NF-κB (p65) Transcription Factor Assay Kit

Item No. 10007889

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA
**Materials Supplied**

Kit components may be stored at -20°C prior to use. For long-term storage, the Transcription Factor NF-κB (human p65) Positive Control should be thawed on ice, aliquoted at 25 µl/vial, and stored at -80°C. After opening the kit, we recommend each kit component be stored according to the temperature listed below.

<table>
<thead>
<tr>
<th>Item Number</th>
<th>Item</th>
<th>Quantity/Size</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>10006880</td>
<td>Transcription Factor Binding Assay Buffer (4X)</td>
<td>1 vial/3 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>10007472</td>
<td>Transcription Factor Reagent A</td>
<td>1 vial/120 µl</td>
<td>-20°C</td>
</tr>
<tr>
<td>10007924</td>
<td>Transcription Factor NF-κB (human p65) Positive Control</td>
<td>1 vial/150 µl</td>
<td>-80°C</td>
</tr>
<tr>
<td>10006882</td>
<td>Transcription Factor Antibody Binding Buffer (10X)</td>
<td>1 vial/3 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>409218</td>
<td>Transcription Factor NF-κB (p65) Primary Antibody</td>
<td>1 vial/120 µl</td>
<td>-20°C</td>
</tr>
<tr>
<td>400062</td>
<td>Wash Buffer Concentrate (400X)</td>
<td>1 vial/5 ml</td>
<td>RT</td>
</tr>
<tr>
<td>400035</td>
<td>Polysorbate 20</td>
<td>1 vial/3 ml</td>
<td>RT</td>
</tr>
<tr>
<td>10007884</td>
<td>Transcription Factor NF-κB Competitor dsDNA</td>
<td>1 vial/120 µl</td>
<td>-20°C</td>
</tr>
<tr>
<td>10006884</td>
<td>Transcription Factor Goat Anti-Rabbit HRP Conjugate</td>
<td>1 vial/120 µl</td>
<td>-20°C</td>
</tr>
<tr>
<td>10007882</td>
<td>Transcription Factor NF-κB 96-Well Strip Plate</td>
<td>1 plate</td>
<td>4°C</td>
</tr>
<tr>
<td>400012</td>
<td>96-Well Cover Sheet</td>
<td>1 cover</td>
<td>RT</td>
</tr>
<tr>
<td>10006888</td>
<td>Transcription Factor Developing Solution</td>
<td>1 vial/12 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>10006889</td>
<td>Transcription Factor Stop Solution</td>
<td>1 vial/12 ml</td>
<td>RT</td>
</tr>
</tbody>
</table>

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.
WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data
This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent via email to your institution.

Precautions
Please read these instructions carefully before beginning this assay.

If You Have Problems
Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888
Fax: 734-971-3640
E-Mail: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

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.

Materials Needed But Not Supplied
1. A plate reader capable of measuring absorbance at 450 nm
2. A source of pure water; glass Milli-Q or HPLC-grade water is acceptable
   NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000)
3. 300 mM Dithiothreitol (DTT)
4. Nuclear Extraction Kit available from Cayman (Item No. 10009277) or buffers for preparation of 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.
INTRODUCTION

Background

The NF-κB/Rel family of transcription factors is comprised of several structurally related proteins that form homodimers and heterodimers, including p50/p105, p52/p100, RelA/p65, c-Rel, and RelB. Members of this family are responsible for regulating over 150 target genes and the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules. In the canonical pathway of NF-κB activation, p65/p50 heterodimers are sequestered in the cytoplasm bound to IκBα. Upon stimulus, such as activation of TNF receptor 1 (TNFR1), IκBα is phosphorylated, ubiquitinated, and degraded, allowing the translocation of p65/p50 to the nucleus. There, the transcription factor binds to specific DNA sequences, called κB sites, and stimulates transcription of target genes. Since NF-κB is a powerful activator of proinflammatory transcriptional programs, its activity is tightly regulated. IκBα is a transcriptional target of p65/p50, resulting in a negative feedback loop that turns off NF-κB activation. The importance of NF-κB/Rel transcription factors in human inflammation and certain diseases makes them attractive targets for potential therapeutics.

About This Assay

Cayman’s NF-κB (p65) Transcription Factor Assay is a non-radioactive, sensitive method for detecting specific NF-κB (p65) DNA binding activity in nuclear extracts. It replaces the cumbersome radioactive electrophoretic mobility shift assay (EMSA) with an ELISA, as shown in Figure 1 on page 8. This kit is an ideal way to measure NF-κB transcriptional activity downstream of drug treatment or manipulation of cells in vitro or in vivo. Cayman’s NF-κB (p65) Transcription Factor Assay detects human, mouse, and rat NF-κB (p65). It does not cross react with NF-κB (p50).
Reagent Preparation

1. Transcription Factor Antibody Binding Buffer (1X) Preparation
Dilute the Transcription Factor Antibody Binding Buffer (10X) (ABB; Item No. 10006882) 1:10 by adding 27 ml of UltraPure water. Store at 4°C for up to six months.

2. Wash Buffer (1X) Preparation
Dilute the Wash Buffer (400X) (Item No. 400062) to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035). Scale as necessary. 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. Store at 4°C for up to two months.

3. Complete Transcription Factor Binding Assay Buffer Preparation
Prepare 10 ml of Complete Transcription Factor Binding Assay Buffer (CTFB) by adding 2.5 ml of Transcription Factor Binding Assay Buffer (4X) (Item No. 10006880), 0.1 ml of Transcription Factor Reagent A (Item No. 10007472), and 0.1 ml of 300 mM DTT to 7.3 ml of UltraPure water. Scale as necessary. It is recommended that the CTFB be used the same day it is prepared.

Figure 1. Schematic of the NF-κB (p65) Transcription Factor Binding Assay Kit
4. Transcription Factor NF-κB (human p65) Positive Control Preparation

Transcription Factor NF-κB (human p65) Positive Control (Item No. 10007924) contains 150 μl of clarified cell lysate. This lysate is provided as a positive control (PC) for NF-κB (p65) activation; it is not intended to be used as a standard for quantitative measurements. The positive control provided will produce a strong signal (>0.5 AU at 450 nm) when used at 10 μl/well. Serial two-fold dilutions of this PC can be used for monitoring the dynamic range of the assay. A decrease in signal may occur with repeated freeze/thaw cycles. It is recommended that the Transcription Factor NF-κB (human p65) Positive Control be aliquoted at 50 μl per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw cycles.

**Positive Control Dilution Set Up**

To prepare the PC for use in the ELISA: Obtain six clean test tubes and label them #PC1-PC6. Dilute 45 μl of Transcription Factor NF-κB (human p65) Positive Control with 405 μl of CTFB. This dilution is positive control 1 (PC1). Add 220 μl of CTFB to the tubes that correspond to PC2-PC6. Transfer 220 μl of the PC1 to tube PC2 and mix gently. Transfer 220 μl from PC2 to PC3 and mix gently. Repeat this process for the remaining tubes.

![Diagram of Positive Control Dilution Set Up](image)

Figure 2. Preparation of the NF-κB (human p65) positive controls
Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout for the PC1-PC6 serial dilutions and unknown samples of nuclear extracts (S1-S40) to be measured in duplicate is given below in Figure 3. We suggest you record the contents of each well on the template sheet provided (see page 22).

A suggested plate format is shown in Figure 3, below. The user may vary the location and type of wells present as necessary for each particular experiment.

---

<table>
<thead>
<tr>
<th>Plate Set Up</th>
</tr>
</thead>
<tbody>
<tr>
<td>There is no specific pattern for using the wells on the plate. A typical layout for the PC1-PC6 serial dilutions and unknown samples of nuclear extracts (S1-S40) to be measured in duplicate is given below in Figure 3. We suggest you record the contents of each well on the template sheet provided (see page 22). A suggested plate format is shown in Figure 3, below. The user may vary the location and type of wells present as necessary for each particular experiment.</td>
</tr>
</tbody>
</table>

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General Information

- Plate strips can be used in separate experiments if stored at 4°C properly in the resealable pouch.
- A minimum of two Blk, two zero wells, and two PC wells should be included in each assay.
- We recommend using Cayman’s Nuclear Extraction Kit (Item No. 10009277) for preparing your samples.

Performing the Assay

**Binding of active NF-κB (p65) 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.
2. Add the appropriate amount of reagents listed below to the designated wells as follows:
   - **Blk** - add 100 μl of CTFB to designated wells.
   - **Zero Well** - add 100 μl of CTFB to designated wells.
   - **PC1-PC6** - Add 100 μl of PC dilutions to the appropriate wells.
   - **S1-S40** - Add 90 μl of CTFB followed by 10 μl of sample to designated wells.
   - **Competitor (optional)** - Add 80 μl of CTFB prior to adding 10 μl of Transcription Factor NF-κB Competitor dsDNA (Item No. 10007884) to designated wells, followed by 10 μl of control cell lysate or sample.
3. Use the 96-Well Cover Sheet (Item No. 400012) provided to seal the plate. Incubate overnight at 4°C without shaking or one hour at room temperature on an orbital shaker.

4. Empty the wells and wash five times with 200 μl of Wash Buffer (1X). After the final wash, tap the plate on a paper towel to remove any residual Wash Buffer.

**Addition of Transcription Factor NF-κB (p65) Primary Antibody**

5. Dilute the Transcription Factor NF-κB (p65) Primary Antibody (Item No. 409218) 1:100 in ABB (1X). Add 100 μl to each well except the Blk wells.

6. Seal the plate with the cover sheet.
7. Incubate the plate for one hour at room temperature on an orbital shaker.
8. Empty the wells and wash each well five times with 200 μl of Wash Buffer (1X). After the final wash, 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**

9. Dilute the Transcription Factor Goat Anti-Rabbit HRP Conjugate (Item No. 10006884) 1:100 in ABB (1X). Add 100 μl to each well except the Blk wells.

10. Seal the plate with the cover sheet.
11. Incubate for one hour at room temperature on an orbital shaker.
12. Empty the wells and wash five times with 200 μl of Wash Buffer (1X). After the final wash, tap the plate three to five times on a paper towel to remove any residual wash buffer.

**Develop and Read the Plate**

13. Add 100 μl of Transcription Factor Developing Solution (Item No. 10006888), to each well.
14. Seal the plate with the cover sheet, and incubate the plate for 30 minutes at room temperature on an orbital shaker protected from light.
15. Remove cover sheet and add 100 μl of Transcription Factor Stop Solution (Item No. 10006889) per well. The solution within the wells will change from blue to yellow.
16. Read absorbance at 450 nm within five minutes of adding the Transcription Factor Stop Solution.
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. Add reagents to wells as indicated in Table 1.
2. Incubate overnight at 4°C without shaking or one hour at room temperature on an orbital shaker.
3. Wash each well five times with 200 μl of Wash Buffer (1X).
4. Add 100 μl of diluted Transcription Factor NF-kB (p65) Primary Antibody (1:100) per well (except Blk wells).
5. Incubate one hour at room temperature on an orbital shaker.
6. Wash each well five times with 200 μl of Wash Buffer (1X).
7. Add 100 μl of diluted Transcription Factor Goat Anti-Rabbit HRP Conjugate (1:100) (except Blk wells).
8. Incubate one hour at room temperature on an orbital shaker.
9. Wash each well five times with 200 μl of Wash Buffer (1X).
10. Add 100 μl of Transcription Factor Developing Solution per well.
11. Incubate 30 minutes at room temperature on an orbital shaker, protected from light.
12. Add 100 μl of Transcription Factor Stop Solution per well.
13. Read the absorbance at 450 nm.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blk</th>
<th>NSB</th>
<th>PC1-PC6</th>
<th>S1-S40</th>
<th>Competitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTFB</td>
<td>100 μl</td>
<td>100 μl</td>
<td>90 μl</td>
<td>80 μl</td>
<td></td>
</tr>
<tr>
<td>Positive Control Dilutions</td>
<td>100 μl</td>
<td></td>
<td>10 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samples</td>
<td>100 μl</td>
<td></td>
<td>10 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Competitor dsDNA</td>
<td>10 μl</td>
<td></td>
<td>10 μl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Plate Set Up Summary
**Interferences**

The following reagents were tested for interference in the assay.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Will Interfere (Yes or No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA (≤1 mM)</td>
<td>No</td>
</tr>
<tr>
<td>EDTA (≤0.5 mM)</td>
<td>No</td>
</tr>
<tr>
<td>ZnCl (any concentration)</td>
<td>Yes</td>
</tr>
<tr>
<td>DTT (between 1 and 5 mM)</td>
<td>No</td>
</tr>
<tr>
<td>Dimethylsulfoxide (≤1.5%)</td>
<td>No</td>
</tr>
</tbody>
</table>

**Troubleshooting**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Causes</th>
<th>Recommended Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>No signal or weak signal in control wells</td>
<td>A. Omission of key reagent</td>
<td>A. Check that all reagents have been added and in the correct order; perform the assay using the positive control</td>
</tr>
<tr>
<td></td>
<td>B. Plate reader settings not correct</td>
<td>B. Check wavelength setting on plate reader and change to 450 nm</td>
</tr>
<tr>
<td></td>
<td>C. Reagent expired</td>
<td>C. Check kit expiration date</td>
</tr>
<tr>
<td></td>
<td>D. Developing reagent used cold</td>
<td>D. Prewarm the developing solution to room temperature prior to use</td>
</tr>
<tr>
<td>High signal in all wells</td>
<td>A. Incorrect dilution of antibody</td>
<td>A. Check antibody dilutions and use amounts outlined in instructions</td>
</tr>
<tr>
<td></td>
<td>B. Improper/inadequate washing of wells</td>
<td>B. Follow the protocol for washing wells using the correct number of times and volumes</td>
</tr>
<tr>
<td></td>
<td>C. Overdeveloping</td>
<td>C. Decrease the incubation time when using the developing reagent</td>
</tr>
<tr>
<td>High background (Zero Values)</td>
<td>Incorrect dilution of antibody</td>
<td>Check antibody dilutions and use amounts outlined in the instructions</td>
</tr>
<tr>
<td>Problem (cont.)</td>
<td>Possible Causes (cont.)</td>
<td>Recommended Solutions (cont.)</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Weak signal in sample wells</td>
<td>A. Sample concentration too low</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>B. Incorrect dilution of antibody</td>
<td>B. Check antibody dilutions and use amounts outlined in the instructions</td>
</tr>
<tr>
<td></td>
<td>C. Salt concentrations affecting binding between DNA and protein</td>
<td>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)</td>
</tr>
</tbody>
</table>

References

Warranty and Limitation of Remedy

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