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Protocol

NF-κB (p65) Transcription Factor Assay Kit

Rockland's NF-κB (p65) Transcription Factor Assay (KAA065) 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. NF-κB contained in a nuclear extract specifically binds to the NF-κB response element. NF-κB (p65) is detected by addition of a specific primary antibody directed against NF-κB (p65). NF-κB (p65) is detected by addition of a specific primary antibody directed against NF-κB (p65). A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. Rockland's NF-κB (p65) Transcription Factor Assay detects human NF-κB (p65). It will not cross-react with NF-κB (p50).

I. Kit Components

Product	Preparation	Storage
Transcription Factor Binding Assay Buffer (4X)	Prepare TFB immediately prior to use in 1.5 mL centrifuge tubes or 15 mL conical tubes as outlined in Table 1. This buffer is then referred to as Complete Transcription Factor Binding Assay Buffer (CTFB). **Note:** It is recommended that the CTFB be used the same day it is prepared.	4°C
Transcription Factor Reagent A		-20°C
Transcription Factor NF-κB (p65) Positive Control[1]		-80°C
Transcription Factor Antibody Binding Buffer (10X)	For preparing 1X ABB, dilute to 1X by adding 27 mL of UltraPure water.	4°C (up to 2 months)
Transcription Factor NF-κB (p65) Primary Antibody		-20°C
Wash Buffer Concentrate (400X)[2]	Dilute the contents of the vial to a total volume of 2 liters with UltraPure water and add 1 mL of Tween 20.	4°C (up to 2 months)
TWEEN 20		RT
Transcription Factor NF-κB Specific Competitor dsDNA		-20°C
HRP Goat Anti-Rabbit Secondary Antibody Conjugate		-20°C
Transcription Factor NF-κB 96-well Plate		4°C
Plate Cover		N/A
Transcription Factor Developing Solution		4°C
Transcription Factor Stop Solution		4°C

[1]This lysate is provided as a positive control for NF-κB (p65) activation; it is not intended for plate-to-plate comparisons. The cell lysate 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 control, a decrease in signal may occur with repeated freeze/thaw cycles. It is recommended that the clarified cell lysate be aliquoted at 20 µL per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw.

[2]Tween 20 is a viscous liquid and cannot be measured by a pipet. 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°C for up to two months.

II. Reagents Required but Not Provided

Product	Preparation	Storage
10X PBS	a. Dissolve 80 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 800 ml distilled H_20. b. Adjust pH to 7.4 with HCl. c. Adjust volume to 1 L with H_2O.	
1X PBS	Add 100 mL of 10X stock into 900 mL distilled H_20	
Phosphatase Inhibitor Solution (50X)	• 1 M NaF • 0.05 M β-glycerophosphate • 0.05 M Na3VO4	-80°C
PBS/Phosphatase Inhibitor Solution	a. Add 250 µL of 50X Phosphatase Inhibitor Solution to 10 mL of 1X PBS b. Mix well and keep on ice	Make fresh daily
Hypotonic Buffer (pH 7.5)	• 20 mM HEPES (pH 7.5) • 5 mM NaF • 10 µM Na_2MoO_4 • 0.1 mM EDTA	4°C
Extraction Buffer	• 10 mM HEPES (pH 7.9) • 0.1 mM EDTA • 1.5 mM $MgCl_2$ • 420 mM NaCl • 0.5 mM DTT • 0.5 mM PMSF • µg/ml Pepstatin A • µg/ml Leupeptin • 10 µg/mL Aprotinin • 20 mM NaF • 1 mM ß-glycerophosphate • 10 mM Na_3VO_4 25% glycerol (v/v)	This buffer cannot be stored and must be made fresh on the day of use.

III. Additional Supplies

- A plate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes and a repeat pipettor
- A source of UltraPure water. Deionized or HPLC-grade water is acceptable
- 300 mM dithiothreitol (DTT)

- Buffers for preparation of nuclear extracts.
- Nuclear extracts

Table 1. Preparation of Complete Transcription Factor Binding Assay Buffer

Component	Volume Required Per Well (µL)	Volume Required Per Strip (µL)	Volume Required Per 96-well Plate (µL)
UltraPure water	73	584	7,008
4X Transcription Factor Binding Assay Buffer	25	200	2,400
Transcription Factor Reagent A	1	8	96
300 mM DTT	1	8	96
Total	**100**	**800**	**9,600**

IV. Procedure for Purification of Cellular Nuclear Extract

This procedure 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 5 minutes at 4°C.
3. Discard the supernatant. Resuspend cell pellet in 5 mL of ice-cold PBS/Phosphatase Inhibitor Solution and centrifuge at 300 x g for 5 minutes at 4°C. Repeat one time.
4. Discard the supernatant. Add 500 µL ice-cold Hypotonic buffer.
5. Mix gently by pipetting and transfer resuspended pellet to pre-chilled 1.5 mL microcentrifuge tube.
6. Incubate cells on ice for 15 minutes allowing cells to swell.
7. Add 50 µL of 10% Nonidet P-40. Mix gently by pipetting.
8. Centrifuge for 30 seconds (pulse spin) at 4°C in a microcentrifuge.
9. Transfer the supernatant containing the cytosolic fraction to a new tube and store at -80°C.
10. Resuspend the pellet in 50 µL ice-cold extraction buffer (with protease inhibitors).
11. Vortex 15 seconds at highest setting then gently rock the tube on ice for 15 minutes using a shaking platform.
12. Vortex sample for 30 seconds at highest setting and gently rock for an additional 15 minutes.
13. Centrifuge at 14,000 x g for 10 minutes at 4°C. The supernatant contains the nuclear fraction. Aliquot to clean chilled tubes, flash freeze and store at -80°C. Avoid freeze/thaw cycles. The extracts are ready to use in assay.
14. Keep small aliquot of nuclear extract to quantify the protein concentration.

V. Procedure for Transcription Factor Binding Assay Method

Pipetting Tips

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps to maintain more precise times of incubation.

- Before pipetting each reagent, equilibrate the pipet tip in that reagent (i.e. fill the tip and expel the contents; repeat several times).
- Do not expose the pipet tip to the reagent(s) already in the well.
- 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 blanks (Blk), two non-specific binding (NSB), and two positive control wells be included.

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.
   Note: If you are not using all the strips at once, place the unused strips back in the plate packet and store at 2–4°C. Be sure that the packet is sealed with the desiccant inside.
2. Prepare the complete transcription factor buffer (CTFB) as outlined in Table 2 (page 5).
3. Add appropriate amount of reagent(s) listed below to the designated wells as follows:
   - Blank wells (Blk): add 100 µL of CTFB to designated wells.
   - Non-specific Binding wells (NSB): add 100 µL of CTFB to designated wells. Do not add NF-κB (p65) to these wells.
   - Competitor wells (C1): Add 80 µL of CTFB prior to adding 10 µL of competitor dsDNA to designated wells. Add 10 µL of control cell lysates or unknown sample.
     Note: Competitor dsDNA must be added prior to adding the positive control or nuclear extracts.
   - Sample wells (U1-U44): Add 90 µL of CTFB prior to adding 10 µL of Nuclear extract to designated wells. A protocol for isolation of nuclear extracts is given on page 4-5.
   - Positive Control wells (PC): Add 90 µL of CTFB prior to adding 10 µL of positive control to appropriate wells.
4. Use the cover provided to seal the plate. Incubate overnight at 4°C or 1 hour at room temperature without agitation (incubation for one hour will result in a less sensitive assay).
5. Empty the wells and wash 5 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 Anti-NF-κB (p65) Primary Antibody

1. Dilute the Anti-NF-κB (p65) antibody 1:100 in 1X antibody binding buffer (ABB) as outlined in Table 2 below. Add 100 µL of diluted Anti-NF-κB (p65) antibody to each well except the Blank (Blk) wells.

Table 2. Dilution of Primary Antibody

Component	Volume Required Per Well (µL)	Volume Required Per Strip (µL)	Volume Required Per 96-well Plate (µL)
1X ABB	99	792	9,504
Anti-NF-κB (p65) Primary Antibody	1	8	96
Total	**100**	**800**	**9,600**

2. Use the adhesive cover provided to seal the plate.
3. Incubate the plate for 1 hour at room temperature without agitation.

4. Empty the wells and wash each well 5 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 3 to 5 times on a paper towel to remove any residual wash buffer.

Addition of HRP Goat anti-Rabbit conjugated Secondary Antibody

1. Dilute the HRP-conjugated secondary antibody (kit component #7) 1:100 in 1X Antibody binding buffer (ABB) as outlined in Table 4 below. Add 100 µL antibody to each well except the Blank (Blk) wells.

Table 3. Dilution of Secondary Antibody

Component	Volume Required Per Well (µL)	Volume Required Per Strip (µL)	Volume Required Per 96-well Plate (µL)
1X ABB	99	792	9,504
HRP Goat anti-Rabbit	1	8	96
Total	**100**	**800**	**9,600**

2. Use the adhesive cover provided to seal the plate.
3. Incubate for 1 hour at room temperature without agitation.
4. Empty the wells and wash 5 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 3 to 5 times on a paper towel to remove any residual wash buffer.

Develop and Read the Plate

1. To each well, add 100 µL of developing solution, which has been equilibrated to room temperature.
2. Incubate the plate for 15–45 minutes at room temperature with gentle agitation protected from light. Allow the wells to turn medium to dark blue prior to adding stop solution. Taking absorbance measurements at 655 nm prior to stopping the reactions can monitor this reaction. An OD655 of 0.4–0.5 yields an OD450 of approximately 1.0. Monitor development of sample wells to ensure adequate color development prior to stopping the reaction.
   Note: Do not overdevelop the plate. However positive control wells (only) 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 5 minutes of adding the stop solution. Blank the plate reader according to the manufacturer's requirements using the blank wells.

VI. 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 Complete Transcription Factor Binding Buffer (CTFB) as described in the Pre-Assay Preparation section, Table 2.
2. Add 90 µL CTFB per well (80 µL if adding Competitive dsDNA, 100 µL to Blank (Blk) and non-specific binding (NSB) wells).
3. Add 10 µL of Competitive dsDNA (optional) to appropriate wells.
4. Add 10 µL of Positive Control to appropriate wells.

5. Add 10 µL of Sample containing NF-κB (p65) to appropriate wells.
6. Incubate overnight at 4°C without agitation.
7. Wash each well five times with 200 µL of 1X Wash Buffer.
8. Add 100 µL of diluted Anti-NF-κB (p65) antibody per well (except Blank wells).
9. Incubate 1 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 conjugated secondary antibody (except Blank wells).
12. Incubate 1 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 –45 min with gentle agitation.
16. Add 100 µL of Stop Solution per well.
17. Measure the absorbance at 450 nm.

Quick Protocol Guide (Steps 1-17 listed above)

Steps	Reagent	Blk (µL)	NSB (µL)	PC (µL)	C1 (µL)	U1-U44 (µL)
(1-5) Add reagents	CTFB	100	100	90	80	90
	Competitive dsDNA				10	
	Positive Control			10	10	
	Samples					10
(6) Incubate	Cover plate and incubate overnight at 4°C without agitation					
(7) Wash	Wash all wells five times					
(8) Add reagent	Primary Antibody		100	100	100	100
(9) Incubate	Cover plate and incubate 1 hour at room temperature without agitation					
(10) Wash	Wash all wells five times					
(11) Add Reagent	Goat Anti-Rabbit HRP		100	100	100	100
(12) Incubate	Cover plate and incubate 1 hour at room temperature without agitation					
(13) Wash	Wash all wells five times					
(14) Add Reagent	Developer	100	100	100	100	100
(15) Incubate	Monitor development in wells					
(16) Add Reagent	Stop Solution	100	100	100	100	100
(17) Read Plate	Read plate at wavelength of 450 nm					

Interferences

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 Guide

Issue	Cause	Solution
No signal or weak signal in all wells	Omission of key reagent	• Check that all reagents have been added and in the correct order. Perform the assay using the positive control • Check wavelength setting on plate reader and change to 450 nm • Check expiration date on reagents
	Plate reader settings not correct	
	Reagent/reagents expired	
	Salt concentrations affected binding between DNA and protein	• 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) • Prewarm the developing solution to room temperature prior to use. • Check pipettes to ensure correct amount of developing solution was added to wells
	Developing reagent used cold	
	Developing reagent not added at correct volume	
High signal in all wells	Incorrect dilution of antibody (too high)	• Check antibody dilutions and use amounts outlined in instructions • Follow the protocol for washing wells using the correct number of times and volumes • Decrease the incubation time when using the developing reagent
	Improper/inadequate washing of wells	
	Overdeveloping	
High background (NSB)	Incorrect dilution of antibody (too high)	Check antibody dilutions and use amounts outlined in the instructions
Weak signal in sample wells	Sample concentration is too low	• 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 • Check antibody dilutions and use amounts outlined in the instructions • 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)
	Incorrect dilution of antibody	
	Salt concentrations affecting binding between DNA and protein	

References

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