NF-κB (p65) Transcription Factor Assay Kit

Item No. 10007889

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Customer Service 800.364.9897
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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

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.

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

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.[1] 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.[3] 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.[4-6]

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).

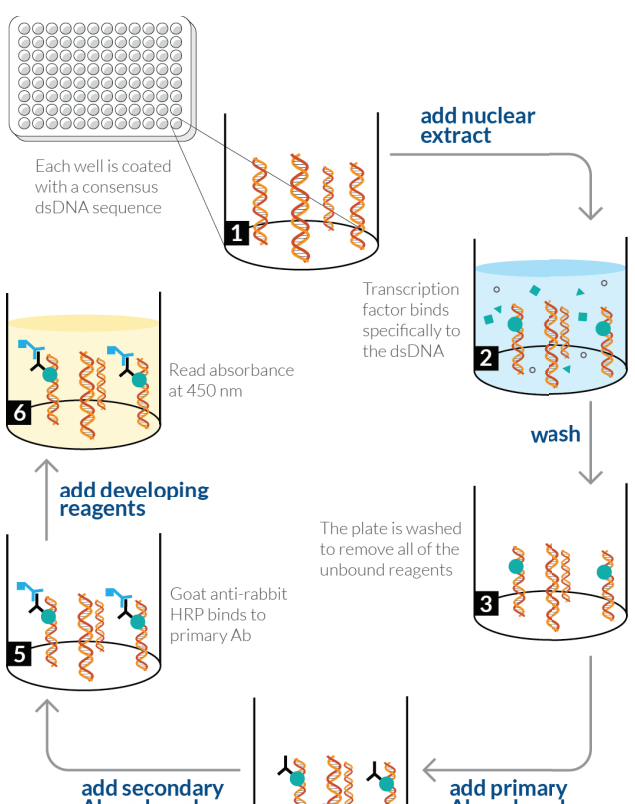

Figure 1. Schematic of the NF-κB (p65) Transcription Factor Binding Assay Kit

PRE-ASSAY PREPARATION

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.*

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.

ASSAY PROTOCOL

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.

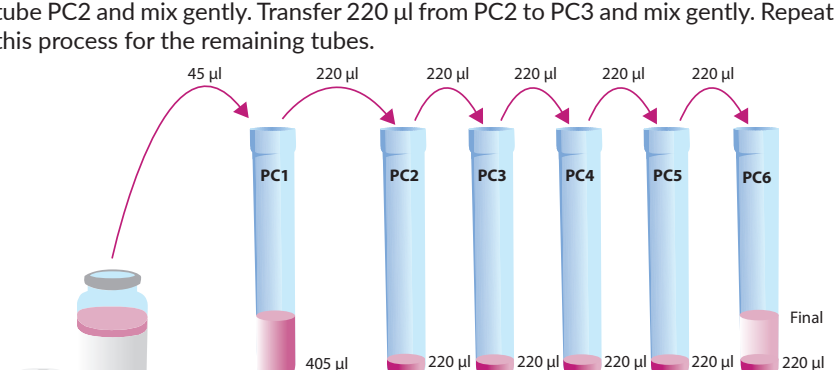

Figure 2. Preparation of the NF-κB (human p65) positive controls

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.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC1	PC1	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	PC2	PC2	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	PC3	PC3	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	PC4	PC4	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	PC5	PC5	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	PC6	PC6	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	0	0	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	Blk	Blk	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

Blk - Blank Wells
0 - Zero Wells
PC1-PC6 - Positive Control Wells
S1-S40 - Sample Wells

Figure 3. Sample plate format

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-κB (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.

Reagent	Blk	NSB	PC1-PC6	S1-S40	Competitor
CTFB	100 µl	100 µl		90 µl	80 µl
Positive Control Dilutions			100 µl		10 µl
Samples				10 µl	
Competitor dsDNA					10 µl

Table 1. Plate Set Up Summary

ANALYSIS

Performance Characteristics

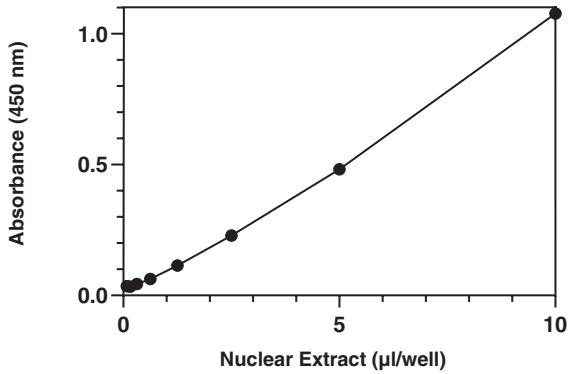

Figure 4. Typical data generated using Transcription Factor NF-kB (human p65) Positive Control

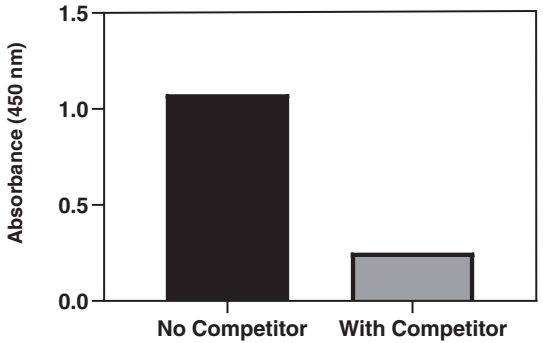

Figure 5. Typical decrease in absorbance of positive control by dsDNA competitor (Item No. 10007884)

RESOURCES

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 control wells	A. Omission of key reagent B. Plate reader settings not correct C. Reagent expired D. Developing reagent used cold	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 kit expiration date D. Prewarm the developing solution to room temperature prior to use
High signal in all wells	A. Incorrect dilution of antibody B. Improper/inadequate washing of wells C. Overdeveloping	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 (Zero Values)	Incorrect dilution of antibody	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 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 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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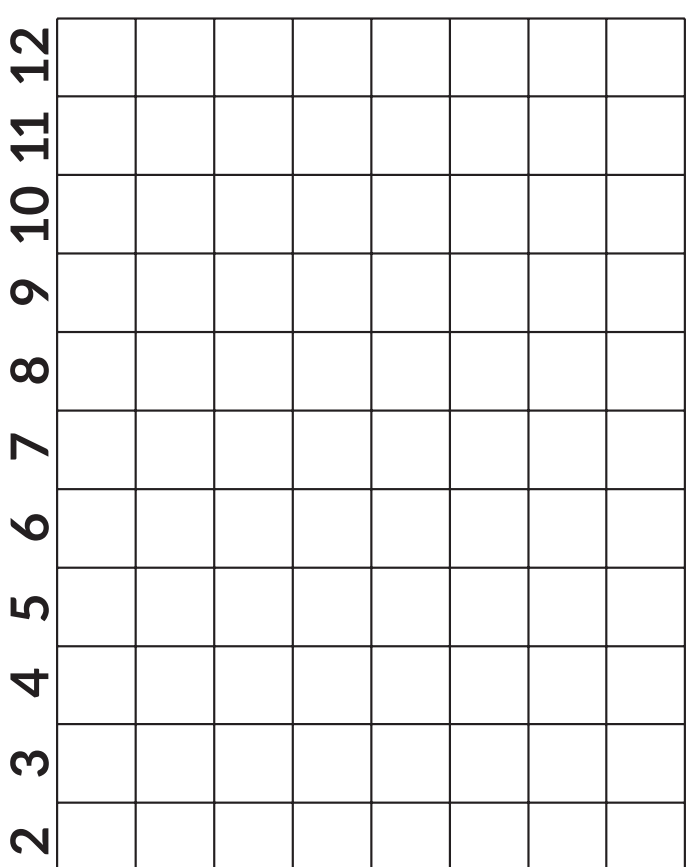
2. Pahl, H.L. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* **18**, 6853-6866 (1999).
3. Karin, M. The beginning of the end: IκB kinase (IKK) and NF-κB activation. *J. Biol. Chem.* **274(39)**, 27339-27342 (1999).
4. Gilroy, D.W., Lawrence, T., Perretti, M., *et al.* Inflammatory resolution: New opportunities for drug discovery. *Nature Reviews* **3(5)**, 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(5710)**, 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).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.