

**TransAM™**  
NFκB p65 / NFκB p50 /NFκB p52  
Transcription Factor Assay Kits

(version G3)

Catalog Nos. 40096 & 40596 (p65)

Catalog Nos. 41096 & 41596 (p50)

Catalog Nos. 48196 & 48696 (p52)

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## Overview

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The transcription factor NFκB (nuclear factor κB) is a key component for the inducible expression of a wide variety of cellular and viral genes. Therefore, accurate monitoring of NFκB activation in cells, tissues or animals is crucial for drug development and signal transduction pathway studies. To date, such research projects are time consuming, tedious and lack high-throughput screening systems.

With its patented TransAM™ method\*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM NFκB p65, p50 and p52 Kits are designed specifically for the study of NFκB subunits. They contain a 96-well plate to which oligonucleotide containing an NFκB consensus binding site has been immobilized. The activated NFκB homodimers and hetero-dimers contained in nuclear or whole-cell extracts specifically binds to this oligonucleotide. By using an antibody that is directed against either the NFκB p65, p50 or p52 subunit, the activated NFκB subunit bound to the oligonucleotide is detected. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout that is easily quantified by spectrophotometry. The 96-well plate with detachable wells is suitable for manual use or high-throughput screening applications. TransAM NFκB p65, p52 and NFκB p50 Kits are available in two sizes:

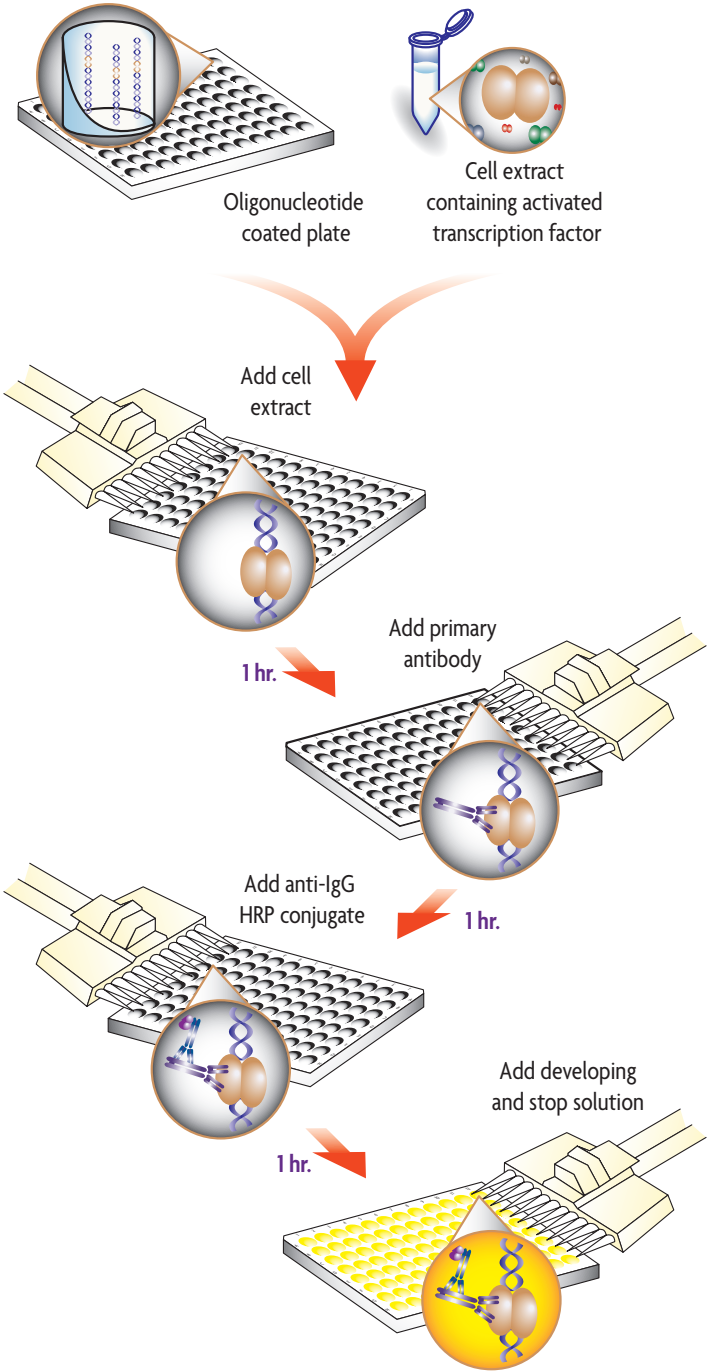
product	format	catalog no.
TransAM™ NFκB p65	1 x 96 rxns	40096
	5 x 96 rxns	40596
TransAM™ NFκB p50	1 x 96 rxns	41096
	5 x 96 rxns	41596
TransAM™ NFκB p52	1 x 96 rxns	48196
	5 x 96 rxns	48596

The procedure outlined in this manual can be used for TransAM NFκB p65, TransAM NFκB p50 or TransAM NFκB p52 Kits.

Active Motif also offers chemiluminescent versions of the TransAM NFκB p65, p50 and p52 Kits that provide increased sensitivity and dynamic range, as well as the TransAM NFκB/Rel Family Kit for the study of NFκB p50, p52, p65, c-Rel and RelB. See these and other Active Motif products related to the NFκB signaling pathway in Appendix, Section C.

\*Technology covered by AAT-filed patents and licensed to Active Motif. Use of TransAM 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).

# Flow Chart of Process



# Introduction

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## NFκB Transcription Factor

The transcription factor NFκB is implicated in the regulation of many genes that code for mediators of the immune, acute phase and inflammatory responses<sup>1</sup>. The DNA-binding protein complex recognizes a discrete nucleotide sequence (5'-GGGACTTCC-3') in the upstream region of a variety of cellular and viral response genes<sup>2</sup>. NFκB is composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family. There are five subunits of the NFκB family in mammals: p50, p65 (RelA), c-Rel, p52 and RelB3. These proteins share a conserved 300 amino acid sequence in the N-terminal region, known as the Rel homology domain, that mediates DNA binding, protein dimerization and nuclear localization. This domain is also a target of the IκB inhibitors, which include IκBα, IκBβ, IκBγ, Bcl-3, p105 and p1004. Various dimer combinations of the NFκB subunits have distinct DNA binding specificities and may serve to activate specific sets of genes such as adhesion molecules, immunoreceptors and cytokines. The p50/p65 (NFκB1/RelA) heterodimers and the p50 homodimers are the most common dimers found in the NFκB signaling pathway. In the majority of cells, NFκB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins<sup>5</sup>. Treatment of cells with various inducers results in the phosphorylation, ubiquitination and subsequent degradation of IκB proteins<sup>6</sup> (For studying the phosphorylation state of IκBα, see Active Motif's FunctionELISA™ IκBα Kit). Proteolytic cleavage of p105 results in two proteins: p50, which has DNA-binding activity but no transactivation domain, and its antagonist, the inhibitory IκBγ protein. This results in the release of NFκB dimers, which subsequently translocate to the nucleus, where they activate appropriate target genes. NFκB can be activated by a number of stimuli, including components of bacterial cell walls, such as lipopolysaccharide, or inflammatory cytokines, such as TNF-α or IL-1β.

## Transcription Factor Assays

To date, three methods are widely used to measure NFκB activation, either directly or indirectly:

1. NFκB expression or cytoplasmic IκB degradation can be measured by Western blot, using antibodies raised against NFκB subunits or IκB (see Appendix, Section C for related products). This method is time consuming (up to 2 days once the cell extracts are prepared), and is not suitable for processing large numbers of samples.
2. The DNA-binding capacity of NFκB can be assayed by gel retardation, also called electrophoretic mobility shift assay (EMSA). In this method, cell extracts are incubated with a radioactive double-stranded oligonucleotide probe containing the consensus sequence for NFκB binding. If NFκB is active in the cell extract, it will bind to the probe. Samples are then resolved by electrophoresis on a native polyacrylamide gel, followed by autoradiography. This method is sensitive, but like the previous procedure, it is time consuming (multiple days of gel exposure may be required to achieve sufficient sensitivity) and it cannot be applied to high-throughput screening. Gelshift assays also require special precautions and equipment for handling radioactivity.
3. Another method used to assay NFκB activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing the NFκB consensus sequence. This promoter can be artificial, made of several NFκB cis-elements

and a TATA box, or natural, like the HIV long terminal repeat (LTR) sequence. Limitations of this procedure are: (i) reporter gene assays have to be repeated several times to obtain statistically reliable data; and (ii) reporter gene assays are sensitive to confounding factors that may influence the expression level of the reporter gene. Therefore, assays have to be carefully standardized. This method is sensitive and easy to perform with a large number of samples but requires efficient cell transfection with the reporter plasmid.

### **TransAM NFκB**

NFκB is a key factor in various immune processes and represents an excellent pharmacological target. However, pharmaceutical research in this field has been hampered by the lack of convenient assays suitable for large numbers of samples.

To overcome this, Active Motif offers a high-throughput assay to quantify NFκB activation<sup>7</sup>. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM NFκB Kits contain a 96-well plate to which an oligonucleotide containing the NFκB consensus site (5'-GGGACTTCC-3') has been immobilized. The active form of NFκB contained in nuclear or whole-cell extract specifically binds to this oligonucleotide. The primary antibodies used to detect NFκB recognize an epitope on p65, p50 or p52 that is accessible only when NFκB is activated and bound to its target DNA. An HRP-conjugated secondary antibody provides a sensitive colorimetric readout that is easily quantified by spectrophotometry. Once the cellular extract is prepared, this assay is completed in less than 3.5 hours. As this assay is performed in a 96-well plate, a large number of samples can be handled simultaneously, allowing for high-throughput automation. This assay is specific for NFκB activation and has been shown to be 10-fold more sensitive and 40-fold faster than the gel retardation technique. With the 3.5-hour procedure of TransAM, we could detect NFκB activation with 0.5 μg of nuclear extract from Jurkat cells stimulated with TPA and calcium ionophore. A comparable assay using EMSA required 5 μg of extract and a 5-day autoradiography.

TransAM has many applications including the study of drug potency, inhibitor or activator proteins, and/or protein structure/function in the NFκB signaling pathway.

## Kit Performance and Benefits

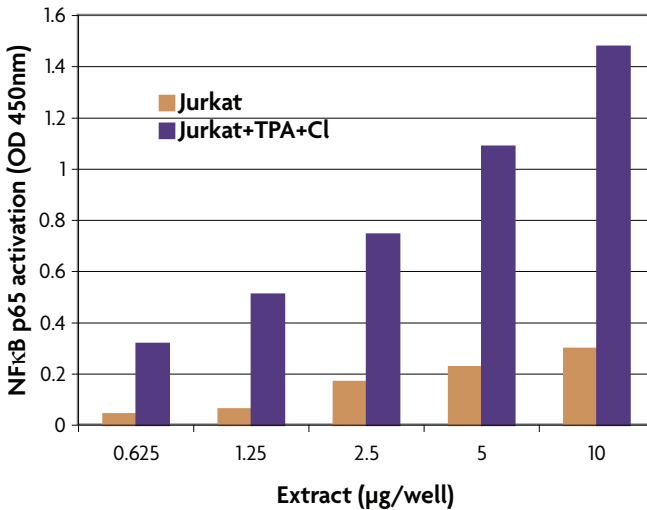
Detection limit: < 0.5 µg nuclear extract/well. For whole-cell extract, twice as much input may be needed. Alternatively, < 0.4 ng recombinant p50 or p65 protein/well may be used. TransAM NFκB is 10-fold more sensitive than EMSA. (For increased sensitivity, the TransAM NFκB p50 Chemi, TransAM NFκB p52 Chemi and TransAM NFκB p65 Chemi Kits utilize a chemiluminescent detection method that can detect 5 ng extract/well. See Appendix, Section C, Related Products.)

**Range of detection:** TransAM provides quantitative results from 0.2 to 10 µg of nuclear or whole-cell extract per well or 0.1 to 40 ng of recombinant p50 or p65 protein/well.

**Cross-reactivity:**

	p65	p50	p52
Human	+	+	+
Mouse	+	+	+
Rat	+	-	-

**Assay time:** 3.5 hours.



### Monitoring NFκB activation with the TransAM NFκB Kit:

Nuclear extracts from Jurkat cells (Catalog No. 36014) and Jurkat cells stimulated with TPA and calcium ionophore (Catalog No. 36013) were assayed from 0.625-10 µg/well for NFκB p65 activation using the TransAM NFκB p65 Kit. Data shown are the results from wells assayed in duplicate. These results are provided for demonstration only.



## Kit Components and Storage

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TransAM NFκB Kits are for research use only. Not for use in diagnostic procedures. Except for the nuclear extract that must be kept at -80°C, kit components can be stored at -20°C prior to first use. Then, we recommend storing each component at the temperature indicated in the table below. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity 1 plate / 5 plates	Storage
NFκB p52, p65 or p50 antibody	11 μl / 55 μl	-20°C for 6 months
Anti-rabbit HRP-conjugated antibody	11 μl / 55 μl	4°C for 6 months
Wild-type oligonucleotide AM20	100 μl / 500 μl (10 pmol/μl)	-20°C for 6 months
Mutated oligonucleotide AM20	100 μl / 500 μl (10 pmol/μl)	-20°C for 6 months
Positive control nuclear extract	40 μl / 200 μl (2.5 μg/μl)	-80°C for 6 months
Dithiothreitol (DTT) (1 M)	100 μl / 500 μl	-20°C for 6 months
Protease Inhibitor Cocktail	100 μl / 500 μl	-20°C for 6 months
Herring sperm DNA	100 μl / 500 μl (1 μg/μl)	-20°C for 6 months
Lysis Buffer AM2	10 ml / 50 ml	4°C for 6 months
Binding Buffer AM3	10 ml / 50 ml	4°C for 6 months
10X Wash Buffer AM2	22 ml / 110 ml	4°C for 6 months
10X Antibody Binding Buffer AM2	2.2 ml / 11 ml	4°C for 6 months
Developing Solution	11 ml / 55 ml	4°C for 6 months
Stop Solution	11 ml / 55 ml	4°C for 6 months
96-well NFκB assay plate	1 / 5	4°C for 6 months
Plate sealer	1 / 5	Room temperature

### Additional materials required

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform
- Nuclear or whole-cell extracts
- Microplate spectrophotometer capable of reading at 450 nm (655 nm as an optional reference wavelength)

### Buffer Preparation and Recommendations

#### Preparation of Complete Lysis Buffer

We provide an excess of Lysis Buffer AM2 in order to perform the assay AND to prepare customized nuclear extracts. Please refer to the Appendix Section A for a protocol to prepare a nuclear extract. Our Nuclear Extract Kit can also be purchased separately (Cat. Nos. 40010 & 40410). When preparing extracts, it is suggested to perform the final lysis step using the Lysis Buffer AM2 provided in the TransAM Kit. All subsequent dilutions should also be performed using Lysis Buffer AM2 from the TransAM Kit. Prepare the amount of Complete Lysis Buffer required for the assay by adding 5  $\mu$ l of 1 M DTT and 10  $\mu$ l Protease Inhibitor Cocktail per ml of Lysis Buffer AM2 (see the Quick Chart for Preparing Buffers in this section). Some of the protease inhibitors lose their activity after a few hours once diluted. Therefore, we recommend adding protease inhibitors immediately prior to use. Any remaining Complete Lysis Buffer should be discarded if not used in the same day.

#### Preparation of Complete Binding Buffer

Prepare the amount of Complete Binding Buffer AM3 required for the assay by adding 2  $\mu$ l of DTT and 10  $\mu$ l of 1  $\mu$ g/ $\mu$ l Herring sperm DNA per ml of Binding Buffer AM3 (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Complete Binding Buffer.

#### Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 100 ml of 1X Wash Buffer required, dilute 10 ml 10X Wash Buffer AM2 with 90 ml distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in the 10X Wash Buffer AM2 may form clumps, therefore it is necessary to completely resuspend any precipitates by incubating at 50°C for 2 minutes and mixing prior to use.

#### Preparation of 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer AM2 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section)\*. Mix gently to avoid foaming. Discard remaining 1X Antibody Binding Buffer after use. The BSA contained in the 10X Antibody Binding Buffer AM2 may form clumps, therefore it is necessary to completely resuspend the buffer by warming to room temperature and vortexing for 1 minute prior to use.

\* Volumes listed refer to the preparation of buffer for diluting both the primary & secondary antibodies.

## Developing Solution

The Developing Solution should be warmed to room temperature before use. The Developing Solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. A blue color present in the Developing Solution indicates that it has been contaminated and must be discarded. Prior to use, place the Developing Solution at room temperature for at least 1 hour. Transfer the amount of Developing Solution required for the assay into a secondary container before aliquoting into the wells (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Developing Solution.

## Stop Solution

Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Stop Solution.

**WARNING:** The Stop Solution is corrosive. Wear personal protective equipment when handling, i.e. safety glasses, gloves and labcoat.

## Nuclear extract

The Jurkat (TPA + CI) nuclear extract is provided as a positive control for NFκB p50 and p65 activation. This extract is optimized to give a strong signal when used at 2.5 μg/well. The Raji nuclear extract is provided as a positive control for NFκB p52 activation and should be used at 5 μg/well. We recommend aliquoting the extract in 5 μl fractions and storing at -80°C. Avoid multiple freeze/thaw cycles of the extract. Various cell extracts are available from Active Motif (see Appendix, Section C. Related Products).

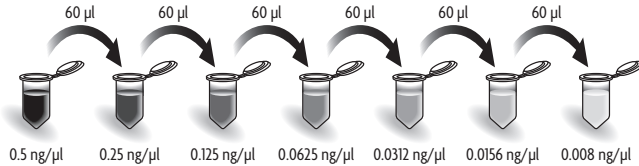
## Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for NFκB binding in order to monitor the specificity of the assay. This competition assay will confirm that the protein subunits binding to the plate are specific for the NFκB consensus binding sequence. Used at 20 pmol/well, the oligonucleotide will prevent NFκB binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on NFκB binding. Prepare the required amount of wild-type and/or mutated consensus oligonucleotide by adding 2 μl of appropriate oligonucleotide to 31.8 μl of Complete Binding Buffer per well being used (see the Quick Chart for Preparing Buffers in this section). To allow for optimum competition, add the oligonucleotide to the well first, then add the cell extract. It is not necessary to perform an incubation step of the oligonucleotide in the well prior to addition of the cell extract. The oligonucleotide competition only needs to be performed as a control. It is suggested to test the oligonucleotide competition each new cell type used.

## OPTIONAL – Preparation of standard curve

For those who wish to quantify the amount of NFκB in their samples, Active Motif offers recombinant NFκB p50 and p65 for use as protein standards (see Appendix, Section C. Related Products). Each protein is supplied with a lot specific Certificate of Analysis. Please refer to this before preparing the standard curve as the concentration of the protein varies from lot to lot.

1. Begin with a 100 ng/μl working stock of recombinant protein (use the TransAM Complete Lysis Buffer to dilute the protein). Set up a standard curve in duplicate using the following concentrations: 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.008 and 0 ng/μl. Note: The preceding range is provided as guidance, a broader range of values may be used. Refer to the protein's lot specific data sheet for details.
2. Make up a 0.5 ng/μl solution by adding 1 μl of the 100 ng/μl working stock to 199 μl of Complete Lysis Buffer. Next, pipette 60 μl of Complete Lysis Buffer into the 7 remaining tubes. Use the 0.5 ng/μl solution to prepare a dilution series as indicated below. Mix tubes thoroughly before each transfer. The 0.5 ng/μl standard is the high standard and Lysis Buffer alone is the 0.0 ng/μl.



3. 20 μl from each tube will be aliquoted to the wells in Step 1, No. 2 of the protocol and will correspond to the following quantities of NFκB: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.0 ng/well.

## Quick Chart for Preparing Buffers

Reagents to prepare	Components	For 1 well	For 1 strip (8 wells)	For 6 strips (48 wells)	For 12 strips (96 wells)
Complete Lysis Buffer	DTT	0.11 μl	0.9 μl	5.4 μl	10.8 μl
	Protease Inhibitor Cocktail	0.23 μl	1.8 μl	10.8 μl	21.6 μl
	Lysis Buffer AM2	22.2 μl	177.3 μl	1,064 ml	2,128 ml
	<b>TOTAL REQUIRED</b>	<b>22.5 μl</b>	<b>180 μl</b>	<b>1.08 ml</b>	<b>2.16 ml</b>
Complete Binding Buffer	DTT	0.07 μl	0.54 μl	3.2 μl	6.5 μl
	Herring sperm DNA	0.34 μl	2.7 μl	16.2 μl	32.4 μl
	Binding Buffer AM3	33.4 μl	267 μl	1.6 ml	3.2 ml
	<b>TOTAL REQUIRED</b>	<b>33.8 μl</b>	<b>270 μl</b>	<b>1.62 ml</b>	<b>3.24 ml</b>
Complete Binding Buffer with NFκB wild-type or mutated oligonucleotide	NFκB wild-type or mutated oligo	2 μl	16 μl	96 μl	N/A
	Complete Binding Buffer	31.8 μl	254 μl	1,524 ml	N/A
	<b>TOTAL REQUIRED</b>	<b>33.8 μl</b>	<b>270 μl</b>	<b>1.62 ml</b>	<b>N/A</b>
1X Wash Buffer	Distilled water	2,025 ml	16.2 ml	97.2 ml	194.4 ml
	10X Wash Buffer AM2	225 μl	1.8 ml	10.8 ml	21.6 ml
	<b>TOTAL REQUIRED</b>	<b>2.25 ml</b>	<b>18 ml</b>	<b>108 ml</b>	<b>216 ml</b>
1X Antibody Binding Buffer*	Distilled water	202.5 μl	1.62 ml	9.72 ml	19.44 ml
	10X Ab Binding Buffer AM2	22.5 μl	180 μl	1.08 ml	2.16 ml
	<b>TOTAL REQUIRED</b>	<b>225 μl</b>	<b>1.8 ml</b>	<b>10.8 ml</b>	<b>21.6 ml</b>
Developing Solution	<b>TOTAL REQUIRED</b>	<b>112.5 μl</b>	<b>900 μl</b>	<b>5.4 ml</b>	<b>10.8 ml</b>
Stop Solution	<b>TOTAL REQUIRED</b>	<b>112.5 μl</b>	<b>900 μl</b>	<b>5.4 ml</b>	<b>10.8 ml</b>

\* Volumes listed refer to the preparation of buffer for diluting both the primary & secondary antibodies.

# NFκB Transcription Factor Assay

Determine the appropriate number of microwell strips required for testing samples, controls and blanks in duplicate. If less than 8 wells in a strip need to be used, cover the unused wells with a portion of the plate sealer while you perform the assay. The content of these wells is stable at room temperature if kept dry and, therefore, can be used later for a separate assay. Store the unused strips in the aluminum pouch at 4°C. Use the strip holder for the assay.

Prepare the Complete Lysis Buffer, Complete Binding Buffer, 1X Wash Buffer and 1X Antibody Binding Buffer as described above in the section Buffer Preparation and Recommendations. Multi-channel pipettor reservoirs may be used for dispensing the Complete Binding Buffer, Wash Buffer, Antibody Binding Buffer, Developing Solution and Stop Solution into the wells being used.

## Step 1: Binding of NFκB to its Consensus Sequence

1. Add 30 µl Complete Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 30 µl Complete Binding Buffer that contains 20 pmol (2 µl) of the wild-type or mutated oligonucleotide (see the Buffer Preparation section above for a description of competitive binding).
2. **Sample wells:** Add 20 µl of sample diluted in Complete Lysis Buffer per well. We recommend using 2-20 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts is provided on page 13. For whole-cell extracts we recommend using 4-40 µg per well.

**Positive control wells:** For p65 or p50 add 2.5 µg of the provided Jurkat nuclear extract (1 µl of nuclear extract in 19 µl of Complete Lysis Buffer per well). For p52 use 5 µg of the provided Raji nuclear extract diluted in 20 µl of Complete Lysis Buffer per well (2 µl of nuclear extract in 18 µl of Complete Lysis Buffer per well).

**Blank wells:** Add 20 µl Complete Lysis Buffer only per well.

**OPTIONAL – Protein standard wells for p50 and p65:** Add 20 µl of the appropriate protein standard diluted in Complete Lysis Buffer to each well being used (see page 8, for details)

Reagents	Blank wells	Positive Control no competition	Sample no competition	(Optional) wild-type	(Optional) mutated	(Optional) Recombinant protein
Complete Binding Buffer	30 µl	30 µl	30 µl	28 µl	28 µl	30 µl
Wild-type oligonucleotide	–	–	–	2 µl	–	–
Mutated oligonucleotide	–	–	–	–	2 µl	–
Complete Lysis Buffer	20 µl	–	–	–	–	–
Sample in Complete Lysis Buffer	–	20 µl	20 µl	20 µl	20 µl	–
Recombinant protein sample	–	–	–	–	–	20 µl

3. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform). Keep any unused wells covered during the remaining steps in order to preserve those wells for future assays. Any unused strips from the stripwell plate can be placed in the foil bag, sealed with tape and stored at 4°C.

4. Wash each well 3 times with 200  $\mu$ l 1X Wash Buffer. For each wash, flick the plate over a sink to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.

## Step 2: Binding of Primary Antibody

1. Add 100  $\mu$ l of diluted NF $\kappa$ B antibody (1:1,000 dilution in 1X Antibody Binding Buffer) to each well being used, including blank wells.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. Wash the wells 3 times with 200  $\mu$ l 1X Wash Buffer (as described in Step 1, No. 4).

## Step 3: Binding of Secondary Antibody

1. Add 100  $\mu$ l of diluted HRP-conjugated antibody (1:1,000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. During this incubation, place the Developing Solution at room temperature.
4. Wash the wells 4 times with 200  $\mu$ l 1X Wash Buffer (as described in Step 1, No. 4).

## Step 4: Colorimetric Reaction

1. Add 100  $\mu$ l Developing Solution to all wells being used.
2. Incubate 30 seconds to 5 minutes at room temperature protected from direct light. Please read the Certificate of Analysis supplied with this kit for optimal development time for this specific kit lot. Monitor the blue color development in the sample wells until it turns medium to dark blue. Do not overdevelop. **NOTE: Recombinant standards develop quickly.**
3. Add 100  $\mu$ l Stop Solution. In presence of the acid, the blue color turns yellow.
4. Read absorbance on a spectrophotometer within 5 minutes at 450 nm with an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

### OPTIONAL – Calculation of results using the standard curve

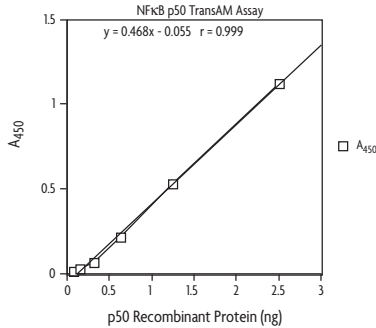
If you have generated a standard curve using Active Motif's recombinant NF $\kappa$ B p50 or p65 protein, average the duplicate readings for each standard, control, and sample and subtract the optical density (OD) obtained from the zero standard.

Plot the OD for the standards against the quantity (ng/well) of the standards and draw the best fit curve. The data can be linearized using log/log paper and regression analysis may also be applied.

To quantify the amount of NF $\kappa$ B in the samples, find the absorbance value for the samples on the y-axis and extend a horizontal line to the standard curve. At the intersection point extend a vertical line to the x-axis and read the corresponding standard value. Note: If the samples have been diluted, the value read from the standard curve must be multiplied by the dilution factor.

### Example curve:

The following standard curve is provided for demonstration only. A standard curve should be made every time an experiment is performed.



## References

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### Section A. Preparation of Nuclear Extract

For your convenience, Active Motif offers a Nuclear Extract Kit (Cat. Nos. 40010 & 40410). This kit contains buffers optimized for use in TransAM Kits, which serves to reduce inconsistencies in the assay that may arise from using homemade or other buffers. If you prefer to make your own buffers, please refer to the following protocol.

This procedure can be used for a confluent cell layer of 75 cm<sup>2</sup> (100-mm dish). The yield is approximately 0.15 mg of nuclear proteins for 9 x 10<sup>6</sup> cells.

1. Wash cells with 10 ml ice-cold PBS/PIB. Discard PBS/PIB.
2. Add 10 ml ice-cold PBS/PIB and scrape the cells off the dish with a cell lifter. Transfer cells into a pre-chilled 15 ml tube and spin at 300 x g for 5 minutes at 4°C. Do not use Trypsin to remove cells as it may alter transcription factor activation states.
3. Resuspend the pellet in 1 ml ice-cold HB buffer by gentle pipetting and transfer the cells into a pre-chilled 1.5 ml tube.
4. Allow the cells to swell on ice for 15 minutes.
5. Add 50 µl 10% Nonidet P-40 (0.5 % final) and vortex the tube vigorously for 10 seconds. Check the cells under a microscope to monitor the cell lysis. The cell membrane should be completely lysed, while the nuclear membrane remains intact.
6. Centrifuge the homogenate for 30 seconds at 4°C in a microcentrifuge. Remove the supernatant (cytoplasmic fraction) and, if you wish to save this for other uses, transfer it into a pre-chilled microcentrifuge tube. (Store the cytoplasmic fraction at -80°C.)
7. Resuspend the nuclear pellet in 50 µl Complete Lysis Buffer and rock the tube gently on ice for 30 minutes on a shaking platform.
8. Centrifuge for 10 minutes at 14,000 x g at 4°C and save the supernatant (nuclear extract). Aliquot and store at -80°C. Avoid freeze/thaw cycles. Discard the debris pellet.
9. Determine the protein concentration of the extract by using a Bradford-based assay. It is recommended to perform a 1:50 dilution of your samples for protein determination. A blank sample consisting of Complete Lysis Buffer diluted 1:50 should be run as control. Standard controls should also be generated in Complete Lysis Buffer diluted 1:50.



## Preparation of Buffers for Nuclear Extract

### 10X PBS

0.1 M phosphate buffer, pH 7.5
1.5 M NaCl
27 mM KCl

### For 250 ml, mix:

3.55 g $\text{Na}_2\text{HPO}_4$ + 0.61 g $\text{KH}_2\text{PO}_4$
21.9 g
0.5 g

Adjust to 250 ml with distilled water. Prepare a 1X PBS solution by adding 10 ml 10X PBS to 90 ml distilled water. Sterilize the 1X PBS by filtering through a 0.2  $\mu\text{m}$  filter. The 1X PBS is at pH 7.5. Store the filter-sterilized 1X PBS solution at 4°C.

### PIB (Phosphatase Inhibitor Buffer)

125 mM NaF
250 mM $\beta$ -glycerophosphate
250 mM p-nitrophenyl phosphate (PNPP)
25 mM $\text{NaVO}_3$

### For 10 ml, mix:

52 mg
0.55 g
1.15 g
31 mg

Adjust to 10 ml with distilled water. Mix the chemicals by vortexing. Incubate the solution at 50°C for 5 minutes. Mix again. Store at -20°C.

### PBS/PIB

Prior to use, add 0.5 ml PIB to 10 ml 1X PBS.

### HB (Hypotonic Buffer)

20 mM Hepes, pH 7.5
5 mM NaF
10 $\mu\text{M}$ $\text{Na}_2\text{MoO}_4$
0.1 mM EDTA

### For 50 ml, mix:

0.24 g
12 mg
5 $\mu\text{l}$ of a 0.1 M solution
10 $\mu\text{l}$ of a 0.5 M solution

Adjust pH to 7.5 with 1 N NaOH. Adjust volume to 50 ml with distilled water. Sterilize by filtering through a 0.2  $\mu\text{m}$  filter. Store the filter-sterilized solution at 4°C.

## Section B: Troubleshooting Guide

Problem/question	Possible cause	Recommendation
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in all wells in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity by mixing HRP and Developing Solution together
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction. Follow our recommendations to prepare buffers
	Plate reader settings not optimal	Verify the wavelength and filter settings
	Incorrect assay temperature	Bring Developing Solution and Stop Solution to room temperature before using
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette
High background in all wells	Measurement time too long	Stop enzymatic reactions as soon as the positive wells turn medium-dark blue
	Concentration of antibodies is too high	Increase antibody dilutions
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
Uneven color development	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Well cross-contamination	Follow washing recommendations
High background in sample wells	Too much nuclear extract per well	Decrease amount of nuclear extract down to 1-2 µg/well
	Concentration of antibodies is too high	Perform antibody titration to determine optimal working concentration. Start using 1:2,000 for primary antibody and 1:5,000 for the secondary antibody. The sensitivity of the assay will be decreased
No signal or weak signal in sample wells	Not enough nuclear extract per well	Increase amount of nuclear extract. Not to exceed 50 µg/well
	Too many freeze/thaw cycles of extract	Aliquot extract into 5 µl aliquots and store at -80°C to avoid multiple freeze/thaws
	NFκB is poorly activated or inactivated in nuclear fractions	Perform a time course for NFκB activation in the studied cell line
	Nuclear extracts are not from correct species	The p65, p50 and p52 antibodies work in human and mouse samples. The p65 antibody also cross-reacts with rat.
	Salt concentration too high in binding reaction	Reduce amount of extract per well or dialyze extract before use

## Technical Services

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If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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