TransAM® MAPK Family

Transcription Factor Assay Kits

(version B2)

Catalog No. 47296

Revision	Date	Description of Change
B2	August, 2023	c-Myc antibody has changed to new monoclonal antibody. However, dilution used in protocol and species reactivity are the same as previous.

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Overview

The activation of mitogen-activated protein kinase (MAPK) signal transduction pathways is responsible for the phosphorylation of transcription factors by the terminal kinase in these cascades. MAPK pathways play major roles in converting mitogenic and stress stimuli into nuclear responses,[1] and therefore, accurate monitoring of MAPK substrates in cells, tissues and animals is crucial for many biomedical research and drug development projects. To date, such research projects are tedious and time consuming, and lack high-throughput screening methods.

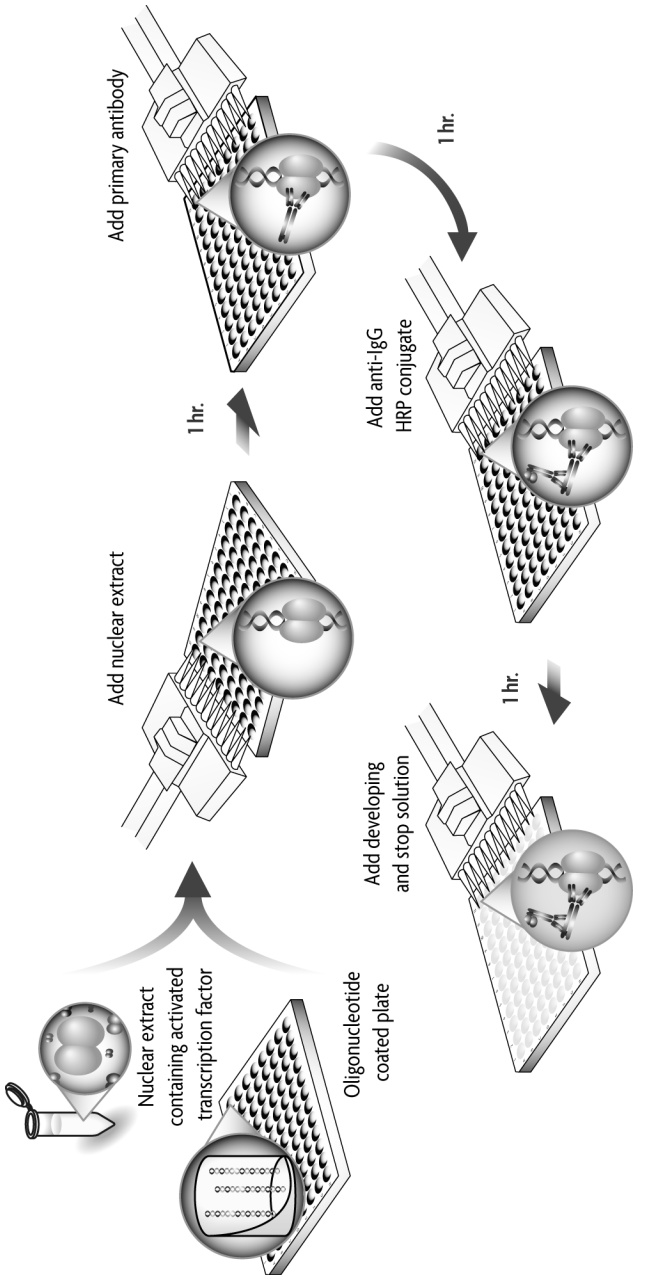
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM MAPK Family Kits are designed specifically for the study of the MAPK regulated transcription factors ATF-2, c-Jun, c-Myc, MEF2 and STAT1. The kit contains a 96-well plate to which a mixture of oligonucleotides containing the consensus-binding sites for ATF-2, c-Jun, c-Myc, MEF2 and STAT1 have been immobilized. The activated form of the protein contained in nuclear extracts specifically binds to this oligonucleotide. By using an antibody that is directed against phosphorylated ATF-2, phosphorylated c-Jun, c-Myc, MEF2 or STAT1α, the complex bound to the oligonucleotide is detected. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) 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.

product	format	catalog no.
TransAM MAPK Family	2 x 96-well plates	47296

See Active Motif products related to MAPK signaling pathways in Appendix, Section B.

* Technology covered by AAT-filed patents and licensed to Active Motif.

Flow Chart of Process



MAPK Regulated Transcription Factors

The transmission of extracellular signals into intracellular responses is a complex process that often involves the activity of mitogen-activated protein kinases (MAPKs) (for review, see 2). The MAPK pathway is a three kinase cascade consisting of a MAPK kinase (MAPKKK or MEKK) that activates a MAP/ERK kinase (MEK or MAPKK). This stimulates a phosphorylation-dependent increase in the activity of the MAP kinase. Upon activation, MAPKs phosphorylate a variety of intracellular targets including transcription factors, transcription adaptor proteins, membrane and cytoplasmic substrates as well as other protein kinases, as illustrated below.[2]

MAP kinase	Activator	Nuclear Substrate
ERK1/2	Growth Factors Serum Hormones Cytokines Small molecules	ATF-2, Elk-1, c-Fos, c-Myc, SAPs, c-Jun, NeuroD1, PDX-1, STAT3, RSKs, Mnks, MSK
ERK5	Growth factors Serum Hormones Osmotic stress	MEF2, RSKs
p38	Hormones Cytokines Osmotic stress Heat shock	ATF-2, Elk-1, MEF2, SAPs, STAT1, STAT3, MAPKAPs, Mnks, MSK
JNK	Hormones Cytokines DNA and protein synthesis inhibitors Osmotic stress	ATF-2, c-Jun, Elk-1, STAT1, STAT3

At least three parallel MAPK pathways exist in humans. The extracellular signal-regulated protein kinase (ERK) pathway primarily transmits mitogenic and differentiation stimuli, while the c-Jun N-terminal kinase (JNK) and p38 pathways predominantly transmit stress and cytokine stimuli.[1]

c-Myc, an ERK substrate, is a transcription factor that regulates cell growth and differentiation, glycolysis and apoptosis. Deregulation of c-Myc has been implicated in the origin of diverse human cancers. Elk-1 is a member of the ternary complex factor (TCF) sub-family of the ETS domain family. Elk-1 can be stimulated by all three MAPK pathways, and its main function is the regulation of the activity of the c-Fos promoter in response to extracellular stimuli.[2] MEF2, a member of the MADS box family, is mainly involved in muscle differentiation, but also plays roles in muscle hypertrophy, neuronal survival and T-cell apoptosis.[3] MEF2 is activated by both the p38 and ERK5 pathways.[1] STAT1 is involved in activation of IFNα and γ genes, and is activated by p38 and JNK pathways.[4] c-Jun is a member of the activator protein-1 (AP-1) family and is activated by both ERK1/2 and JNK pathways.[2] AP-1 members play roles in the expression of genes involved in proliferation and cell cycle progression. ATF-2 is a member of the ATF/CREB family that binds to the cAMP response element (CRE). ATF-2 is activated by ERK1/2, JNK and p38.[2, 5]

Transcription Factor Assays

To date, three methods are widely used to measure MAPK regulated transcription factors, either directly or indirectly:

1. Expression can be measured by Western blot, using antibodies raised against the transcription factor of interest. 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 MAPK regulated transcription factors can be assayed by gel retardation, also called electrophoretic mobility shift assay (EMSA). In this method, nuclear extracts are incubated with a radioactive double-stranded oligonucleotide probe containing the consensus sequence for binding. If the transcription factor is active in the nuclear 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 transcription factor activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing the consensus sequence. This promoter can be artificial, made of several cis-elements and a TATA box, or natural. 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 MAPK

Transcription factors that are activated by MAP kinases convert mitogenic and stress stimuli into nuclear responses, and these diverse transcription factors play important roles in many areas of cell proliferation and survival. However, this field has been hampered by the lack of convenient, high-throughput assays suitable for detection of transcription factors activated by MAPK.

To overcome this, Active Motif is introducing a high-throughput assay to quantify the MAPK regulated transcription factors ATF-2, c-Jun, c-Myc, MEF2 and STAT1α. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM MAPK Family Kits contain a 96-well plate on which has been immobilized a mixture of oligonucleotides that contains the consensus binding site for each transcription factor. The active form of each transcription factor contained in the nuclear extract specifically binds to this oligonucleotide mixture. The primary antibodies used to detect the MAPK regulated transcription factors will recognize an epitope on phosphorylated ATF-2, phosphorylated c-Jun, c-Myc, MEF2 and STAT1α that is accessible only when they are activated and bound to their target DNA. Addition of a secondary HRP-conjugated antibody provides a sensitive colorimetric readout easily quantified by spectrophotometry. Once the nuclear extracts are prepared, this assay is completed in less than 3.5 hours. As this assay is performed in 96-well plates, a large number

of samples can be handled simultaneously, enabling high-throughput automation. This assay is specific for ATF-2, c-Jun, c-Myc, MEF2 and STAT1α activation and has been shown to be 10-fold more sensitive and 20-fold faster than the gel-retardation technique. With the 3.5-hour TransAM procedure, we could detect activation using as little as 0.5 µg of nuclear extract. A comparable assay using EMSA required 5 µg of nuclear extract and a 3-day autoradiography.

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

Kit Performance and Benefits

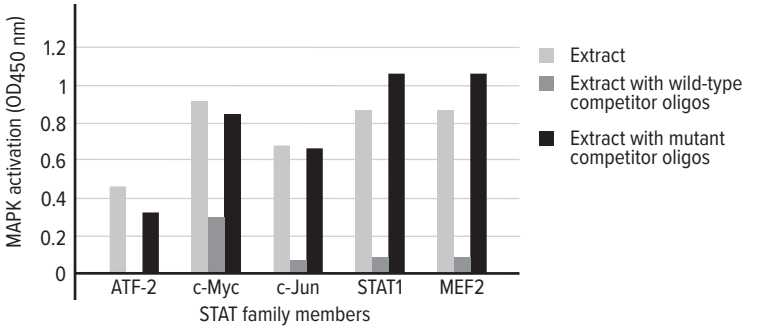
The TransAM MAPK Family Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: < 0.5 µg nuclear extract/well. The TransAM MAPK Family Kit is up to 10-fold more sensitive than EMSA.

Range of detection: TransAM provides quantitative results from 0.5 to 5 µg of nuclear extract/well for c-Myc, MEF2 and STAT1 and from 1 to 10 µg nuclear extract/well for ATF-2 and c-Jun.

Cross-reactivity: TransAM MAPK Family detects phosphorylated ATF-2, MEF2 and STAT1α from human origin and c-Myc from mouse and human origin. TransAM MAPK Family also detects mouse and human c-Jun phosphorylated at Ser-73 and JunD phosphorylated at Ser-100, as this site is conserved between c-Jun and JunD.

Assay time: 3.5 hours. TransAM is 20-fold faster than EMSA.



Monitoring MAPK regulated transcription factor activity with the TransAM MAPK Family Kit: Nuclear extracts from HeLa (Anisomycin) for ATF-2, Jurkat (1 day growth) for c-Myc, K-562 (TPA) for c-Jun, U-937 (TPA + IFNγ) for STAT1 and C2C12 for MEF2 were assayed for activity using the TransAM MAPK Family 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 2 plates	Storage / Stability
Phosphorylated ATF-2 antibody	11 µl	4°C for 6 months
Phosphorylated c-Jun antibody	22 µl	-20°C for 6 months
c-Myc antibody	11 µl	-20°C for 6 months
MEF2 antibody	11 µl	4°C for 6 months
STAT1α antibody	11 µl	-20°C for 6 months
HRP-rabbit conjugated IgG	2 x 11 µl (0.2 µg/µl)	4°C for 6 months
HRP-mouse conjugated IgG	11 µl / 55 µl (0.2 µg/µl)	4°C for 6 months
AP-1 wild-type oligonucleotide AM7	100 µl (10 pmol/µl)	-20°C for 6 months
AP-1 mutated oligonucleotide AM7	100 µl (10 pmol/µl)	-20°C for 6 months
ATF-2 wild-type oligonucleotide AM2	100 µl (10 pmol/µl)	-20°C for 6 months
ATF-2 mutated oligonucleotide AM2	100 µl (10 pmol/µl)	-20°C for 6 months
c-Myc wild-type oligonucleotide AM3	100 µl (10 pmol/µl)	-20°C for 6 months
c-Myc mutated oligonucleotide AM3	100 µl (10 pmol/µl)	-20°C for 6 months
MEF2 wild-type oligonucleotide AM5	100 µl (10 pmol/µl)	-20°C for 6 months
MEF2 mutated oligonucleotide AM5	100 µl (10 pmol/µl)	-20°C for 6 months
STAT wild-type oligonucleotide AM6	100 µl (10 pmol/µl)	-20°C for 6 months
STAT mutated oligonucleotide AM6	100 µl (10 pmol/µl)	-20°C for 6 months
K-562 nuclear extract (TPA 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

Lysis Buffer AM1	10 ml	4°C for 6 months
Binding Buffer AM6 months	10 ml	4°C for 6
10X Wash Buffer AM2 months	60 ml	4°C for 6
10X Antibody Binding Buffer AM3 months	2 x 2.2 ml	4°C for 6
Developing Solution months	2 x 11 ml	4°C for 6
Stop Solution months	60 ml	4°C for 6
96-well assay plate months	2	4°C for 6
Plate sealer	2	

Additional materials required

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

For Nuclear Extract preparation

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

Buffer Preparation and Recommendations

Preparation of Complete Lysis Buffer

We provide an excess of Lysis Buffer AM1 in order to perform the assay AND to prepare customized nuclear extracts. Our Nuclear Extract Kit can also be purchased separately (Cat. Nos. 40010 & 40410). Prepare the amount of Complete Lysis Buffer required for the assay by adding 1 µl of 1 M DTT and 10 µl of Protease Inhibitor Cocktail per ml of Lysis Buffer AM1 (see the Quick Chart for Preparing Buffers in this section). Some of the protease inhibitors lose their activity after 24 hours once diluted. Therefore, we recommend using the Complete Lysis Buffer immediately for cell lysis. The remaining amount should be discarded if not used in the same day.

Preparation of Complete Binding Buffer

Prepare the amount of Complete Binding Buffer required for the assay by adding 2 µl of 1 M DTT per ml of Binding Buffer AM6 (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 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 AM3 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 AM3 may form clumps, therefore homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use. Dilute the phosphorylated c-Jun antibody to 1:500 and all other 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.

Nuclear extract

K-562 (TPA stimulated) nuclear extract is provided as a positive control to ensure the kit reagents are functional. The K-562 (TPA stimulated) extract is optimized to give a strong signal for both
c-Jun and ATF-2 when used at 5 µg/well. Sufficient extract is supplied for 20 reactions. We recommend aliquoting the extract in 5 µl fractions and storing at -80°C to avoid multiple freeze/thaw cycles of the extract.

In addition, positive control nuclear extract for c-Myc, MEF2 and STAT1 can be purchased separately. Jurkat nuclear extract (1 day growth) is recommended as a control for c-Myc. C2C12 (Undifferentiated) nuclear extract is suitable for MEF2 and COS-7 nuclear extract (IFNγ treated) is suitable for STAT1 (see Appendix, Section B. Related Products).

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotides are provided as competitors for binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on binding. Prepare the required amount of wild-type and/or mutated consensus oligonucleotide by adding 2 µl of appropriate oligonucleotide to 43 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 prior to addition of the cell extract.

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	0.01 µl	0.1 µl	0.6 µl	1.2 µl
	Protease Inhibitor Cocktail	0.12 µl	0.9 µl	5.4 µl	10.8 µl
	Lysis Buffer AM1	11.12 µl	89.0 µl	534.0 µl	1.068 ml
	TOTAL REQUIRED	**11.25 µl**	**90.0 µl**	**540.0 µl**	**1.08 ml**
Complete Binding Buffer	DTT	0.09 µl	0.72 µl	4.32 µl	8.64 µl
	Binding Buffer AM6	44.9 µl	359.3 µl	2.15 ml	4.31 ml
	TOTAL REQUIRED	**45 µl**	**360 µl**	**2.16 ml**	**4.32 ml**
Complete Binding Buffer with wild-type or mutated oligonucleotide	Wild-type or mutated oligo	2 µl	16 µl	96 µl	N/A
	Complete Binding Buffer	43 µl	344 µl	2.158 ml	N/A
	TOTAL REQUIRED	**45 µl**	**360 µl**	**2.16 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	202.5 µl	1.62 ml	9.72 ml	19.44 ml
	10X Ab Binding Buffer AM3	22.5 µl	180 µl	1.08 ml	2.16 ml
	TOTAL REQUIRED	**225 µl**	**1.8 ml**	**10.8 ml**	**21.6 ml**
Developing Solution	**TOTAL REQUIRED**	**112.5 µl**	**900 µl**	**5.4 ml**	**10.8 ml**
Stop Solution	**TOTAL REQUIRED**	**112.5 µl**	**900 µl**	**5.4 ml**	**10.8 ml**

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

MAPK 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 MAPK-regulated transcription factors to their consensus sequence

1. Add 40 µl Complete Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 40 µl Complete Binding Buffer that contains 20 pmol (2 µl) of the wild-type or mutated consensus oligonucleotide (see the Buffer Preparation section above for a description of competitive binding).
2. **Sample wells:** Add 10 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 can be found on page 12.

   Positive control wells: Add 5 µg of the positive control nuclear extract diluted in 10 µl of Complete Lysis Buffer per well (2 µl of extract in 8 µl of Complete Lysis Buffer per well). Please refer to page 9 for specific positive control recommendations.

   Blank wells: Add 10 µl Complete Lysis Buffer only per well.
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).
4. Wash each well 3 times with 200 µl 1X Washing 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 of one of the diluted antibodies (1:500 dilution for phosphorylated c-Jun and 1:1000 dilution for all other antibodies in 1X Antibody Binding Buffer) to each well.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. Wash the wells 3 times with 200 µl 1X Washing Buffer (as described in Step 1, No. 4).

Step 3: Binding of secondary antibody

1. Add 100 µl diluted HRP-conjugated antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used. Use the mouse HRP with the phosphorylated ATF-2 antibody; all other antibodies require the rabbit HRP.
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 Washing 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. Monitor the blue color development in the sample and positive control wells until it turns medium to dark blue. Do not overdevelop.
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 reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

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 0.5 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.

References

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3. Youn H.D. *et al.* (2000) *J. Biol. Chem.* 275(29): 22563-22567.
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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 nuclear extract per well	Decrease amount of nuclear 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 nuclear extract per well	Increase amount of nuclear extract not to exceed 40 µg/well
	The factor is poorly activated or inactivated	Perform a time course for activation in the studied cell line
	Extracts are not from correct species	Refer to cross-reactivity information on page 5

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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Notes