TransAM™

NFκB p65 Chemi/ NFκB p50 Chemi/ NFκB p52 Chemi

(version E3)

Catalog Nos. 40097 & 40597 (NFκB p65)
Catalog Nos. 41097 & 41597 (NFκB p50)
Catalog Nos. 48197 & 48697 (NFκB p52)

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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.

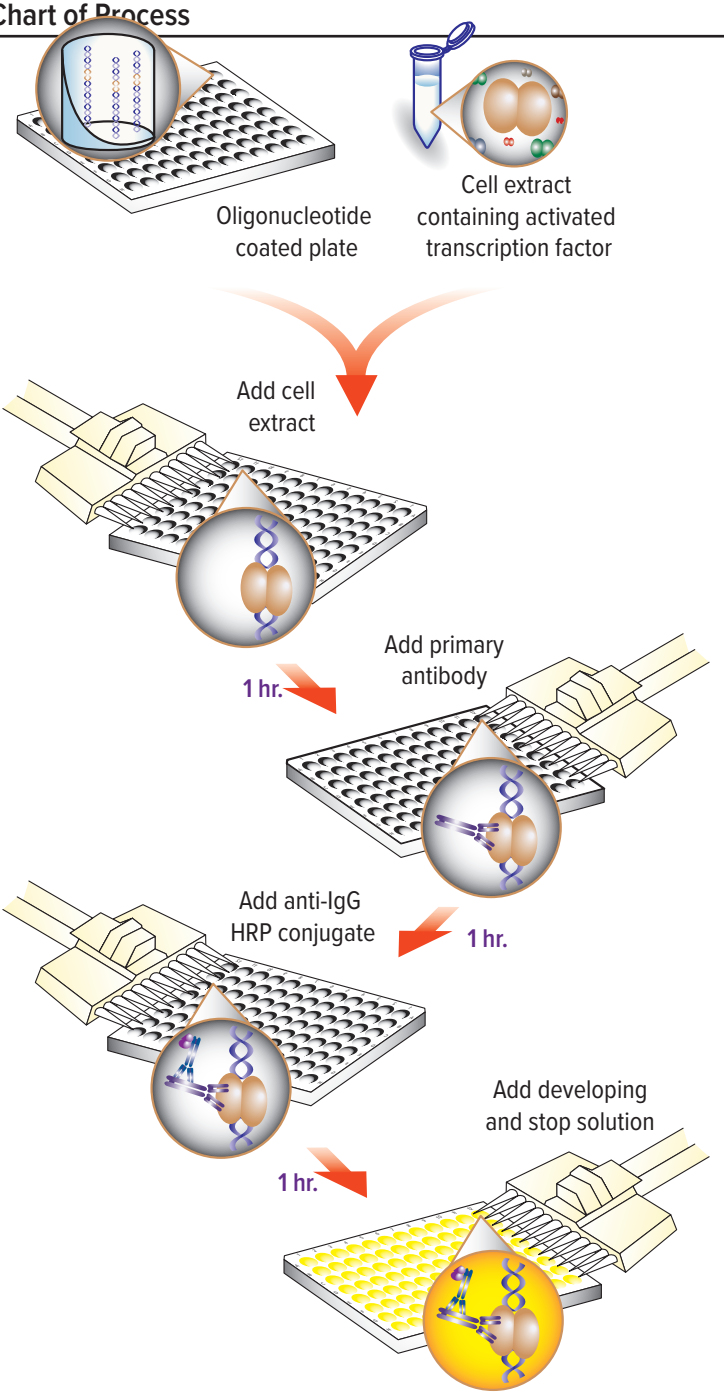
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM NFκB p65, p52 and p50 Chemi Kits are designed specifically for the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

product	format	catalog no.
TransAM™ NFκB p65 Chemi	1 x 96 rxns 5 x 96 rxns	40097 40597
TransAM™ NFκB p50 Chemi	1 x 96 rxns 5 x 96 rxns	41097 41597
TransAM™ NFκB p52 Chemi	1 x 96 rxns 5 x 96 rxns	48197 48697

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

* 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

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 (NFκB/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[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 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[7]. 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´-GGGACTTTCC-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 chemiluminescent readout that is easily quantified by luminescence. 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 125-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 40 ng 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

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

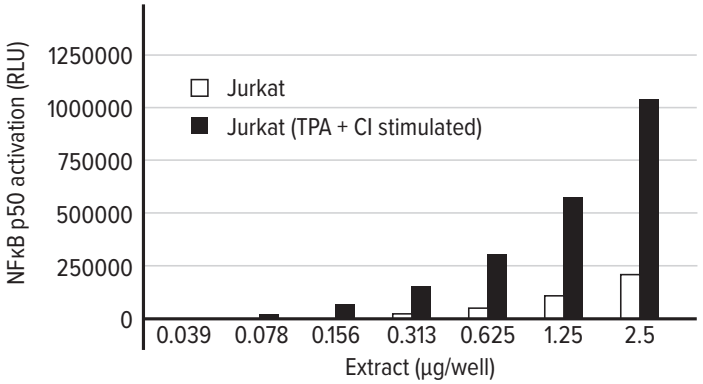
Detection limit: < 40 ng nuclear extract/well. For whole-cell extract, twice as much input may be needed. TransAM NFκB Chemi is 125-fold more sensitive than EMSA.

Range of detection: TransAM Chemi Kits provide quantitative results from 0.039 to 2.5 µg of nuclear or whole-cell extract per well.

Cross-reactivity:

	p65	p50	p52
Human	+	+	+
Mouse	+	+	+
Rat	+	–	–

Assay time: 3.5 hours.



TransAM NFκB p50 Chemi sensitivity and dynamic range.

Nuclear extracts from Jurkat cells (Catalog No. 36014) and Jurkat cells stimulated with TPA and calcium ionophore (Catalog No. 36013) were assayed from 0.039 to 2.5 µg/well for NFκB p50 activation using the TransAM NFκB p50 Chemi Kit.

Kit Components and Storage

TransAM NFκB Kits are for research use only. Not for use in diagnostic procedures. 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. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity 1 plate / 5 plates	Storage
NFκB p65 or p50 antibody	11 µl / 55 µl	-20°C for 6 months
NFκB p52 antibody	11 µl / 55 µl	4°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
Chemiluminescent Reagent	2 ml / 10 ml	4°C for 6 months
Reaction Buffer	4 ml / 20 ml	4°C for 6 months
96-well NFκB chemi 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 luminometer or CCD camera-couple imaging system (*e.g.* Tecan GENios microplate reader)

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 µl of 1 M DTT and 10 µl 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 µl of DTT 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 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.

Preparation of Antibody Solutions

Dilute the primary antibody with the 1X Antibody Binding Buffer to 1:1,000 for NFκB p65, p50 and p52. For example, dilute 0.44 µl antibody in 440 µl 1X Antibody Binding Buffer for 1 strip (8 wells); multiply these values by the number of strips to be used. Dilute the secondary antibody to 1:10,000 with the 1X Antibody Binding Buffer by performing a 1:500 dilution followed by a 1:20 dilution. For example, dilute 0.8 µl antibody in 400 µl 1X Antibody Binding Buffer. Next, take

22 µl of the previous dilution and add it to 418 µl 1X Antibody Binding Buffer. This is sufficient reagent for 1 strip (8 wells); multiply these values by the number of strips to be used. 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.

Preparation of Chemiluminescent Working Solution

The Chemiluminescent Reagent and Reaction Buffer should be warmed to room temperature before use. These components are light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. Prior to use, place the Chemiluminescent Reagent and Reaction Buffer at room temperature for at least 1 hour. In a separate container, mix 1 volume of Chemiluminescent Reagent with 2 volumes of Reaction Buffer to prepare the Chemiluminescent Working Solution (see the Quick Chart for Preparing Buffers in this section). The Chemiluminescent Working Solution is stable for several hours. After the Chemiluminescent Working Solution is aliquoted into the wells, discard the remaining solution.

Nuclear extract

The Jurkat (TPA + CI) nuclear extract is provided as a positive control for NFκB p50 and p65 activation. The Raji nuclear extract is provided as a positive control for NFκB p52 activation. Sufficient extract is supplied for 80 reactions per plate. The positive control extract is optimized to give a strong signal when used at 1.25 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.

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.

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
	TOTAL REQUIRED	**22.5 µl**	**180 µl**	**1.08 ml**	**2.16 ml**
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
	TOTAL REQUIRED	**33.8 µl**	**270 µl**	**1.62 ml**	**3.24 ml**
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
	TOTAL REQUIRED	**33.8 µl**	**270 µl**	**1.62 ml**	**N/A**
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
	TOTAL REQUIRED	**2.25 ml**	**18 ml**	**108 ml**	**216 ml**
1X Antibody Binding Buffer*	Distilled water	157.5 µl	1.26 ml	7.56 ml	15.12 ml
	10X Ab Binding Buffer AM2	17.5 µl	140 µl	840 µl	1.68 ml
	TOTAL REQUIRED	**175 µl**	**1.4 ml**	**8.4 ml**	**16.8 ml**
Chemiluminescent Working Solution	Chemiluminescent Reagent	18.7 µl	150 µl	0.9 ml	1.8 ml
	Reaction Buffer	37.5 µl	300 µl	1.8 ml	3.6 ml
	TOTAL REQUIRED	**56.2 µl**	**450 µl**	**2.7 ml**	**5.4 ml**

* 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 0.2-2 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts is provided on page 11. For whole-cell extracts we recommend using 0.4-4 µg per well.

   Positive control wells: Add 1.25 µg of the provided Jurkat nuclear extract (for p65 or p50) or Raji nuclear extract (for p52) diluted in 20 µl of Complete Lysis Buffer per well (0.5 µl of nuclear extract in 19.5 µl of Complete Lysis Buffer per well).

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

Reagents	Blank wells	Positive Control no competition	Sample no competition	(Optional) wild-type	(Optional) mutated
Complete Binding Buffer	30 µl	30 µl	30 µl	28 µl	28 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

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 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 50 µl of diluted NFκB 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 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 3: Binding of Secondary Antibody

1. Add 50 µl of diluted HRP-conjugated antibody (1:10,000 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 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Chemiluminescent Detection

1. Add 50 µl room-temperature Chemiluminescent Working Solution to all wells being used. Minimize exposure of the plate to light. Pop any bubbles that remain in the wells to ensure accurate measurements and then read the plate immediately.
2. Read chemiluminescence using a luminometer or CCD camera system. You may need to customize the plate geometry to work with your plate reader.

   Plate length = 127,890 µm Plate height = 15,000 µm

   Plate width = 85,500 µm Height tolerance = 500 µm

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Appendix

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^2 (100-mm dish). The yield is approximately 0.15 mg of nuclear proteins for 9 x 10^6 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	**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 p-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 PIB to 10 ml 1X PBS.

HB (Hypotonic Buffer)	**For 50 ml, mix:**
20 mM Hepes, pH 7.5	0.24 g
5 mM NaF	12 mg
0 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.

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 Chemiluminescent Working Solution together
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction. Follow our recommendations to prepare buffers
	Plate reader or CCD camera settings not optimal	Verify the plate reader is set for luminescence measurement mode or check filter settings on the CCD camera
	Incorrect assay temperature	Bring Chemiluminescent Reagent and Reaction Buffer to room temperature before using
	Inadequate volume of Chemiluminescent Working Solution	Check to make sure that correct volume is delivered by pipette
High background in all wells	Measurement time too long	Reduce integration time or exposure time on luminometer or CCD camera
	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 0.01-0.05 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 1:50,000 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 40 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 species
	Salt concentration too high in binding reaction	Reduce amount of extract per well or dialyze extract before use

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.

Active Motif North America

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