TransAM™

ATF-2

Transcription Factor Assay Kits

(version B)

Catalog Nos. 42396 & 42896)

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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, time consuming and lack high-throughput screening methods.

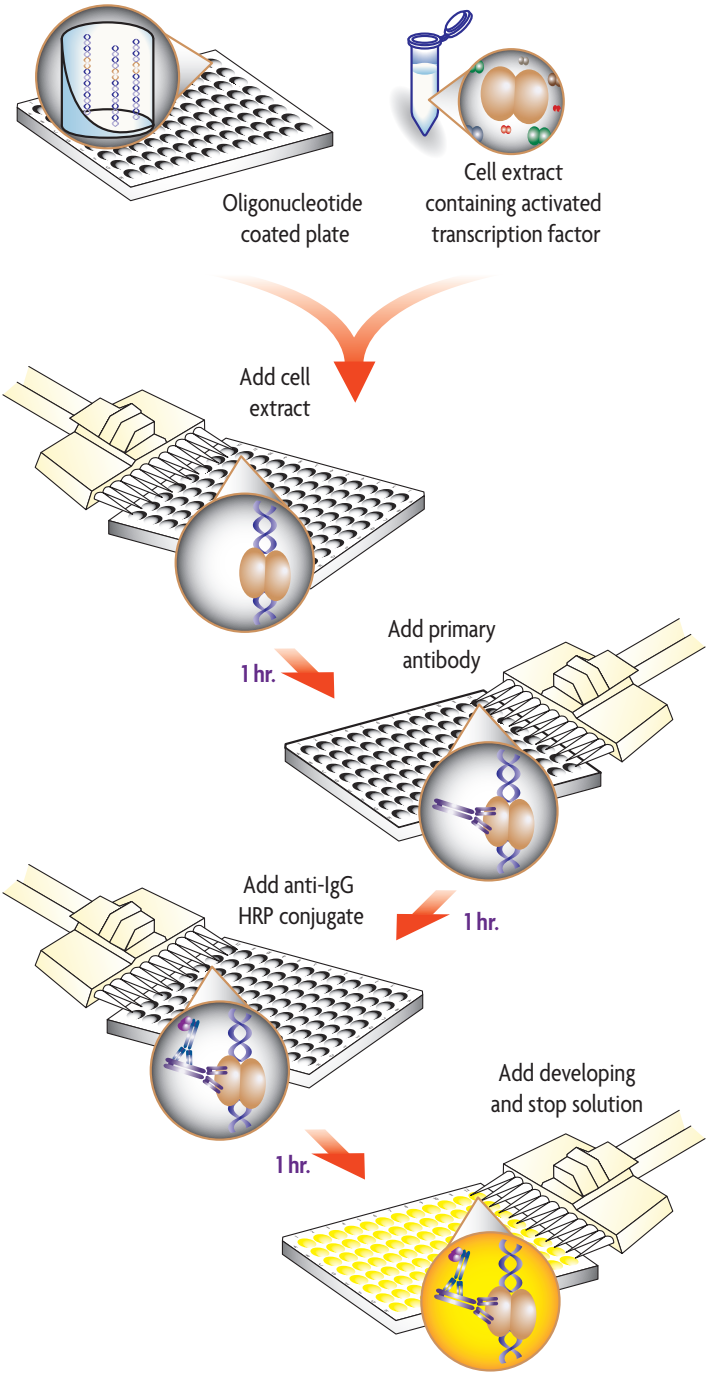
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM ATF-2 Kits are designed specifically for the study of the MAPK regulated transcription factor ATF-2. The kits contain a 96-well plate to which oligonucleotide containing an ATF-2 consensus binding site has been immobilized. The activated ATF-2 contained in nuclear extracts specifically binds to this oligonucleotide. By using an antibody that is directed against phosphorylated ATF-2, the activated complex 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 ATF-2 Kits are available in two sizes:

product	format	catalog no.
TransAM™ ATF-2	1 x 96 rxns	42396
	5 x 96 rxns	42896

Active Motif also offers TransAM MAPK Family Kit for the study of ATF-2, c-Jun, c-Myc, MEF2 and STAT1, all of which are regulated by MAP kinase cascades. See these and other Active Motif products related to ATF-2 in Appendix, Section C.

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

Flow Chart of Process



MAPK Regulated Transcription Factor

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, cell 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 such as ATF-2, either directly or indirectly:

1. ATF-2 expression can be measured by Western blot, using antibodies raised against ATF-2. 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 ATF-2 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 ATF-2 binding. If ATF-2 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 ATF-2 activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing the ATF-2 consensus sequence. This promoter can be artificial, made of several ATF-2 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 ATF-2

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 factor ATF-2. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM ATF-2 Kits contain a 96-well plate to which an oligonucleotide containing the ATF-2 consensus site has been immobilized. The active form of ATF-2 contained in the nuclear extract specifically binds to this oligonucleotide. The primary antibodies used to detect ATF-2 will recognize an epitope on phosphorylated ATF-2 that is accessible only when ATF-2 is activated and bound to the target DNA. Utilization 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 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 1 µg of nuclear extract. A comparable assay using EMSA required 10 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.

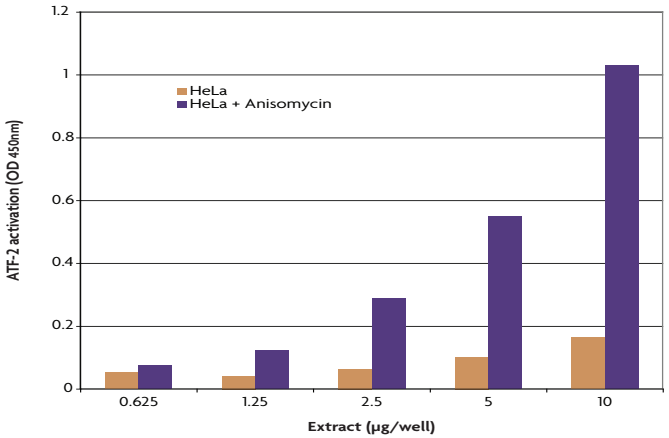
Kit Performance and Benefits

Detection limit: < 1 µg nuclear extract/well. TransAM ATF-2 is 10-fold more sensitive than EMSA.

Range of detection: TransAM provides quantitative results from 1 to 10 µg of nuclear extract/well.

Cross-reactivity: TransAM ATF-2 detects phosphorylated ATF-2 (Thr 71) from human origin. Reactivity with other species has not been determined.

Assay time: 3.5 hours.



Monitoring ATF-2 activation with the TransAM ATF-2 Kit:

Nuclear extracts from HeLa cells (Catalog No. 36010) and HeLa cells stimulated with anisomycin (Catalog No. 36111) were assayed from 0.625-10 µg/well for ATF-2 activation using the TransAM ATF-2 Kit. Data shown are the results from wells assayed in duplicate. These results are provided for demonstration only.

Kit Components and Storage

TransAM ATF-2 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
Phosphorylated ATF-2 antibody	11 µl / 55 µl	4°C for 6 months
Anti-mouse HRP-conjugated antibody	11 µl / 55 µl	4°C for 6 months
Wild-type oligonucleotide AM2	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM2	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
HeLa (Anisomycin) 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
Lysis Buffer AM1	10 ml / 50 ml	4°C for 6 months
Binding Buffer AM6	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 AM3	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 ATF-2 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 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 AM1 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). Prepare the amount of Complete Lysis Buffer required for the assay by adding 1 µl of 1 M DTT and 10 µl 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 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 AM6 required for the assay by adding 2 µl of 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 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 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 it is necessary to completely resuspend the precipitates 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.

Nuclear extract

The HeLa (Anisomycin) nuclear extract is provided as a positive control for ATF-2 activation. 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 C. Related Products).

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for ATF-2 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 ATF-2 consensus binding sequence. Used at 20 pmol/well, the wild-type oligonucleotide will prevent ATF-2 binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on ATF-2 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 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	0.01 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.

ATF-2 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 ATF-2 to its 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 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 1-10 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts is provided on page 13.

   Positive control wells: Add 5 µg of the provided HeLa (Anisomycin) nuclear extract in 10 µl of Complete Lysis Buffer per well (2 µl of nuclear extract in 8 µl of Complete Lysis Buffer per well).

   Blank wells: Add 10 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	40 µl	40 µl	40 µl	38 µl	38 µl
Wild-type oligonucleotide	–	–	–	2 µl	–l
Mutated oligonucleotide	–	–	–	–	2 µl
Complete Lysis Buffer	10 µl	–	–	–	–
Sample in Complete Lysis Buffer	–	10 µl	10 µl	10 µl	10 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 100 µl of diluted phosphorylated ATF-2 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 100 µl 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 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric Reaction

1. Add 100 µl 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 kit lot. Monitor the blue color development in the sample 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 an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

References

1. Yang S.H. *et al.* (1998) *EMBO J.* 17: 1740-1749.
2. Pearson G. *et al.* (2001) *Endocrine Rev.* 22(2): 153-183.
3. Youn H.D. *et al.* (2000) *J. Biol. Chem.* 275(29): 22563-22567.
4. Ramsauer K. *et al.* (2002) *PNAS 99*(20): 12859-12864.
5. Sano Y. *et al.* (1999) *J. Biol. Chem.* 274(13): 8949-8957.

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 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 2.5 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 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
	ATF-2 is poorly activated or inactivated in nuclear fractions	Perform a time course for ATF-2 activation in the studied cell line
	Nuclear extracts are not from correct species	The ATF-2 antibody works in human samples. It has not been tested for cross-reactivity in other species.
	Salt concentration too high in binding reaction	Reduce amount of extract per well or dialyze extract before use

Section C. Related Products

Transcription Factor ELISAs	Format	Catalog No.
TransAM™ AML-1/Runx1	1 x 96-well plate	47396
TransAM™ AML-3/Runx2	1 x 96-well plate	44496
TransAM™ AP-1 Family	2 x 96-well plates	44296
TransAM™ AP-1 c-Fos	1 x 96-well plate	44096
TransAM™ AP-1 c-Jun	1 x 96-well plate	46096
TransAM™ AP-1 FosB	1 x 96-well plate	45096
TransAM™ AP-1 JunD	1 x 96-well plate	43496
TransAM™ ATF-2	1 x 96-well plate	42396
TransAM™ c-Myc	1 x 96-well plate	43396
TransAM™ C/EBP α/β	1 x 96-well plate	44196
TransAM™ CREB	1 x 96-well plate	42096
TransAM™ pCREB	1 x 96-well plate	43096
TransAM™ Elk-1	1 x 96-well plate	44396
TransAM™ ER	1 x 96-well plate	41396
TransAM™ FKHR (FOXO1)	1 x 96-well plate	46396
TransAM™ GATA Family	2 x 96-well plates	48296
TransAM™ GATA-4	1 x 96-well plate	46496
TransAM™ GR	1 x 96-well plate	45496
TransAM™ HIF-1	1 x 96-well plate	47096
TransAM™ HNF Family	2 x 96-well plates	46296
TransAM™ HNF-1	1 x 96-well plate	46196
TransAM™ IRF-3 (Human)	1 x 96-well plate	48396
TransAM™ IRF-3 (Mouse)	1 x 96-well plate	48496
TransAM™ IRF-7	1 x 96-well plate	50196
TransAM™ MAPK Family	2 x 96-well plates	47296
TransAM™ MEF2	1 x 96-well plate	43196
TransAM™ MyoD	1 x 96-well plate	47196
TransAM™ NF-YA	1 x 96-well plate	40396
TransAM™ NFATc1	1 x 96-well plate	40296
TransAM™ NFκB Family	2 x 96-well plates	43296
TransAM™ Flexi NFκB Family	2 x 96-well plates	43298
TransAM™ NFκB p50	1 x 96-well plate	41096
TransAM™ Flexi NFκB p50	1 x 96-well plate	41098
TransAM™ NFκB p50 Chemi	1 x 96-well plate	41097
TransAM™ NFκB p52	1 x 96-well plate	48196
TransAM™ NFκB p52 Chemi	1 x 96-well plate	48197
TransAM™ NFκB p65	1 x 96-well plate	40096
TransAM™ Flexi NFκB p65	1 x 96-well plate	40098
TransAM™ NFκB p65 Chemi	1 x 96-well plate	40097
TransAM™ Nrf2	1 x 96-well plate	50296
TransAM™ Oct-4	1 x 96-well plate	41196
TransAM™ p53	1 x 96-well plate	42496
TransAM™ PPARγ	1 x 96-well plate	40196
TransAM™ Sp1	1 x 96-well plate	41296
TransAM™ Sp1/Sp3	1 x 96-well plate	40496
TransAM™ STAT Family	2 x 96-well plates	42296
TransAM™ STAT3	1 x 96-well plate	45196
TransAM™ T-bet	1 x 96-well plate	51396

Function ELISA	Format	Catalog No.
FunctionELISA IκBα	1 x 96-well plate	48005

Nuclear Extract	Format	Catalog No.
Nuclear Extract Kit	100 rxns	40010
	400 rxns	40410

Recombinant Proteins	Format	Catalog No.
Recombinant c-Fos protein	5 µg	31115
Recombinant c-Jun protein	5 µg	31116
Recombinant c-Myc protein	5 µg	31117
Recombinant CREB protein	25 µg	31107
Recombinant elF2a protein	25 µg	31108
Recombinant NFkB p50 protein	5 µg	31101
Recombinant NFkB p65 protein	5 µg	31102
Recombinant p53 protein	5 µg	31103
Recombinant p300 protein	4 µg	31124
Purified Sp1 protein	2 µg	31137
Recombinant STAT3 protein	10 µg	31140

Cell Extracts	Format	Catalog No.
HeLa	200 µg	36010
HeLa (Anisomycin)	200 µg	36111

Histone Purification	Format	Catalog No.
Histone Purification Kit	10 rxns	40025
Histone Purification Mini Kit	20 rxns	40026

Co-Immunoprecipitation	Format	Catalog No.
Nuclear Complex Co-IP Kit	50 rxns	54001
Universal Magnetic Co-IP Kit	25 rxns	54002

DNA Methylation	Format	Catalog No.
MethylDetector™	50 rxns	55001
MethylCollector™	25 rxns	55002
MethylCollector™ Ultra	30 rxns	55005
UnMethylCollector™	30 rxns	55004

SUMOylation	Format	Catalog No.
SUMOlink™ SUMO-1 Kit	20 rxns	40120
SUMOlink™ SUMO-2/3 Kit	20 rxns	40220

Histone ELISAs	Format	Catalog No.
Histone H3 monomethyl Lys4 ELISA	1 x 96 rxns	53101
Histone H3 dimethyl Lys4 ELISA	1 x 96 rxns	53102
Histone H3 trimethyl Lys4 ELISA	1 x 96 rxns	53103
Histone H3 phospho Ser28 ELISA	1 x 96 rxns	53100

ChIP-validated Antibodies	Application	Format	Catalog No.
AP-2 pAb	ChIP, EMSA	17 rxns	39304
C/EBPα pAb	ChIP, EMSA, IF, WB	100 µg	39306
c-Jun pAb	ChIP, EMSA, IF	100 µg	39309
DNMT1 mAb	ChIP, IHC, IP, WB	100 µg	39204
DNMT3A mAb	ChIP, IF, IHC, WB	100 µg	39206
DNMT3B mAb	ChIP, IF, IP, WB	100 µg	39207
E2F-1 pAb	ChIP, EMSA	17 rxns	39313
E2F-6 mAb	ChIP, WB	100 µl	39509
EZH2 pAb	ChIP, IF, IP, WB	200 µl	39103
HBP-1 mAb	ChIP, IF, WB	100 µl	39511
HDAC1 mAb (Clone 10E2)	ChIP, IF, IHC, IP, WB	200 µl	39531
HDAC2 mAb (Clone 3F3)	ChIP, IF, IHC, IP, WB	200 µl	39533
HDAC3 pAb	ChIP, WB	100 µg	40968
HDAC4 pAb	ChIP, WB	100 µg	40969
HDAC5 pAb	ChIP, WB	100 µg	40970
HDAC6 pAb	ChIP, WB	100 µg	40971
Histone H2A pAb	ChIP, WB	200 µl	39235
Histone H2A phospho Ser129 pAb	ChIP, IF, IP, WB	200 µl	39271
Histone H2B pAb	ChIP, WB	200 µl	39237
Histone H3 acetyl Lys27 pAb	ChIP, IF, WB	200 µg	39133
Histone H3 acetyl Lys27 pAb	ChIP, WB	200 µl	39135
Histone H3 dimethyl Lys4 pAb	ChIP, WB	200 µl	39141
Histone H3 dimethyl Lys9 pAb	ChIP, IF, WB	200 µl	39239
Histone H3 trimethyl Lys4 pAb	ChIP, WB	200 µl	39159
Histone H3 trimethyl Lys9 pAb	ChIP, WB	200 µl	39161
Histone H3 trimethyl Lys27 mAb	ChIP, WB	200 µl	39535
Histone H3, C-terminal pAb	ChIP, WB	200 µl	39163
Histone H4 acetyl Lys5 pAb	ChIP, IF, WB	200 µl	39169
Histone H4 acetyl Lys12 pAb	ChIP, WB	200 µl	39165
Histone H4 acetyl Lys16 pAb	ChIP, WB	200 µl	39167
Histone H4 monomethyl Lys20 pAb	ChIP, IF, WB	200 µl	39175
Histone H4 trimethyl Lys20 pAb	ChIP, IF, WB	200 µl	39180
Histone H4 tetra-acetyl pAb	ChIP, WB	50 µl	39179
HP1 alpha pAb	ChIP, IP, WB	200 µl	39295
IRF-3 pAb	ChIP, WB	100 µl	39033
JunB pAb	ChIP, EMSA	17 rxns	39326
JunD pAb	ChIP, EMSA	100 µl	39328
L3MBTL1 pAb	ChIP, IP, WB	200 µl	39182
Mi-2 beta pAb	ChIP, IF, IP, WB	200 µl	39289
p53 pAb	ChIP, EMSA	17 rxns	39334
PP2A pAb	ChIP, IP, WB	200 µl	39192
RbAp46/48 pAb	ChIP, WB	200 µl	39198
RNA pol II mAb	ChIP, ELISA, IF, IP, WB	200 µl	39097
SNF2h mAb	ChIP, IF, IP, WB	200 µl	39543
Sp1 pAb	ChIP, WB	100 µl	39058
TRF2 Goat pAb	ChIP, IP, WB	100 µg	39223

Application Key: ChIP = Chromatin Immunoprecipitation; **EMSA** = Electrophoretic Mobility Shift Assay; **IF** = Immunofluorescence; **IHC** = Immunohistochemistry; **IP** = Immunoprecipitation; **WB** = Western blot;

For an up-to-date list of ChIP-validated antibodies, please visit **www.activemotif.com/chipabs**

ChIP-IT™ Kits	Format	Catalog No.
ChIP-IT™ Express	25 rxns	53008
ChIP-IT™ Express Enzymatic	25 rxns	53009
ChIP-IT™ Express HT	96 rxns	53018
ChIP-IT™ Protein G Magnetic Beads	25 rxns	53014
ChIP-IT™	25 rxns	53001
ChIP-IT™ w/o controls	25 rxns	53004
ChIP-IT™ Shearing Kit	10 rxns	53002
ChIP-IT™ Enzymatic	25 rxns	53006
ChIP-IT™ Enzymatic w/o controls	25 rxns	53007
Enzymatic Shearing Kit	10 rxns	53005
Salmon Sperm DNA/Protein G agarose	25 rxns	53003
ChIP-IT™ Control Kit – Human	5 rxns	53010
ChIP-IT™ Control Kit – Mouse	5 rxns	53011
ChIP-IT™ Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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