INSTRUCTIONS

Transcription Factor Kits for NFκB p50 and p65

89858 89859

1421.5

Number	Description
89858	**NFκB p50 Transcription Factor Kit,** sufficient for two 96-well ELISA strip plates
89859	**NFκB p65 Transcription Factor Kit,** sufficient for two 96-well ELISA strip plates

Box 89858X, 89859X Contents:

Wild Type NFκB Competitor Duplex, 72µL, 10pmol/µL in 10mM Tris, 1mM EDTA; pH 7.5; store at -20°C

5′-CACAGTTGAG**GGGACTTTCC**CAGGC-3′
3′-GTGTCAACTC**CCCTGAAAGG**GTCCG-5′

Mutant NFκB Competitor Duplex, 72µL, 10pmol/µL in 10mM Tris, 1mM EDTA; pH 7.5; store at -20°C

5′-CACAGTTGAG**G***CC***ACTTTCC**CAGGC-3′
3′-GTGTCAACTC**C***GG***TGAAAGG**GTCCG-5′

5X NFκB Binding Buffer, 2 × 1.3mL, store at -20°C
20X Poly dI•dC, 700µL, 1mg/mL in 10mM Tris, 1mM EDTA; pH 7.5; store at -20°C
TNFα Activated HeLa Cell Nuclear Extract (positive control), 20µL, store at -70°C

Box 89858Y, 89859Y Contents:

96-well NFκB Assay Plate, two strip plates coated with NFκB consensus duplex, store at 4°C in foil pouch with desiccant
NFκB p50 (Box 89858Y) or p65 (Box 89859Y) Primary Antibody, 24µL, store at 4°C
HRP-conjugated Secondary Antibody, 20µL, store at 4°C
Antibody Dilution Buffer, 60mL, store at 4°C
10X Wash Buffer, 48mL, store at 4°C
Luminol/Enhancer Solution, 12mL, store at 4°C (1 year stability) or room temperature (6 months)
Stable Peroxide Solution, 12mL, store at 4°C (1 year stability) or room temperature (6 months)
Plate Sealer, 2 each

Storage: Upon receipt store individual components as indicated. The TNFα Activated HeLa Cell Nuclear Extract must be stored at -70°C immediately upon receipt. Box X is shipped on dry ice. Box Y is shipped with an ice pack.

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Introduction

The Thermo Scientific™ NFκB p50 and p65 Transcription Factor Kits are complete systems to measure active forms of these proteins. Each kit contains two streptavidin-coated 96-well plates with bound NFκB biotinylated-consensus sequence. Because only the active form of NFκB binds to the DNA sequence, nonspecific binding is minimized, providing greater signal-to-noise ratios than a traditional ELISA. The active transcription factor bound to the consensus sequence is incubated with specific primary antibody (NFκB p50 or p65) and then with a secondary HRP-conjugated antibody. Primary antibodies included in these kits have demonstrated reactivity to human, mouse, and rat samples. A chemiluminescent substrate that uses Thermo Scientific™ SuperSignal™ Technology is added to the wells and the resulting signal is detected using a luminometer or CCD camera.

The Rel/Nuclear Factor kappa B (NFκB) family of transcription factors is implicated in the regulation of genes involved in immune responses, developmental processes, inflammation and diseases such as cancer.[1-3] The Rel/NFκB proteins are structurally related and form a variety of homo- and heterodimers, with the p50/p65 heterodimer and p50 homodimers being the most common and well-characterized in non-lymphomyeloid cells. NFκB (p50 or p65) is sequestered in the cytoplasm in an inactive form bound to the inhibitor protein (IκB). Activation may occur by a variety of stimuli such as inflammatory cytokines, bacterial/viral products and agents, that cause cell damage. Upon stimulation, IκB is phosphorylated, ubiquitinated and degraded, enabling rapid translocation of NFκB into the nucleus where it regulates the expression of specific genes.[4]

Additional Materials Required

- Whole cell or nuclear extracts
- CCD camera or luminometer
- Orbital or plate shaker

Material Preparation

Working Binding Buffer: Prepare Working Binding Buffer as described in Table 1. Prepare just before use for each assay.

Table 1. Working Binding Buffer preparation.

Components	Volume per well	Volume per strip	Volume per 96-well plate
Ultrapure Water	45µL	360µL	4.32mL
5X NFκB Binding Buffer*	12µL	96µL	1.152mL
20X Poly dI•dC	3µL	24µL	288µL
Total Required	60µL	480µL	5.76mL

*The 5X NFκB Binding Buffer may not freeze while stored at -20°C

Wash Buffer: Dilute 10X Wash Buffer to 1X by adding 48mL of 10X Wash Buffer to 432mL of ultrapure water. Remove any precipitate in the 10X Wash Buffer by heating at 37°C until no precipitate remains. Store the 1X solution at 4°C for up to 1 year.

Procedure Summary for the Detection of Active NFκB p50 or p65

Note: This procedure is provided as a quick reference for experienced users. When initially performing the assay, follow the detailed protocol.

1. Prepare Working Binding Buffer as described in the Material Preparation section.
2. Add 50µL of Working Binding Buffer to each well.
3. (Optional) Add 4µL of Wild Type or Mutant Competitor Duplex.
4. Add Extract or positive control TNFα Activated HeLa Cell Nuclear Extract.
5. Incubate for 1 hour with mild agitation.
6. Wash three times with 200µLof 1X Wash Buffer.
7. Add 100µL of diluted Primary Antibody.
8. Incubate for 1 hour without agitation.
9. Wash three times with 200µL of 1X Wash Buffer.
10. Add 100µL of diluted Secondary Antibody.
11. Incubate for 1 hour without agitation.
12. Wash four times with 200µL of 1X Wash Buffer.
13. Add 100µL of chemiluminescent substrate.
14. Measure the chemiluminescence using a luminometer or a CCD camera system.

Detailed Procedure for the Detection of Active NFκB p50 or p65

A. Binding Active NFκB p50 or p65 Protein to the Consensus Sequence on the Assay Plate

1. Equilibrate the plate to room temperature before opening. Remove the plate from the foil bag and select the number of strips needed. Seal any remaining strips in the foil pouch with the desiccant provided and store at 4°C.
2. Prepare the Working Binding Buffer as described in the Material Preparation Section.
3. Add 50µL of Working Binding Buffer to each well.
4. (Optional) Add competitor duplex to the appropriate wells.
   - **Wild Type NFκB Competitor Duplex wells:** Add 4µL (40pmol) of Wild Type NFκB Competitor Duplex; then add the desired amount of extract. **Note:** Wild Type NFκB Competitor Duplex is provided to ensure signal specificity. Including Wild Type NFκB Competitor Duplex in the reaction will prevent NFκB from binding to the sequence attached to the plate and, therefore, eliminate specific signal. When using the Wild Type NFκB Competitor Duplex, it must be added before adding the extract.
   - **Mutant NFκB Competitor Duplex wells:** Add 4µL (40pmol) of Mutant NFκB Competitor Duplex; then add the desired amount of extract. **Note:** Mutant NFκB Competitor Duplex is provided to ensure signal specificity. Including Mutant NFκB Competitor Duplex will not affect specific NFκB binding or specific signal. When using the Mutant NFκB Competitor Duplex, it must be added before adding the extract.
5. Add cellular extract to the appropriate wells.
   - **Positive control wells:** Add 2µL of TNFα Activated HeLa Cell Nuclear Extract (optional positive control). The positive control is not intended for use as a standard.
   - **M-PER Extract sample wells:** If using extracts prepared with Thermo Scientific™ M-PER™ Mammalian Protein Extraction Reagent (Product No. 78501), add up to 25µL of extract.
   - **NE-PER Nuclear Extract sample wells:** If using extracts prepared with Thermo Scientific™ NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Product No. 78833), add up to 10µL of nuclear extract. Additional NE-PER Nuclear Extract may be added, but linear response decreases when greater than 10µL is used.
   - **RIPA extract sample wells:** If using extracts prepared with RIPA (Product No. 89901) extraction reagents, add up to 10 µl of extract. If using extracts prepared with other extraction reagents, use a negative control with an equal volume of cell lysis buffer without protein to ensure no interference occurs from the lysis buffer. High salt in the binding reaction may interfere with transcription factor binding.
   - **Blank wells:** Add neither extract nor competitor duplex. This is a negative control to establish baseline chemiluminescence.
6. Place the transparent plate sealer over the strips. Incubate with mild agitation for 1 hour at room temperature.

   Note: The assay will function without agitating the plate during transcription factor binding, but optimal performance is achieved when the plate is agitated. Approximately 50% signal reduction is observed without plate agitation.
7. Flick the strip contents into a sink and tap the plate 3-5 times on a stack of paper towels.
8. Wash plate three times with 200µL of 1X Wash Buffer. After each wash, flick the strip contents into a sink and tap the plate 3-5 times on a stack of paper towels.

B. Binding of the Primary Antibody

1. Dilute the Primary Antibody 1:1000 in Antibody Dilution Buffer by adding 1µL of Primary Antibody to 1mL of Antibody Dilution Buffer (Table 2). Each strip requires 1mL total.

Table 2. Primary Antibody dilution.

Components	Volume per strip	Volume per 96-well plate
Primary Antibody	1µL	12µL
Antibody Dilution Buffer	1mL	12mL
Total Required	1mL	12mL

2. Add 100µL of the diluted Primary Antibody to each well.
3. Incubate for 1 hour at room temperature without agitation.
4. Flick the strip contents into a sink and tap the plate 3-5 times on a stack of paper towels.
5. Wash the strips three times with 200µL of 1X Wash Buffer. After each wash, flick the strip contents into a sink and tap the plate 3-5 times on a stack of paper towels.

C. Binding of the Secondary Antibody

1. Dilute the Secondary Antibody 1:10,000 in Antibody Dilution Buffer.
   - **For a Full Plate:** Add 1.2µL Secondary Antibody to 12mL of Antibody Dilution Buffer.
   - **For a Partial Plate:** Dilute the Secondary Antibody in two steps. First dilute the Secondary Antibody 1:500 in Antibody Dilution Buffer by adding 1µL Secondary Antibody to 500µL Antibody Dilution Buffer. Second, make an additional 20-fold dilution using Table 3 as a guideline (each strip requires 1mL total).

Table 3. Final dilution of Secondary Antibody for partial plate

Components	Volume per strip
1:500 Secondary Antibody	50µL
Antibody Dilution Buffer	950µL
Total Required	1mL

2. Add 100µL of the diluted Secondary Antibody (1:10,000) to each well.
3. Incubate for 1 hour at room temperature without agitation.
4. Flick the strip contents into a sink and tap the plate 3-5 times on a stack of paper towels.
5. Wash the strips four (4) times with 200µL of 1X Wash Buffer. After each wash, flick the strip contents into a sink and tap the plate 3-5 times on a stack of paper towels.

D. Chemiluminescent Detection

1. Prepare the substrate working solution by combining equal amounts of Luminol/Enhancer Solution and Stable Peroxide Solution. Each strip requires 0.5mL of each reagent (1mL total volume of the substrate working solution).

   Note: The substrate working solution is stable for 8 hours at room temperature. Exposure to the sun or any other intense light can harm the working solution. For best results keep the working solution in an amber bottle and avoid prolonged exposure to any intense light. Typical laboratory lighting will not harm the working solution.

2. Add 100µL of the substrate working solution to each well.
3. Measure the chemiluminescence using a luminometer or a CCD camera system.

   Note: If using a luminometer, refer to the instrument manual for optimal settings. If using a CCD camera system, be aware that longer exposures increase background signal disproportionately to specific signal. Expose plate to the CCD camera for as short a time as possible to avoid background signal and signal saturation. Exposure to film is **not** recommended and may result in all wells producing the same strong signal on the film. Preventing qualitative or quantitative distinctions between different samples.

   Note: The amount of chemiluminescent signal decreases with time (Figure 1); however, signal-to-noise ratios are stable for at least one hour (Figure 2).

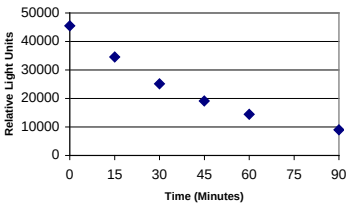

Figure 1. Signal decrease with time.

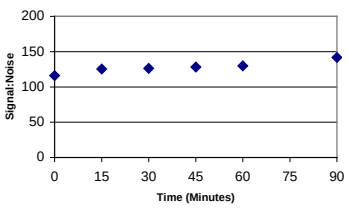

Figure 2. Signal-to-noise stability with time.

Troubleshooting

Problem	Cause	Solution
Weak or no signal	Omission of key reagent or step	Perform assay using positive control extract
	Insufficient cell extract per well	Perform cell extract titration to determine optimal concentration
	Poor activation or inactivation of NFκB	Perform activation time course to determine optimal activation time
	Cell extract is not of human, mouse or rat origin	NFκB p50 and p65 antibody detects human, mouse and rat protein
	Luminometer settings are not optimal	Refer to luminometer manual
	Salt concentration too high in binding reaction	Reduce amount of extract per well
		Reduce salt in extraction buffer
High background	Luminometer settings are not optimal	Refer to luminometer manual
	CCD camera exposure is too long	Reduce exposure to avoid increased background signal levels **Note:** Longer exposures increase background signal disproportionately to specific signal
	Inadequate washing	Wash as described in protocol

Related Thermo Scientific Products

20148 **LightShift™ Chemiluminescent EMSA Kit,** sufficient components for 100 binding reactions and detection reagents for ~800cm^2 of membrane

23235 **Micro BCA™ Protein Assay Reagent,** sufficient reagents to perform 480 standard tube assays or 3200 microplate assays

78501 **M-PER Mammalian Protein Extraction Reagent,** 250mL

78833 **NE-PER Nuclear and Cytoplasmic Extraction Reagents**

References

1. Karin, M., *et al.* (2002). NF-kappaB in cancer: from innocent bystander to major culprit. *Nature Rev Cancer* **2(4):**301-10.
2. Karin, M. and Lin, A. (2002). NF-κB at the crossroads of life and death. *Nature Immunol* **3(3):**221-7.
3. Baldwin, A.S., Jr. (1996). The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14:**649-83.
4. Thanos, D. and Maniatis, T. (1995). NF-κB: A lesson in family values. *Cell* **80:**529-32.