TransAM® AP-1 Family

Transcription Factor Assay Kit

(version D5)

Catalog Nos. 44296

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Overview

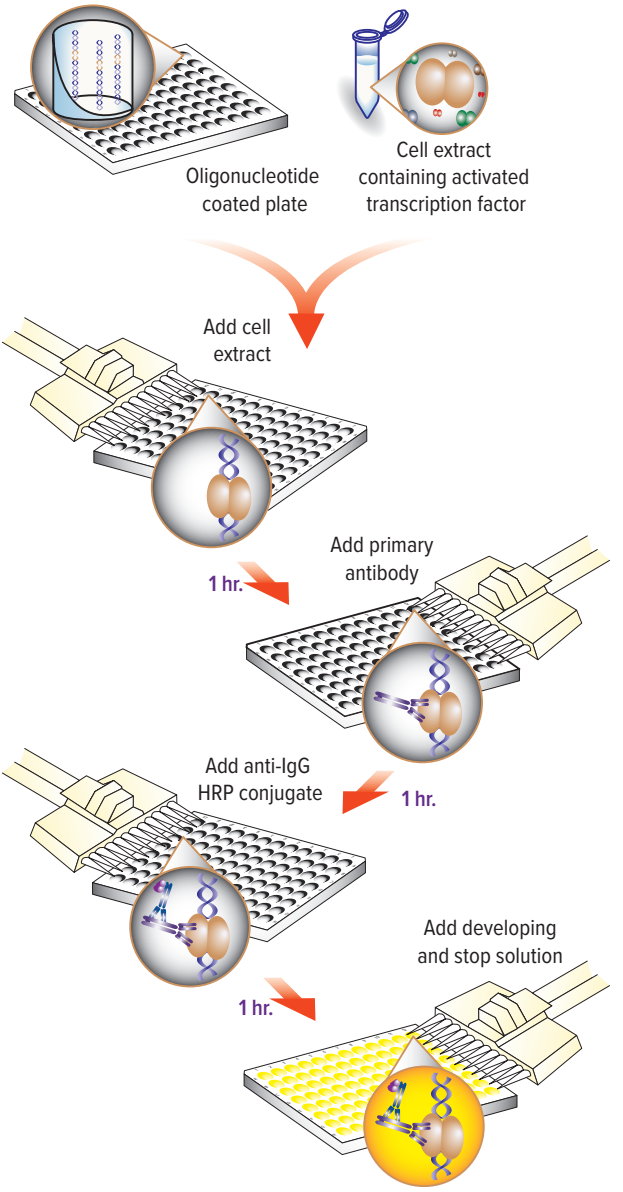
Activator protein-1 (AP-1) is a transcription factor that is activated during the cell cycle to promote cell survival, differentiation and adaptive responses. Therefore, accurate monitoring of AP-1 activation in cells, tissues and animals is crucial for many biomedical research and drug development projects. 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 AP-1 Family Kits are designed specifically to detect and quantify AP-1 activation. They contain a 96-well plate to which oligonucleotide containing a TPA-responsive element (TRE) has been immobilized. AP-1 dimers contained in nuclear cell extracts bind specifically to this oligonucleotide and are detected through use of an antibody directed against c-Fos, FosB, Fra-1, c-Jun, JunB or JunD. 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 individual strips of 8 wells is suitable for manual use or for high-throughput screening applications. TransAM AP-1 Family Kits are available in one convenient format:

product	format	catalog no.
TransAM® AP-1 Family	2 x 96 rxns	44296

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

Flow Chart of Process



Introduction

AP-1 Transcription Factor

The activator protein-1 (AP-1) transcription factors belong to a large family of structurally related transcription factors that includes ATF1-4, c-Fos, c-Jun, c-Myc and C/EBP (see 1-2 for review). The members of this family, named bZIP, share a dimerization domain with a leucine zipper motif and a DNA binding domain rich in basic residues (lysines and arginines). AP-1 is composed of a mixture of heterodimeric complexes of proteins derived from the Fos and Jun families including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD. Only Jun proteins can form transcriptionally active homodimers within AP-1 members, or heterodimers with CREB/ATF members, to bind the CRE element (5´-TGACGTCA-3´)[1]. Primarily, AP-1 dimers bind to DNA on a TPA-response element (TRE) with the 5´-TGA(C/G)TCA-3´ sequence[3]. Jun-Fos heterodimers form more stable complexes with TREs. These complexes display stronger transactivating activity than Jun-Jun homodimers[4].

Phosphorylation of AP-1 family members by kinases is required for transactivation activity. In the case of c-Jun, the activation domain is regulated to a large extent by the JNK family of MAP kinases[5]. JNK kinases phosphorylate c-Jun at Ser-63, resulting in the binding of c-Jun to the CBP/p300 family of transcriptional co-activators[6]. For the Fos proteins, both N- and C-terminal domains flanking the bZIP domain require phosphorylation for biological activity. The kinases responsible for activation remain to be determined[7-8].

AP-1 expression is induced by multiple stimuli such as serum, growth factors, phorbol esters and oncogenes[2]. These include peptide growth factors, cytokines of the TGF-β, TNF, and interferon families, neuronal depolarization and cellular stress. The total increase in AP-1 DNA binding activity after any stimulus is often much less impressive than would be expected based on analysis of mRNA expression. This increase is accompanied by a change in the composition of AP-1 complexes shortly after stimulation in order to modulate transcriptional control[9]. Upon serum starvation of human fibroblast cells, Fos and Jun protein production can be induced for up to 4 hours by adding serum. Interestingly, serum starvation lowers basal expression of FosB and c-Fos but has no significant effect on c-Jun.

AP-1 proteins play a role in the expression of many genes involved in proliferation and cell cycle progression including neuronal apoptosis, learning process, drug-induced behavioral responses, bone growth and differentiation, and embryo development. For instance, cell transformation by oncogenes that function in the growth factor signal transduction pathway, such as *ras*, *ras*F and *mek*, results in a high increase in AP-1 component protein expression (see 10 for review). Therefore, AP-1-regulated genes support the invasive process observed during malignancy and metastasis.

Transcription Factor Assays

To date, three methods are widely used to measure AP-1 activation, either directly or indirectly:

1. AP-1 activation can be determined by Western blot by using antibodies specific for AP-1 proteins. This method is time consuming (up to 2 days once the nuclear cell extracts are prepared), and is not suitable for processing large numbers of samples.

2. The DNA-binding capacity of AP-1 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 AP-1 binding. If AP-1 is active in the nuclear extract, it will bind to the probe. To detect phosphorylated AP-1 proteins, a supershift step in the presence of phospho-specific antibodies is required. 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 AP-1 activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing the TRE sequences. The promoter can be artificial, made of several TRE cis-elements and a TATA box, or natural, like the collagenase promoter sequence. However, the procedure is limited by the following issues: (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, and therefore assays have to be carefully standardized. Reporter gene assays are sensitive and easy to perform with a large number of samples, but require efficient cell transfection with the reporter plasmid.

TransAM AP-1

AP-1 is a key factor in various and distinct biological functions such as cell proliferation and survival, and therefore represents an excellent pharmacological target. However, pharmaceutical research in this field has been limited by the lack of convenient assays suitable for high sample volume.

To overcome this, Active Motif is introducing a high-throughput assay to quantify AP-1 activation[11]. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM AP-1 Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains a TRE (5´-TGAGTCA-3´). AP-1 contained in nuclear extract specifically binds to this oligonucleotide. The primary antibodies used in TransAM AP-1 Kits recognize accessible epitopes on c-Fos, FosB, Fra-1, c-Jun, JunB or JunD proteins upon DNA binding. Addition of a secondary HRP-conjugated antibody provides a sensitive colorimetric read-out 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 AP-1 activation and has been shown to be 5-fold more sensitive and 20-fold faster than the gel-retardation technique. With the 3.5-hour TransAM procedure, we could detect AP-1 activation using as little as 1.25 µg of nuclear extract from TPA-stimulated cells. A comparable assay using EMSA required 2.5 µg of nuclear extract and a 3-day autoradiography.

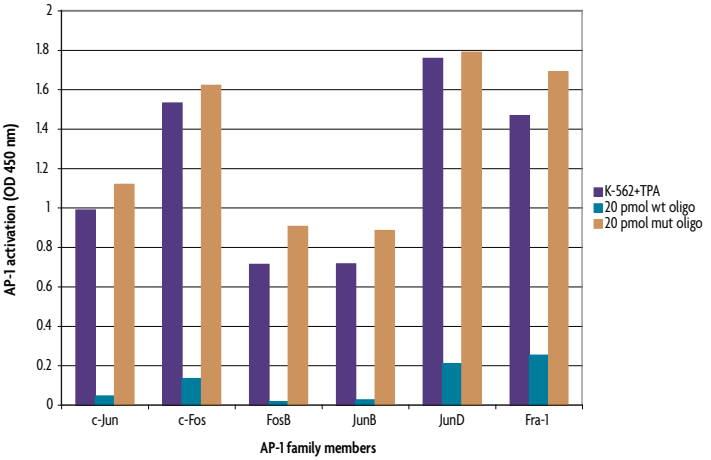
TransAM has many applications including the study of drug potency toward AP-1 related kinase, AP-1 transcriptional activity regulation and protein structure/function studies of AP-1 and its cofactors in areas such as cell differentiation and survival in tumorigenesis, hematopoiesis, learning process, drug-induced behavioral responses, and many more.

Kit Performance and Benefits

TransAM AP-1 Kits are for research use only. Not for use in diagnostic procedures.

Detection limit: < 1.25 µg nuclear extract/well. TransAM AP-1 is 5-fold more sensitive than EMSA.

Range of detection: TransAM provides quantitative results from 0.1 to 20 µg of nuclear extract/well.



AP-1 family profiling for DNA binding activation upon addition of TPA. Nuclear extracts from K-562 cells stimulated with TPA were assayed for activity of AP-1 family members c-Jun, c-Fos, FosB, JunB, JunD and Fra-1. Each antibody was tested with 5 µg/well of nuclear extract in the absence or presence of wild-type or mutated consensus binding oligonucleotides using the TransAM AP-1 Family Kit.

Cross-reactivity:

	FosB	c-Fos	Fra-1	c-Jun*	JunB	JunD
Human	+	+	+	+	+	+
Mouse	+	+	-	+	-	+
Rat	+	+	-	nt	-	+

nt = not tested

* c-Jun antibody detects c-Jun phosphorylated at S73 and JunD phosphorylated at S100.

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

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
c-Fos or Fra-1 antibodies	11 µl	4°C for 6 months
JunB or JunD antibodies	11 µl	-20°C for 6 months
FosB or Phospho-c-Jun antibodies	22 µl	-20°C for 6 months
Anti-rabbit HRP-conjugated antibody	2 x 11 µl	4°C for 6 months
Wild-type oligonucleotide AM7	100 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM7	100 µl (10 pmol/µl)	-20°C for 6 months
K-562 (TPA) nuclear extract	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
Poly [d(I-C)]	100 µl (17 µg/µl)	-20°C for 6 months
Lysis Buffer AM1	10 ml	4°C for 6 months
Binding Buffer AM2	10 ml	4°C for 6 months
10X Wash Buffer AM2	60 ml	4°C for 6 months
10X Antibody Binding Buffer AM3	2 x 2.2 ml	4°C for 6 months
Developing Solution	2 x 11 ml	4°C for 6 months
Stop Solution	60 ml	4°C for 6 months
96-well AP-1 assay plate	2	4°C for 6 months
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)

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). Lysis Buffer AM1 contains phosphatase inhibitors to prevent dephosphorylation of AP-1 during the extract preparation and the assay. The presence of these inhibitors gives a yellow coloration to Lysis Buffer AM1. 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 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 1 µl of 1 M DTT and 10 µl of 17 µg/ml poly[d(I-C)] per ml of Binding Buffer AM2 (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 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 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 primary antibody with the 1X Antibody Binding Buffer to 1:500 for the FosB and Phospho-c-Jun antibodies or to 1:1000 for the c-Fos, Fra-1, JunB, JunD and HRP-conjugated secondary antibodies. 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 K-562 (TPA) nuclear extract is provided as a positive control for AP-1 activation. This extract is optimized to give a strong signal when used at 5 µg/well for c-Fos, FosB, c-Jun, JunB, JunD and Fra-1. Sufficient extract is supplied for 20 reactions. We recommend aliquoting the extract in 5 µl fractions and storing at -80ºC. Avoid multiple freeze/thaw cycles of the extract.

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for AP-1 binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent AP-1 binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on AP-1 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 prior to addition of the nuclear 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	0.02 µl	0.2 µl	1.2 µl	2.4 µl
	Protease inhibitor cocktail	0.23 µl	1.8 µl	10.8 µl	21.6 µl
	Lysis Buffer AM1	22.25 µl	178.0 µl	1.068 ml	2.136 ml
	TOTAL REQUIRED	**22.5 µl**	**180.0 µl**	**1.08 ml**	**2.16 ml**
Complete Binding Buffer	DTT	0.03 µl	0.3 µl	1.8 µl	3.6 µl
	Poly[d(I-C)]	0.34 µl	2.7 µl	16.2 µl	32.4 µl
	Binding Buffer AM2	33.4 µl	267.0 µl	1.602 ml	3.2 ml
	TOTAL REQUIRED	**33.8 µl**	**270.0 µl**	**1.62 ml**	**3.24 ml**
Complete Binding Buffer with wild-type or mutated oligonucleotide	Wild-type or mutated oligo	2.0 µl	16.0 µl	96.0 µl	N/A
	Complete Binding Buffer	31.8 µl	254.0 µl	1.524 ml	N/A
	TOTAL REQUIRED	**33.8 µl**	**270.0 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	225.0 µl	1.8 ml	10.8 ml	21.6 ml
	TOTAL REQUIRED	**2.25 ml**	**18.0 ml**	**108.0 ml**	**216.0 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	180.0 µl	1.08 ml	2.16 ml
	TOTAL REQUIRED	**225.0 µl**	**1.8 ml**	**10.8 ml**	**21.6 ml**
Developing Solution	**TOTAL REQUIRED**	**112.5 µl**	**900.0 µl**	**5.4 ml**	**10.8 ml**
Stop Solution	**TOTAL REQUIRED**	**112.5 µl**	**900.0 µl**	**5.4 ml**	**10.8 ml**

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

AP-1 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 AP-1 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 consensus oligonucleotide (see the Buffer Preparation section above for a description of competitive binding).

2. **Sample wells:** Add 20 µl of sample diluted in Complete Lysis Buffer per well. We recommend using 2-20 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts can be found on page 12.

   Positive control wells: Add 5 µg of the positive control nuclear extract diluted in 20 µl of Complete Lysis Buffer per well (2 µl of nuclear extract in 18 µl of Complete Lysis Buffer per well). Please refer to page 9 for recommended positive control information.

   Blank wells: Add 20 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 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 one of the diluted AP-1 antibodies (FosB and Phospho-c-Jun antibodies diluted to 1:500 in 1X Antibody Binding Buffer, or a 1:1000 dilution in 1X Antibody Binding Buffer for the c-Fos, Fra-1, JunB or JunD antibodies) to each well 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 of diluted HRP-conjugated 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 Developing Solution to all wells being used.
2. Incubate 2-20 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.

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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.
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.
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.
9. Determine the protein concentration of the extract by using a Bradford-based assay.

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
	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 is too high	Increase antibody dilutions
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
Uneven color development	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Well cross-contamination	Follow washing recommendations
High background in sample wells	Too much nuclear extract per well	Decrease amount of nuclear extract down to 1-2 µg/well
	Concentration of antibodies is too high	Perform antibody titration to determine optimal working concentration. Start using 1:1000 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 to 50 µg/well
	AP-1 is poorly activated or inactivated in nuclear fractions	Perform a time course for AP-1 activation in the studied cell line
	Nuclear extracts are not from correct species	

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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