TransAM™ Flexi

NFκB p50 / NFκB p65
Transcription Factor Assay Kits

(version A2)

Catalog No. 41098 (p50)
Catalog No. 40098 (p65)

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Overview

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 original TransAM™ method, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. These kits contain a 96-well plate that is pre-coated with a consensus binding site oligonucleotide for the factor of interest. This enables convenient assaying of transcription factor binding at this consensus site, but does not allow the study of alternate binding sites. Because of this, Active Motif has developed the TransAM Flexi Kits, which enable the study of transcription factor binding to any DNA-binding site. The TransAM Flexi NFκB p50 and p65 Kits are designed specifically to study NFκB binding at alternative sites. During the assay, a biotinylated oligonucleotide or PCR product, which contains the transcription factor-binding site of choice, is incubated with a nuclear extract that has been treated to activate NFκB. When incubated together, the active form of NFκB contained in the extract binds to the biotinylated probe. After incubation, the extract/probe mixture is then added to the provided streptavidin-coated plate. The biotinylated probe bound by active NFκB protein is immobilized and any inactive, unbound material is washed away. The bound NFκB transcription factor subunits are detected with specific primary antibody, for either NFκB p50 or p65. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) followed by developing and stop solutions provides a sensitive colorimetric readout that is easily quantified by spectrophotometry. The 96-well plate with individual strips of 8 wells is suitable for manual use or high-throughput screening applications. TransAM Flexi has many applications including the study of variant transcription factor binding sites, analysis of native promoters, confirmation of chromatin immunoprecipitation results and determination of isoform-binding affinity.

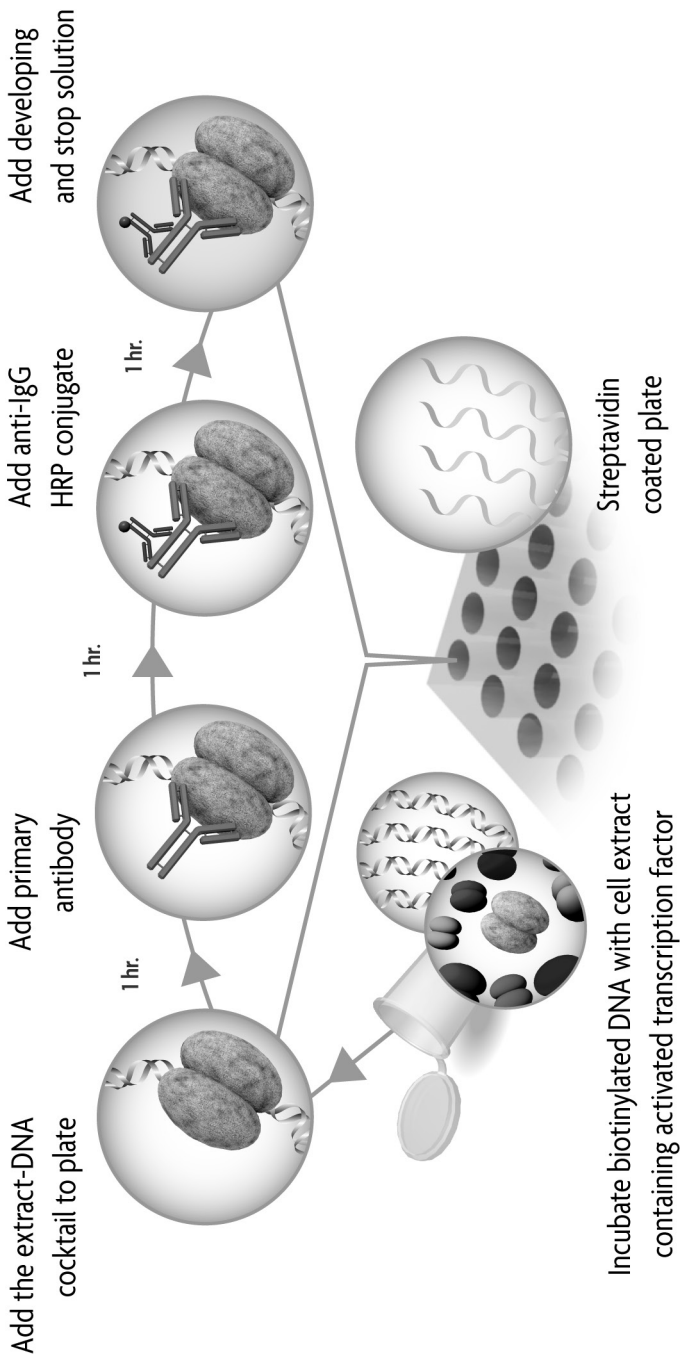
product	format	catalog no.
TransAM Flexi NFκB p50	1 x 96-well plate	41098
TransAM Flexi NFκB p65	1 x 96-well plate	40098

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

Active Motif also offers the original TransAM Kits, which contain the consensus binding site for NFκB bound to the wells of a 96-well plate. These original TransAM Kits are available in both colorimetric and chemiluminescent formats. See these and other Active Motif products related to the NFκB signaling pathway in Appendix, Section B.

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

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[1]. The DNA-binding protein complex recognizes a discrete nucleotide sequence (5´-GGGACTTTCC-3´) in the upstream region of a variety of cellular and viral response genes[2]. 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 RelB[3]. 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 p100[4]. 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 NFκB signaling pathway. In the majority of cells, NFκB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins[5]. Treatment of cells with various inducers results in the phosphorylation, ubiquitination and subsequent degradation of IκB proteins[6]. (For studying the phosphorylation state of IκBα, see Active Motif's FunctionELISA™ IκBα Kit). Proteolytic cleavage of p105 results in two antagonist proteins: p50, which has DNA-binding activity but no transactivation domain, and 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 B 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.

Kit Performance and Benefits

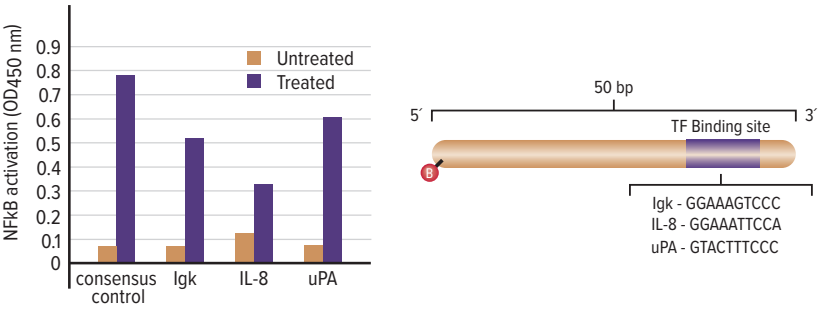
The TransAM Flexi NFκB Kits are for research use only. Not for use in diagnostic procedures.

Detection limit: > 0.5 µg nuclear or whole-cell extract/well or > 0.4 ng recombinant p50 or p65 protein/well. TransAM Flexi is 10-fold more sensitive than EMSA.

Range of detection: TransAM Flexi provides quantitative results from 0.5 to 10 µg of nuclear or whole-cell extract/well.

Cross-reactivity: TransAM NFκB specifically detects bound NFκB p65 in human, mouse and rat extracts and bound p50 in human extracts. The p50 antibody has limited reactivity with rat samples.

Assay time: 3.5 hours. TransAM is 40-fold faster than EMSA.



Monitoring NFκB binding affinity to natural binding sites using TransAM NFκB p65 Flexi.
Five µg of nuclear extract from untreated and TNF-α-treated HeLa cells were used to assay for NFκB binding affinity to four different 50-mer oligonucleotides. Three oligos were synthesized to represent the natural binding sites on various promoters regulated by NFκB[7]: Igκ (Accession NG000833), IL-8 (Accession M28130) and uPA (Accession X12641). Binding affinity from the natural binding sites was compared with the biotinylated consensus binding site control provided in the TransAM Flexi Kit. This data is provided for demonstration only.

Kit Components and Storage

Except for the nuclear extract that must be kept at -80ºC, kit components can be stored at -20ºC prior to first use. Then, we recommend storing each component at the temperature indicated in the table below.

Reagents	Quantity	Storage / Stability
NFκB p50 or p65 antibody	11 µl	-20°C for 6 months
Anti-rabbit HRP-conjugated IgG	11 µl	4°C for 6 months
Biotinylated oligonucleotide	40 µl (1 pmol/µl)	-20°C for 6 months
Wild-type oligonucleotide AM20	100 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM20	100 µl (10 pmol/µl)	-20°C for 6 months
Jurkat nuclear extract (TPA + CI stimulated)	40 µl (2.5 µg/µl)	-80°C for 6 months
Dithiothreitol (DTT)	100 µl (1 M)	-20°C for 6 months
Protease Inhibitor Cocktail	100 µl	-20°C for 6 months
Herring sperm DNA	100 µl (1 µg/µl)	-20°C for 6 months
Binding Buffer AM3	10 ml	4°C for 6 months
10X Wash Buffer AM2	22 ml	4°C for 6 months
10X Antibody Binding Buffer AM2	2.2 ml	4°C for 6 months
Developing Solution	11 ml	4°C for 6 months
Stop Solution	11 ml	4°C for 6 months
96-well assay plate	1	4°C for 6 months
Plate sealer	1	

Additional materials required

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform
- 1.5 ml microcentrifuge tubes
- Microplate spectrophotometer capable of reading at 450 nm (655 nm as optional reference wavelength)

For Nuclear Extract preparation

- Lysis Buffer
- Hypotonic Buffer
- Phosphatase Inhibitor Buffer
- 10X PBS
- Detergent (NP-40)

Buffer Preparation and Recommendations

Preparation of Complete Binding Buffer

Prepare the amount of Complete Binding Buffer required for the assay by adding 1 µl of 1 M DTT, 10 µl Protease Inhibitor Cocktail (PIC) and 10 µl of 1 µg/µl 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 10X Wash Buffer AM2 may form clumps, therefore homogenize the buffer by vortexing for 2 minutes 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 homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use. Dilute both primary and secondary antibodies to 1:1000 with the 1X Antibody Binding Buffer. Depending on the particular assay, the signal:noise ratio may be optimized by using higher dilutions of both antibodies. This may decrease the sensitivity of the assay.

* 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.

Positive control - Nuclear extract

The Jurkat nuclear extract (TPA + CI stimulated) is provided as a positive control for NFκB activation. Sufficient extract is supplied for 20 reactions per plate. This extract is optimized to give a strong signal when 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 B. Related Products).

Positive control - Biotinylated oligonucleotide

The biotinylated oligonucleotide is provided as a positive control for NFκB binding and is a 50 bp oligonucleotide that contains the consensus binding site 5´-GGGACTTTCC-3´ for NFκB. This oligo is supplied at 1 pmol/µl and 1 pmol per reaction should be used.

Design of test biotinylated oligonucleotides or PCR fragments

The advantage of the TransAM Flexi Kit is that it enables the evaluation of different binding sites for NFκB within the same kit (see Figure on page 4). For optimal results, probes should be a minimum of 50 bp with a maximum length of 2 kb. Probes can either be synthesized or generated by PCR.

a. Once the binding site of interest is determined, synthesize two complementary oligos. In all cases the oligo probes should be biotinylated on one end. Biotinylation can be on either the 5´ or 3´ end of the probe. It is recommended that the binding site of interest be located distal to the biotinylated end. For example, if the oligo is biotinylated on the 5´ end, the binding site should be located closer to the 3´ end. We recommend leaving a minimum of 5 bp between the binding site and the end of the oligo.

b. Combine the two complementary oligos at an equimolar ratio and an approximate concentration of 100 µM, in a volume of 100-500 µl.

c. Heat the oligo mixture at 95°C for 10 minutes in a microcentrifuge tube on a heating block and allow the block containing the mixture to cool down slowly to room temperature.

d. After diluting the double-stranded oligo to its desired concentration (we recommend 1 pmol/µl), it is ready to use.

Wild-type and mutated consensus oligonucleotides

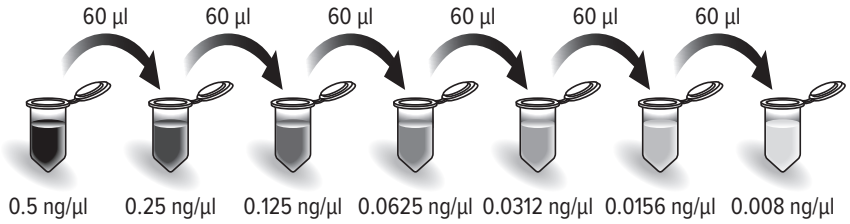
Active Motif provides unlabeled wild-type and mutated oligonucleotides to perform competition experiments against the provided biotinylated oligonucleotide. The wild-type consensus oligonucleotide, when used at 10 pmol/well, will prevent NFκB binding to the probe immobilized on the plate. Conversely, 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 1 µl of appropriate oligonucleotide to the appropriate sample per well being used. To allow for optimum competition, add the oligonucleotide to the Complete Binding Buffer prior to addition of the cell extract.

If you have synthesized a biotinylated probe for use with TransAM Flexi and would like to perform competition experiments, unlabeled wild-type and mutated competitor oligonucleotides should also be prepared. As a general rule, it is best to limit the number of nucleotides that are altered in the mutated oligo to no more than 4 within a given DNA binding site.

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 B. Related Products).

1. Begin with a 100 ng/µl working stock of recombinant protein (use the TransAM Complete Binding 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. Please see the lot specific technical sheet that accompanies each protein for specific standard curve determination.
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 Binding Buffer. Next, pipette 60 µl of Complete Binding Buffer into the 7 remaining tubes. Use the 0.5 ng/µl solution to prepare a dilution series as indicated below. Be sure to mix each tube thoroughly before each transfer. The 0.5 ng/µl standard serves as the high standard, while Complete Binding Buffer alone serves as the 0.0 ng/µl.



3. 20 µl from each tube will be diluted into the Complete Binding Buffer and Biotinylated probe used in Step 1, No. 1 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 and Samples*

For sample wells, please add components in the order listed below.

Reagents to prepare	Components	For 1 well	For 1 strip (8 wells)	For 6 strips (48 wells)	For 12 strips (96 wells)
Sample Wells	Binding Buffer AM3	51.965 µl	424.35 µl	2.452 ml	4.904 ml
2 µg extract/well	DTT	0.055 µl	0.45 µl	2.6 µl	5.2 µl
	Protease Inhibitors	0.55 µl	4.5 µl	26 µl	52 µl
	Herring sperm DNA	0.55 µl	4.5 µl	26 µl	52 µl
	Biotinylated oligo (1 pmol/µl)	1.0 µl	9.0 µl	52 µl	104 µl
	2 µg Extract at 2.5 µg/µl	0.88 µl	7.2 µl	41.6 µl	83.2 µl
	TOTAL REQUIRED	**55 µl**	**450 µl**	**2.6 ml**	**5.2 ml**
Sample Wells	Binding Buffer AM3	50.645 µl	413.55 µl	2.389 ml	4.779 ml
5 µg extract/well	DTT	0.055 µl	0.45 µl	2.6 µl	5.2 µl
	Protease Inhibitors	0.55 µl	4.5 µl	26 µl	52 µl
	Herring sperm DNA	0.55 µl	4.5 µl	26 µl	52 µl
	Biotinylated oligo (1 pmol/µl)	1.0 µl	9.0 µl	52 µl	104 µl
	5 µg Extract at 2.5 µg/µl	2.2 µl	18 µl	104 µl	208 µl
	TOTAL REQUIRED	**55 µl**	**450 µl**	**2.6 ml**	**5.2 ml**
Sample Wells	Binding Buffer AM3	48.445 µl	395.55 µl	2.285 ml	4.571 ml
10 µg extract/well	DTT	0.055 µl	0.45 µl	2.6 µl	5.2 µl
	Protease Inhibitors	0.55 µl	4.5 µl	26 µl	52 µl
	Herring sperm DNA	0.55 µl	4.5 µl	26 µl	52 µl
	Biotinylated oligo (1 pmol/µl)	1.0 µl	9.0 µl	52 µl	104 µl
	10 µg Extract at 2.5 µg/µl	4.4 µl	36 µl	208 µl	416 µl
	TOTAL REQUIRED	**55 µl**	**450 µl**	**2.6 ml**	**5.2 ml**
Sample Wells	Binding Buffer AM3	44.045 µl	359.55 µl	2.077 ml	4.155 ml
20 µg extract/well	DTT	0.055 µl	0.45 µl	2.6 µl	5.2 µl
	Protease Inhibitors	0.55 µl	4.5 µl	26 µl	52 µl
	Herring sperm DNA	0.55 µl	4.5 µl	26 µl	52 µl
	Biotinylated oligo (1 pmol/µl)	1.0 µl	9.0 µl	52 µl	104 µl
	20 µg Extract at 2.5 µg/µl	8.8 µl	72 µl	416 µl	832 µl
	TOTAL REQUIRED	**55 µl**	**450 µl**	**2.6 ml**	**5.2 ml**
1X Wash Buffer	Distilled water	2.025 ml	16.2 ml	93.6 ml	187.2 ml
	10X Wash Buffer AM2	225 µl	1.8 ml	10.4 ml	20.8 ml
	TOTAL REQUIRED	**2.25 ml**	**18 ml**	**104 ml**	**208 ml**
1X Antibody	Distilled water	202.5 µl	1.62 ml	9.36 ml	18.72 ml
Binding Buffer**	10X Ab Binding Buffer AM2	22.5 µl	180 µl	1.04 ml	2.08 ml
	TOTAL REQUIRED	**225 µl**	**1.8 ml**	**10.4 ml**	**20.8 ml**
Developing Solution	**TOTAL REQUIRED**	**112.5 µl**	**900 µl**	**5.2 ml**	**10.4 ml**
Stop Solution	**TOTAL REQUIRED**	**112.5 µl**	**900 µl**	**5.2 ml**	**10.4 ml**

* Volumes listed in the Quick Chart contain an excess of components to perform the assay.

** Volumes listed for the 1X Ab Binding Buffer refer to the preparation of buffer for diluting both the primary & secondary antibodies.

NFκB Flexi 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 Binding Buffer, 1X Wash Buffer and 1X Antibody Binding Buffer as described in the section Buffer Preparation and Recommendations. Multi-channel pipettor reservoirs may be used for dispensing the Wash Buffer, Antibody Binding Buffer, Developing Solution and Stop Solution.

Step 1: Binding of NFκB to the biotinylated probe

1. All samples should be diluted to 50 µl with Complete Binding Buffer in microcentrifuge tubes. Multiple repeats of the same binding assay can be multiplexed and mixed in the same tube. For best results, the nuclear extract should be added to the binding reaction mixture last. Please refer to the Quick Chart on page 9 for directions on preparing sample wells.

   Sample wells: Prepare sample by adding 1 pmol of biotinylated probe and the desired amount of nuclear extract diluted to 50 µl in Complete Binding Buffer. We recommend using 2-20 µg (at 2.5 µg/µl) of nuclear extract per well. A protocol for preparing nuclear extracts is provided on page 12.

   Positive control wells: Add 1 pmol (1 µl) of the provided biotinylated oligonucleotide and 5 µg (2 µl) of the provided Jurkat nuclear extract diluted to 50 µl in Complete Binding Buffer.

   Blank wells: Add 1 pmol biotinylated probe diluted to 50 µl in Complete Binding Buffer only.

   OPTIONAL – Competitive binding wells: If you wish to perform competitive binding experiments, add 10 pmol of the appropriate wild-type or mutated oligo to each sample being used (see page 8 for a description of competitive binding). If using the competition oligos provided in the kit, add 1 µl (10 pmol) wild-type or mutated oligo to each sample being used.

   OPTIONAL – Protein standard wells: Add 1 pmol of the biotinylated probe and 20 µl of the appropriate protein standard diluted to 50 µl in Complete Binding Buffer (see page 8, Preparation of standard curve).
2. Mix each tube from above by vortexing and then incubating at room temperature for 30 minutes.
3. Transfer the entire 50 µl reaction to an individual well on the plate. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at room temperature with mild agitation.
4. Wash each well 3 times with 200 µl 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 µl diluted NFκB antibody (1:1000 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. Wash the wells 3 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 3: Binding of secondary antibody

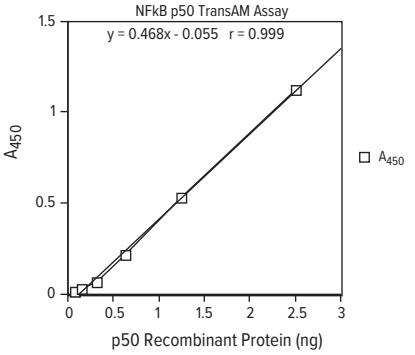
1. Add 100 µl diluted HRP antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. During this incubation, place the Developing Solution at room temperature.
4. Wash the wells 4 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric reaction

1. Add 100 µl room-temperature Developing Solution to all wells being used.
2. Incubate 2-10 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 lot. Monitor the blue color development in the sample until it turns medium to dark blue. Do not over-develop.
3. Add 100 µl 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 a 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 NFκB 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 NFκB 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. The following standard curve is provided for demonstration only.



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 the 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^2 (100 mm dish). The yield is approximately 0.5 mg of nuclear proteins for 10^7 cells.

1. Wash cells with 10 ml of ice-cold PBS/PIB.
2. Add 10 ml of ice-cold PBS/PIB and scrape the cells off the dish with a cell lifter. Transfer the cells into a pre-chilled 15 ml tube and spin at 300 x *g* for 5 minutes at 4°C.
3. Resuspend the pellet in 1 ml of 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 mix by gentle pipetting.
6. Centrifuge the homogenate for 30 seconds at 4°C in a microcentrifuge.
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 cell extract). Aliquot and store at –80°C. Avoid freeze/thaw cycles.
9. Determine the protein concentration of the extract by using a Bradford-based assay.

10X PBS	**For 250 ml, mix:**
0.1 M phosphate buffer, pH 7.5	3.55 g Na_2HPO_4 + 0.61 g KH_2PO_4
1.5 M NaCl	21.9 g
27 mM KCl	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 µm filter. The 1X PBS is at pH 7.5. Store the filter-sterilized 1X PBS solution at 4°C.

PIB (Phosphatase Inhibitor Buffer)	**For 10 ml, mix**
125 mM NaF	52 mg
250 mM β-glycerophosphate	0.55 g
250 mM para-nitrophenyl phosphate (PNPP)	1.15 g
25 mM $NaVO_3$	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 of PIB to 10 ml of 1X PBS.

HB (Hypotonic Buffer)	**For 50 ml, mix**
20 mM Hepes, pH 7.5	0.24 g
5 mM NaF	12 mg
10 µM Na_2MoO_4	5 µl of a 0.1 M solution
0.1 mM EDTA	10 µl 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 µm filter. Store the filter-sterilized solution at 4°C.

Lysis Buffer	**For 50 ml, mix:**
20 mM Hepes, pH 7.5	0.24 g
400 mM NaCl	1.17 g
0.1 mM EDTA	1.5 mg
10 mM NaF	21 mg
10 µM Na_2MoO_4	0.12 mg
1 mM $NaVO_3$	6.1 mg
20% glycerol	10 ml
10 mM PNPP	0.23 g
10 mM beta-glycerophosphate	0.11 g

Adjust pH to 7.5 with 1 N NaOH. Adjust volume to 50 ml with distilled water. Store at 4°C. Just before use, make up Complete Lysis Buffer by adding 1 µl of 1 M DTT and 10 µl of Protease Inhibitor Cocktail (Sigma, Cat. No. P8340) per ml of Lysis Buffer.

References

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Appendix

Section A. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity
	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 in the plate reader
	Incorrect assay temperature	Bring substrate to room temperature
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette
High background in all wells	Developing time too long	Stop enzymatic reaction as soon as the positive wells turn medium-dark blue
	Concentration of antibodies 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 cellular extract per well	Decrease amount of cellular extract down to 1-2 µg/well
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1:2000 for primary antibody and 1:5000 for the secondary antibody. The sensitivity of the assay will be decreased
No signal or weak signal in sample wells	Not enough cellular extract per well	Increase amount of cellular extract not to exceed 50 µg/well
	Using a whole-cell extract	Prepare a nuclear extract according to the protocol
	NFκB is poorly activated or inactivated	Perform a time course for NFκB activation in the studied cell line
	Extracts are not from correct origin	The p65 antibody in TransAM NFκB p65 detects human, mouse and rat p65. The p50 antibody in TransAM NFκB p50 is weakly reactive with rodent p50. You may need to a add double or triple the amount of recommended extract to obtain a signal.

Technical Services

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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