well based enzyme-linked immunosorbent assay (ELISA), enabling manual or high-throughput sample analysis with significantly greater sensitivity than conventional EMSA assays. The entire assay takes less than 4 hours to complete with minimal hands-on time and is extremely easy to perform with the use of its one buffer. The versatile set up allows for a flexible assay design and because the binding reaction occurs in solution, various parameters can be optimized such as probe titration, competitive oligonucleotide concentration, or treatment conditions.

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## Test Principle

The Non-Radioactive Chemiluminescent NFκB p65 Transcription Factor Assay kit is provided in a 96-well format. During the assay, the Capture Probe, a double stranded biotinylated oligonucleotide containing the flanked consensus sequence for NFκB binding (5'-GGGACTTTCC-3'), is mixed with cellular (nuclear) extract in the Transcription Factor Assay Buffer provided. When incubated together, the active form of NFκB contained in the nuclear extract binds to its consensus sequence. After incubation, the extract/probe/buffer mixture is then directly transferred to the streptavidin coated chemiluminescent plate. The biotinylated double stranded oligonucleotide bound by active NFκB protein is immobilized and any inactive, unbound material is washed away. The bound NFκB transcription factor subunit, p65, is detected with a specific Rabbit anti-NFκB p65 primary antibody. An HRP-conjugated secondary antibody is then used for detection with a chemiluminescent substrate that can be read in a microplate luminometer or by a CCD camera-coupled imaging system. Included in the kit are positive cell extract, a non-specific double stranded oligonucleotide, and a specific competitor double stranded oligonucleotide.

The NFκB Transcription Factor Assay was QC tested using nuclear extracts from human (HeLa) cells. Due to the conservation in the NFκB DNA binding site and the fact that the primary antibody contained in this kit cross-reacts with rat and mouse NFκB, this assay is expected to work with samples from rat and mouse as well as human.

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*Use of this assay in NFκB-related drug discovery may be covered under U.S. Patent No. 6,150,090 and require a license from Ariad Pharmaceuticals (Cambridge, MA, USA).*

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## Kit Components

1. Transcription Factor Assay Chemiluminescent Plate (Part No. 90494): One Streptavidin coated 96-well strip plate (12 strips of 8 wells each), sealed in a foil pouch with desiccant.
2. TFA Buffer, 5x (Part No. 90272): One bottle containing 80 mL of 5x Transcription Factor Assay Buffer containing sonicated salmon sperm DNA to block non-specific DNA binding activity.
3. Blocking Reagent (Part No. 90273): One vial containing 12 grams of Blocking Reagent.
4. Primary Antibody (Part No. 90274): One vial containing 15  $\mu$ L Rabbit anti-NF $\kappa$ B p65. Use at 1:1000 dilution in Enhanced Transcription Factor Assay Buffer (see Assay Protocol).
5. Gt X Rb, HRP Secondary Antibody (Part No. 90276): One vial containing 15  $\mu$ L Goat anti-Rabbit, HRP conjugated secondary detection antibody. Use at 1:1000 dilution in Enhanced Transcription Factor Assay Buffer.
6. NF $\kappa$ B Capture Probe (Part No. 90269): One vial containing 110  $\mu$ L biotinylated double stranded oligonucleotide probe (2 pmol/ $\mu$ L) containing the flanked wild-type consensus sequence (5'-GGGACTTCC-3') for NF $\kappa$ B.
7. NF $\kappa$ B Specific Competitor Oligonucleotide (Part No. 90270): One vial containing 20  $\mu$ L (10 pmol/ $\mu$ L) specific competitor oligonucleotide. The NF $\kappa$ B Competitor Oligonucleotide has the same consensus sequence (5'-GGGACTTCC-3') as the NF $\kappa$ B Capture Probe but is not biotinylated and will not bind to the plate. It is provided as a specific competitor for transcription factor binding and can be used to demonstrate specificity. This control does not need to be run with each assay performed.
8. TFA Negative Control Probe (Part No. 90271): One vial containing 20  $\mu$ L biotinylated double stranded oligonucleotide probe (2 pmol/ $\mu$ L). Provided as an internal negative control, this non-specific competitor oligonucleotide does not contain the NF $\kappa$ B consensus sequence.
9. HeLa Whole Cell Extract, TNF $\alpha$  Treated (Part No. 90350): One vial containing 25  $\mu$ L TNF $\alpha$  stimulated HeLa whole cell extract. This extract is provided as a positive control and is not intended for extract-to-extract, nor plate-to-plate comparisons. When using this control, you will notice a decrease in signal with repeated freeze/thaw cycles. This component must be stored at -80°C.
10. Chemiluminescent Detection Reagent (Part No. 90495): One bottle containing 4 mL of Chemiluminescent Detection Reagent.
11. Chemiluminescent Reaction Buffer (Part No. 90496): One bottle containing 8 mL of Chemiluminescent Reaction Buffer.

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## Materials Not Supplied

1. Nuclear Extraction Kit (Catalog Number 2900) or components
2. Pipettor (both single and multi-channel)
3. Pipette tips
4. Distilled water
5. Rotator or Plate shaker (optional)
6. Plate cover (optional)
7. Microplate luminometer or CCD camera-coupled imaging system

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## Storage

The Transcription Factor Assay is a triple temperature storage assay kit.

- Store kit components at the temperatures indicated on the labels and insert until their expiration date.
- Store HeLa Whole Cell Extract at -80°C. Avoid repeated freeze-thaw cycles. Stable at -80°C for 6 months.
- Store the NFκB Capture Probe, Competitor Oligonucleotides, and Negative Control Probe at -20°C. May be stored at 4°C for less than one month.
- Store the Transcription Factor Assay (TFA) Buffer at -20°C. Avoid freeze-thawing of the TFA Buffer. Buffer can be aliquotted.
- Store all other components at 4°C.

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## Sample Preparation

### Cytosolic and Nuclear Extraction Procedure

NFκB Transcription Factor Stimulation and Nuclear Extraction Procedure. Various methods can be utilized to prepare cytosolic and nuclear extracts. The following recommended protocol has been tested and works well with the Chemicon Transcription Factor Assay.

### Materials Required for Nuclear Extraction:

1. PBS (pH 7.5)
2. Protease Inhibitor Cocktail (in DMSO)
3. Syringe with No. 27 needles (small gauge)
4. Materials for buffers: HEPES, MgCl<sub>2</sub>, KCl, EDTA, DTT, Triton X-100, DTT, glycerol, and Igepal CA-630 (NP-40 can be used in place of Igepal CA-630).

Make Buffer A and Buffer B required for the nuclear extraction in advance. Store both buffers on ice.

*Note: Fresh DTT and protease inhibitors should be added just prior to use.*

**Buffer A (Hypotonic Lysis Buffer)**

10 mM HEPES (pH 7.9)  
1.5 mM MgCl<sub>2</sub>  
10 mM KCl  
0.5 mM DTT  
0.1% Triton X-100  
Protease inhibitor cocktail

*Note: Buffer A can be made at a 10x concentration and stored at 2-8°C without the DTT and protease inhibitors added.*

**Buffer B (Extraction Buffer)**

20 mM HEPES (pH 7.9)  
1.5 mM MgCl<sub>2</sub>  
0.42 M NaCl  
0.2 mM EDTA  
0.5 mM DTT  
1.0% Igepal CA-630  
25% (v/v) glycerol  
Protease inhibitor cocktail

*Note: All work done after cell trypsinization/detachment needs to be performed on ice and/or with chilled buffers. It is imperative that the cell pellets and suspension remain as cold as possible without freezing during the extraction process.*

**A. Cell Culture**

1. Grow cells to 80-90% confluency for adherent cells or about 1.5 X 10<sup>6</sup>/mL for suspension cells.
2. Treat cells with 0.2 µg/mL TNF-α (Catalog Number GF023) for 30 minutes. Alternative methods include treatment with IL-1α (Catalog number IL001) for 30 minutes or stimulation with 2 µg/mL of PMA for 2 hours.

**B. Cell Disruption**

1. For adherent cells, wash the cells with HBSS (Hank's Balanced Salt Solution) or PBS and then add warmed trypsin to the culture flask(s). Let the trypsin sit for about 2 minutes and shake the cells off. Alternately, cell lifters may be used instead of trypsin. Collect cells and transfer them to a clean centrifuge tube, rinsing the culture flask with ice cold PBS. Centrifuge the sample at 250 x g for 5 minutes. Discard the supernatant and resuspend the cell pellet in 40 mL of ice-

cold PBS. Centrifuge the suspension at 250 x g as before. Repeat. Pour off supernatant.

2. Estimate the approximate volume of the centrifuged cell pellet. This value will be needed for determining the amount of the various buffers to add for nuclear extraction. (Two T175 tissue culture flasks of HeLa cells will generate a cell pellet of approximately 100 $\mu$ L.)
3. Add 5 cell pellet volumes of ice cold Buffer A (lysis buffer).
4. Resuspend the cell pellet by gently inverting the tube avoiding foam production. Do not vortex.
5. Incubate the cell suspension in ice cold Buffer A for 15 min on ice.
6. Centrifuge the cell suspension for 5 minutes at 250 x g. Discard supernatant and resuspend the cell pellet in two volumes of ice cold Buffer A.

### **C. Cell Lysis**

*Note: All subsequent steps must be performed on ice.*

1. Using a syringe with a small gauge needle (a No. 27 gauge needle works well) slowly draw the cell suspension prepared in Section B, Step 6 into the syringe and then eject the contents with a single stroke. Repeat approximately 5 times (drawing and ejecting). If the cells “clump” and you are not able to draw them into the syringe with a No.27 gauge needle, more non-ionic detergent (Igepal CA-630 or NP40) may need to be added.
2. Centrifuge the disrupted cell suspension at 8,000 x g for 20 minutes.
3. The supernatant contains the cytosolic portion of the cell lysate. Transfer the supernatant to a fresh tube. To keep the cytosolic fraction, snap-freeze and store in aliquots at  $-70^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.
4. The remaining portion (pellet) contains the nuclear portion of the cell lysate.

### **D. Nuclear Extraction**

1. Resuspend the nuclear pellet in 2/3 of the original cell pellet volume (determined in step B.2) ice cold Buffer B.
2. Use a fresh syringe (with a No. 27 gauge needle), repeat Step C.1. to disrupt the nuclei.

*Note: The nuclear extract sample can be stored at  $-80^{\circ}\text{C}$  at this point if needed.*

3. Use a rotator (low speed) to gently agitate the nuclear suspension at  $4^{\circ}\text{C}$  for 30-60 minutes.
4. Centrifuge the nuclear suspension at 16,000 x g for 5 min.

5. Transfer the supernatant to a fresh tube. This fraction is the nuclear extract.
6. Determine protein concentration and adjust the final protein concentration to 2.5 to 5 mg/mL with ice cold Buffer B.
7. Snap-freeze the nuclear extract in aliquots and store at  $-70^{\circ}\text{C}$ . Avoid repeated freezing and thawing of nuclear extract.

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## Assay Protocol

### A. Prepare 1x Transcription Factor Assay Buffer (TFA Buffer)

1. Calculate the volume of 1x TFA Buffer required: Each well requires 3 mL of 1x TFA Buffer (e.g. 8 wells require 24 mL of 1x TFA Buffer).
2. Dilute 1 part 5x TFA Buffer into 4 parts distilled water to obtain the desired amount of buffer.

### B. Prepare Enhanced Transcription Factor Assay Buffer (Enhanced TFA Buffer)

1. Take a portion of the 1x TFA Buffer prepared in Step A and use to prepare 1x Enhanced TFA Buffer by adding blocking reagent.
2. Calculate the volume of Enhanced TFA Buffer required: Each well requires 1.8 mL 1x TFA Buffer supplemented with 0.06 g Blocking Reagent (e.g. 8 wells require 14.4 mL of 1x TFA Buffer supplemented with 0.48 g of Blocking Reagent).
3. Mix Blocking Reagent with 1x TFA Buffer for 15 minutes with gentle agitation. Make sure that the blocking reagent is completely dissolved prior to use.
4. Enhanced TFA Buffer will be used for all steps with the exception of the final wash following secondary antibody incubation. For this wash, use the remaining 1x TFA Buffer prepared in Step A (approximately 1.2 mL per assay).

### C. Sample Incubation

1. Thaw nuclear extract and place on ice.
2. Prepare the test sample, negative control sample, positive control sample, or competitive oligonucleotide control sample in a 0.5 mL microcentrifuge tube (1.5 mL microcentrifuge tube or 96-well plate for multiple tests) as described:

***Note:** Sample extract should always be added to the binding reaction mixture last.*

	Enhanced Transcription Factor Assay Buffer (1x)	NFκB Capture Probe (90269)	NFκB Competitor Oligonucleotide (90270)	TFA Negative Control Probe (90271)	Nuclear Extract*	HeLa Whole Cell Extract (TNFα Treated) (90350)	Total Volume
<b>Transcription Factor Assay (Normal)</b>	48μL	1μL	–	–	1μL	–	50μL
<b>Transcription Factor Assay— Positive Control</b>	47μL	1μL	–	–	–	2μL	50μL
<b>Transcription Factor Assay— Specific Competitor Control</b>	46μL	1μL	2μL	–	1μL	–	50μL
<b>Transcription Factor Assay— Negative Control</b>	48μL	–	–	1μL	1μL	–	50μL

\* Nuclear Extract volume of a typical extract at 5 μg/μL. This amount will ultimately need to be determined by the end user dependent upon cell type and stimulation. Tests show that ng to μg levels of nuclear protein/well can be successfully assayed.

**a. Positive Control:**

The positive control assay utilizes TNFα treated HeLa whole cell extract and should be performed to ensure that the assay is performing correctly. Under normal conditions, this assay will result in signal from the sample well(s). It is not necessary to add the two control probes (the NFκB Competitor Oligonucleotide and the TFA Negative Control Probe) to the positive control extract, unless desired.

**b. Competitor Control:**

The specific NFκB Competitor Control oligonucleotide is provided to ensure that the NFκB complex is binding the probe DNA in a sequence specific manner. It is an unlabeled competitor oligonucleotide containing the identical NFκB consensus sequence as the capture probe. This control will compete with the capture probe for NFκB binding. With a typical nuclear extract, this assay setup should greatly diminish the signal intensity. However, assays may be performed using varying amounts of the NFκB capture probe and/or specific competitor oligonucleotide as desired. Alternatively, the investigator may choose to incorporate other competitor oligonucleotides into the assay. *Keep in mind that any increase in volume of added competitor oligonucleotide must*

*be compensated for by an adjustment in the amount of 1x Enhanced TFA Buffer in the assay setup such that the final volume equals 50 $\mu$ L.*

**c. Negative Control:**

The TFA Negative Control Probe is used without the addition of the NF $\kappa$ B Capture Probe. The TFA Negative Control Probe is used to ensure that the signal obtained from the normal assay setup is specific to NF $\kappa$ B binding to its consensus sequence. Any signal obtained with this assay setup will reflect non-specific binding of NF $\kappa$ B to the capture probe.

3. Rotate sample cocktail for 1 hour at room temperature

*Note: Depending upon cell treatment and nuclear extraction, incubation time may be shortened. Optimal incubation time should be determined by the end user.*

4. Transfer the entire 50  $\mu$ L reaction to an individual well on the plate.  
(Or 50  $\mu$ L per well when running samples in multiplex format.)
5. Incubate for 1 hour at room temperature.

*Note: While our best results were obtained with a one hour incubation, shorter incubation times may be sufficient. Optimal incubation time should be determined by the end user.*

6. Wash wells three times with 150  $\mu$ L of the 1x Enhanced TFA Buffer. Incubate each wash as follows: 1<sup>st</sup> wash, 30 seconds; 2<sup>nd</sup> wash, 2 minutes; 3<sup>rd</sup> wash, 4 minutes. Completely remove the buffer from the wells following the final wash.

**D. Primary Antibody Incubation**

1. Dilute primary antibody in 1x Enhanced Transcription Factor Assay Buffer.
  - a. Prepare enough for 100  $\mu$ L of diluted antibody per well tested.
  - b. Dilute anti-NF $\kappa$ B p65 1:1000.
2. Add 100  $\mu$ L of diluted primary antibody to each assay well. Incubate for 60 minutes at room temperature.
3. Wash wells three times with 150  $\mu$ L of the 1x Enhanced Transcription Factor Assay Buffer (as in step A.6.). Incubate each wash as follows: 1<sup>st</sup> wash, 30 seconds; 2<sup>nd</sup> wash, 2 minutes; 3<sup>rd</sup> wash, 4 minutes. Completely remove the buffer from the wells following the final wash.

### E. Secondary Antibody Incubation

1. Dilute the anti-rabbit IgG-HRP conjugated secondary antibody in 1x Enhanced Transcription Factor Assay Buffer at a 1:1000 dilution.
  - a. Prepare enough for 100  $\mu$ L of diluted secondary antibody per assay well tested.
2. Add 100  $\mu$ L of the diluted secondary antibody to each well and incubate at room temperature for 30 minutes.

*Note: At this time the Chemiluminescent Detection Reagent and Reaction Buffer solutions should be removed from 2-8°C and allowed to equilibrate to room temperature during this 30 minute incubation.*

3. Wash wells four times with 250  $\mu$ L of 1x Transcription Factor Assay Buffer (**WITHOUT BLOCKING REAGENT**) per well. Incubate each wash as follows: 1<sup>st</sup> wash, 30 seconds; 2<sup>nd</sup> wash, 2 minutes; 3<sup>rd</sup> wash, 3 minutes; 4<sup>th</sup> wash, 4 minutes.

*Note: Using 1x Enhanced TFA Buffer, which contains Blocking Reagent, in this step will increase background signal.*

### F. Chemiluminescent Development

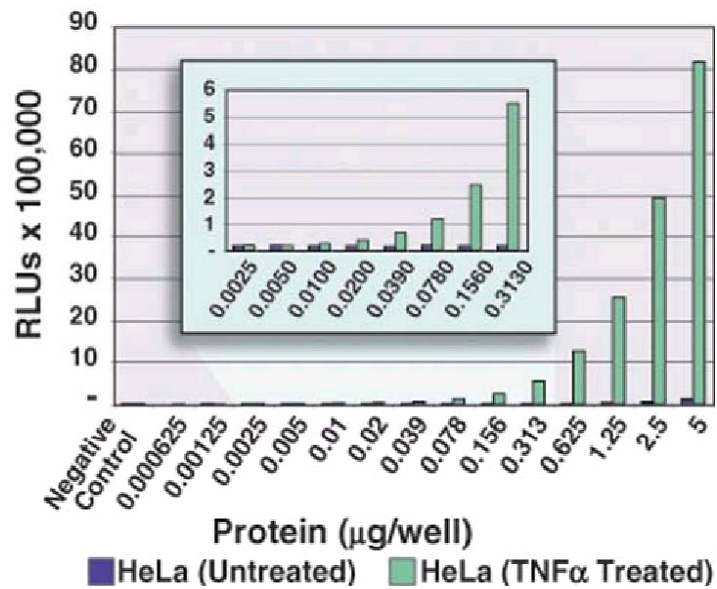
1. Add 1 part Chemiluminescent Detection Reagent to 2 parts Chemiluminescent Reaction Buffer making enough to add 100  $\mu$ L/well.
2. Add 100  $\mu$ L of the pre-mixed chemiluminescent substrate solution to each assay well being tested and incubate at room temperature for approximately 5-10 minutes. The development time may vary according to laboratory conditions.
3. Measure the absorbance of samples according to the directions given with the microplate luminometer (typically 0.2-1.0 seconds integration time) or the CCD camera coupled imaging device. The plate can be read multiple times over a 60-minute period with little change in the signal.

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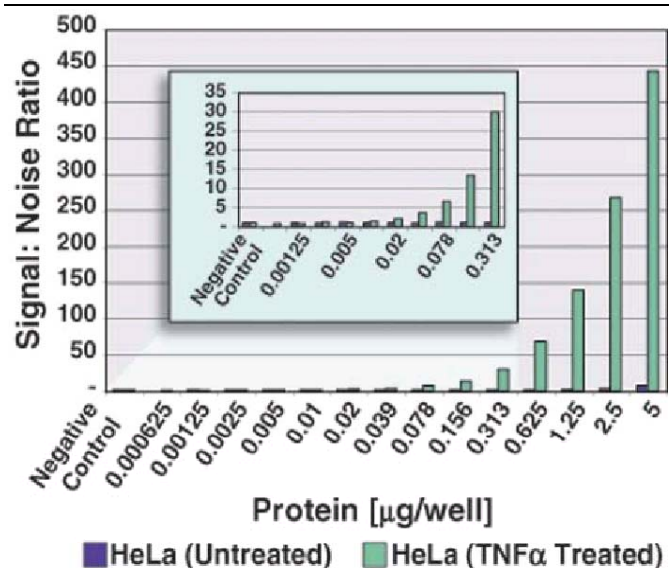
## Calculation of Results

Relative Light Unit (RLU) values obtained using CHEMICON's<sup>®</sup> Transcription Factor Assay Kit may be compared with known standards or other test samples to obtain relative activities. The following graphs represent unstimulated or TNF $\alpha$ -stimulated HeLa nuclear extracts. The data below is for reference use only and the data should not be used to interpret actual assay results.

Figure 1: Demonstration of the sensitivity of the Chemiluminescent NFκB p65 Transcription Factor Assay with lower limits of detection in the ng of nuclear protein/well quantities and the extreme dynamic range of 5 logs of magnitude of detection as demonstrated here assaying serial dilutions of untreated and TNFα-treated HeLa Cell nuclear extracts from 0.000625μg to 5 μg/well.



**Figure 2:** Demonstration of the clean signal: noise ratio obtained from the Chemiluminescent NF $\kappa$ B p65 Transcription Factor Assay as demonstrated here assaying serial dilutions of untreated and TNF $\alpha$ -treated HeLa Cell nuclear extracts from 0.000625 $\mu$ g/well to 5  $\mu$ g/well.



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## Troubleshooting

### 1. Weak or no signal in all wells

Possible explanations:

<u>Cause</u>	<u>Solution</u>
Omission of a reagent and/or step in the protocol	Make sure to follow the protocol accurately. Perform assay using the positive control extract.
Improper preparation or storage of a reagent	Make sure that all reagents are stored at their proper temperatures. Avoid repeat freeze/thawing of frozen materials.
Reagent Expired	Check the date on reagents to make sure they have not expired.
Insufficient cell extract per well resulting in low sample concentration	Perform cell extract titer to determine optimal concentration. The Enhanced Transcription Factor Buffer is tolerant of variations in sample volume.
Salt concentrations affecting DNA: protein binding	Reduce the amount of extract used in assay or reduce the amount of salt in extraction buffer. Alternatively, perform buffer exchange.
Plate reader not working well	Allow plate reader light to warm up. Check Plate reader.
Chemiluminescent Reagents too cold	Pre-warm the two individual components to room temperature.

## 2. High signal in all wells

Possible explanations:

<u>Cause</u>	<u>Solution</u>
Improper or inadequate washing of the wells	Follow the protocol as to the times and volume of each wash. Do not use 1x Enhanced Transcription Factor Assay Buffer for the final wash. 1x Transcription Factor Assay Buffer without Blocking Reagent should be used for the final wash.
Improper antibody dilution	Follow the product insert as to the proper antibody dilutions in the 1x Enhanced Transcription Factor Assay Buffer.
Signal Bleed Over	Adjust machine or space wells. This assay is very sensitive and can result in very intense signals. This can result in signal "bleeding" over into adjacent wells depending on the set up of the luminometer.

## 3. High background in sample wells

<u>Cause</u>	<u>Solution</u>
Sample concentration too high	Adjust the concentration by generating a dose response curve with your sample to determine proper concentration of sample to use.
Improper antibody dilution	Follow the product insert as to the proper antibody dilutions in the 1x Enhanced Transcription Factor Assay Buffer.

#### 4. Weak signal in sample wells

<u>Cause</u>	<u>Solution</u>
Sample concentration too low	Not enough cellular extract was used. Increase volume, or prepare fresh sample as weak signal can result from poor sample preparation.
Improper antibody dilution	Follow the product insert as to the proper antibody dilutions in the 1x Enhanced Transcription Factor Assay Buffer.

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#### References

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