TransAM™

NF-YA Transcription Factor Assay Kits

(version C1)

Catalog Nos. 40396 & 40896

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Overview

NF-Y is an ubiquitously expressed heterodimeric transcription factor composed of three subunits (NF-YA, NF-YB and NF-YC). All three subunits are required for constitution of a functional binding complex to the CCAAT box. There is mounting evidence that NF-YA, whose levels vary in different cell types and/or growth conditions, is a regulatory subunit of the trimeric complex. Therefore, accurate monitoring of NF-YA activity in cells, tissues or animals is crucial for biomedical research and drug development. To date, such research projects are tedious and time consuming, and lack high-throughput screening methods.

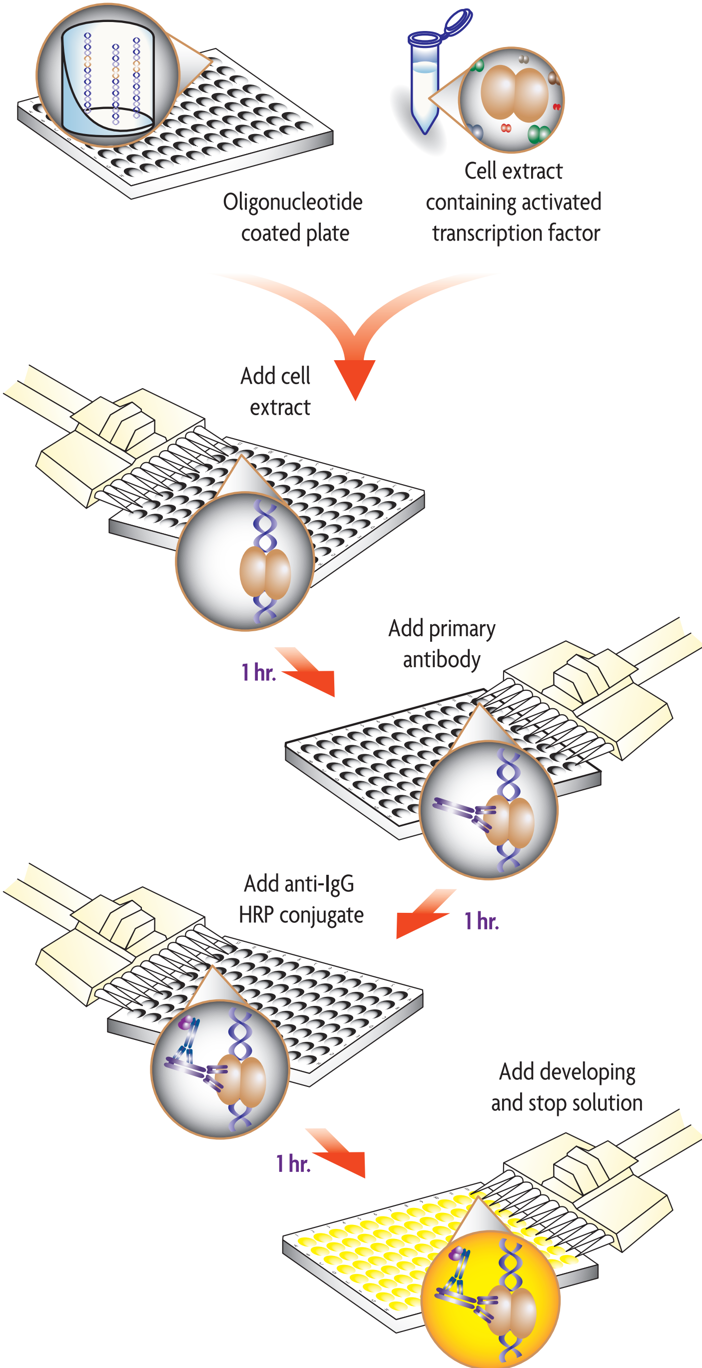
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM NF-YA Kits are designed specifically for the study of NF-YA regulated genes. They contain a 96-well plate to which an oligonucleotide containing the NF-YA consensus binding site has been immobilized. NF-YA contained in nuclear extract binds specifically to this oligonucleotide and is detected through use of an antibody directed against NF-YA. 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 NF-YA Kits are available in two sizes:

product	format	catalog no.
TransAM™ NF-YA	1 x 96 rxns	40396
	5 x 96 rxns	40896

See Active Motif products related to the NF-YA transcription factor in Appendix, Section C.

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

Flow Chart of Process



Introduction

NF-YA Transcription Factor

CCAAT boxes are found in the regulatory region of ~30% of genes. These include so-called house-keeping genes as well as inducible and cell cycle-regulated genes[8]. The role of a CCAAT box as a cis-acting promoter element depends on its ability to interact with the appropriate transcription factor. NF-Y is the major transcription factor that recognizes the CCAAT box[8].

NF-Y is a complex minimally composed of three subunits: NF-YA, NF-YB and NF-YC, which are all required for DNA binding[7, 11]. The NF-YB and NF-YC subunits form a tight heterodimer that offers a complex surface for NF-YA association. The resulting trimer can then bind to DNA with high specificity and affinity[2]. NF-YB and NF-YC contain a histone-like motif and play a basic role in gene activation[1, 8]. Most of the sequence-specific interactions of the trimer are made by NF-YA[8]. The N-terminal part of NF-YA is required for association with the NF-YB/NF-YC heterodimer, while the NF-YA C-terminus is required for DNA binding. Therefore, NF-YA represents the regulatory subunit of the NF-Y trimer[9].

Although NF-Y factors are considered to be ubiquitously expressed, their levels or activities vary depending on cell type, cell stage of growth and degree of cell differentiation[4, 5, 8, 9]. Recent data also suggest a modulation of NF-Y activity during serum starvation or depletion of intracellular calcium[3, 6, 10]. Methods to measure overall levels and modifications of the NF-YA subunit are crucial for understanding transcriptional regulation of the numerous CCAAT box-containing genes.

Transcription Factor Assays

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

1. NF-YA expression can be determined by Western blot by using antibodies specific for NF-YA protein. 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 NF-YA 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 NF-YA binding. If NF-YA 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 NF-YA activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing a NF-YA consensus binding site. The promoter can be artificial, made of a CCAAT box and a TATA box, or natural, like promoter sequences from mammalian gene regulator elements, such as the

h-TERC (human telomerase RNA component) promoter[12]. 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; therefore, assays must 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 NF-YA

NF-YA regulated genes are involved in a variety of cellular pathways that are currently being deciphered by academic and pharmaceutical laboratories for new target discovery. However, this field has been hampered by the lack of convenient assays.

To overcome this, Active Motif is introducing a high-throughput assay to quantify NF-YA activation. TransAM Kits combine a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM NF-YA Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains a NF-YA consensus binding site (5´-CCAAT-3´). NF-YA contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used in the TransAM NF-YA Kit recognizes an epitope on NF-YA protein that is accessible when the protein is bound to 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 NF-YA 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 NF-YA using as little as 0.6 µg of nuclear extract from Jurkat cells. A comparable assay using EMSA would require 3 µg of nuclear extract and a 3-day autoradiography.

TransAM NF-YA has many applications including the study of NF-YA transcriptional activity regulation in a wide range of systems as well as the study of protein structure/function of NF-YA and its mutated variants.

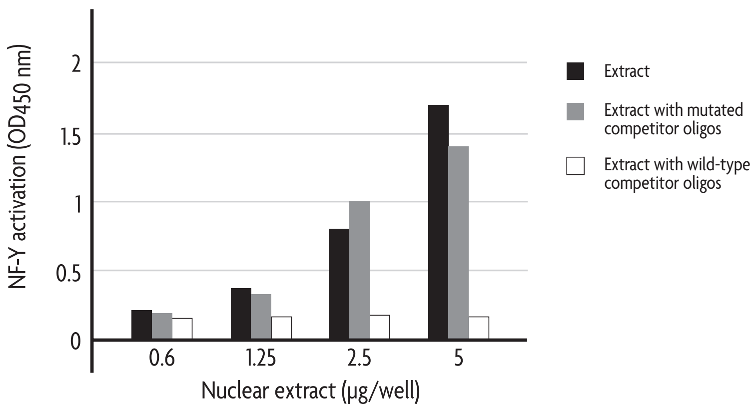
Kit Performance and Benefits

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

Range of detection: TransAM provides quantitative results from 0.6 to 5 µg of nuclear extract/well (see graph below).

Cross-reactivity: TransAM NF-YA detects NF-YA from human, mouse and rat origin.

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



Monitoring NF-YA activation with the TransAM NF-YA Kit:

Different amounts of nuclear extracts from Jurkat cells (Catalog No. 36014) were tested for NF-YA activity by using the TransAM NF-YA Kit. Note that incubation with wild-type NF-Y competitor oligonucleotides reduces NF-YA binding by over 80%, while incubation with mutated NF-Y competitor oligos had no effect on NF-YA binding to DNA. This data is provided for demonstration only.

Kit Components and Storage

TransAM NF-YA 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 below. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity 1 plate / 5 plates	Storage
NF-YA antibody	22 µl / 110 µl (0.2 µg/µl)	4°C for 6 months
Anti-rabbit HRP-conjugated antibody	11 µl / 55 µl (0.4 µg/µl)	4°C for 6 months
Wild-type oligonucleotide AM18	100 µl / 500 µl (20 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM18	100 µl / 500 µl (20 pmol/µl)	-20°C for 6 months
Jurkat 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 AM1	10 ml / 50 ml	4°C for 6 months
Binding Buffer AM1	10 ml / 50 ml	4°C for 6 months
10X Wash Buffer AM1	22 ml / 110 ml	4°C for 6 months
10X Antibody Binding Buffer AM1	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 NF-YA 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 spectrophotometer capable of reading at 450 nm (655 nm as an optional reference wavelength)

For Nuclear Extract preparation

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

Protocols

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). When preparing extracts, it is suggested to perform the final lysis step using the Lysis Buffer AM1 provided in the TransAM Kit. All subsequent dilutions should also be performed using Lysis Buffer AM1 from the TransAM Kit. 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 required for the assay by adding 2 µl of 1 M DTT and 20 µl of 1 µg/µl Herring Sperm DNA per ml of Binding Buffer AM1 (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 AM1 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 AM1 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 AM1 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 AM1 may form clumps, therefore homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use. Dilute the primary antibody to 1:500 and the HRP-conjugated secondary antibody 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. However, 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.

Jurkat nuclear extract

The Jurkat nuclear extract is provided as a positive control for NF-YA activation. Sufficient extract is supplied for 20 reactions per plate. This extract is optimized to give a strong signal when used at 5 µg/well. We recommend aliquoting the extract in 10 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 NF-YA binding in order to monitor the specificity of the assay. Used at 40 pmol/well, the oligonucleotide will prevent NF-YA binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on NF-YA 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 µl	534 µl	1.068 ml
	TOTAL REQUIRED	**11.25 µl**	**90 µl**	**540 µl**	**1.08 ml**
Complete Binding Buffer	DTT	0.09 µl	0.7 µl	4.3 µl	8.6 µl
	Herring sperm DNA	0.89 µl	7 µl	42.7 µl	85.5 µl
	Binding Buffer AM1	44 µl	352 µl	2.112 ml	4.224 ml
	TOTAL REQUIRED	**45 µl**	**360 µl**	**2.160 ml**	**4.32 ml**
Complete Binding Buffer with NF-YA 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	2.064 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 AM1	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 AM1	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.

NF-YA 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 NF-YA 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 40 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 1-5 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts is provided on page 12.

   Positive control wells: Add 5 µg of the provided Jurkat nuclear extract diluted 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.
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 diluted NF-YA antibody (1:500 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. Wash the wells 3 times with 200 µl 1X Washing Buffer (as described in Step 1, No. 4).

Step 3: Binding of Secondary Antibody

1. Add 100 µl of diluted anti-rabbit 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 Washing Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric Reaction

1. Transfer the amount of Developing Solution required for the assay into a secondary container. 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 the optimal development time for this specific kit lot, which varies from lot to lot. 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.

References

1. Baxevanis A.D., Arents G., Moudrianakis E.N. and Landsman D. (1995) *Nucleic Acids Res.* 23(14):2685-91.
2. Bi W., Wu L., Coustry F., de Crombrugghe B. and Maity S.N. (1997) *J Biol Chem.* 272(42):26562-72.
3. Chang Z.F. and Liu C.J. (1994) *J Biol Chem.* 269(27):17893-8.
4. Farina A., Manni I., Fontemaggi G., Tiainen M., Cenciarelli C., Bellorini M., Mantovani R., Sacchi A. and Piaggio G. (1999) *Oncogene* 18(18):2818-27.
5. Good L.F. and Chen K.Y. (1996) *Biol Signals* 5(3):163-9.
6. Kim E.C., Lau J.S., Rawlings S. and Lee A.S. (1997) *Cell Growth Differ.* 8(12):1329-38.
7. McNabb D.S., Tseng K.A. and Guarente L. (1997) *Mol. Cell Biol.* 17(12):7008-18.
8. Mantovani R. (1999) *Gene* 239(1):15-27.
9. Marziali G., Perrotti E., Ilari R., Coccia E.M., Mantovani R., Testa U. and Battistini A. (1999) *Blood* 93(2):519-26.
10. Roy B. and Lee A.S. (1995) *Mol. Cell Biol.* 15(4):2263-74.
11. Sinha S., Maity S.N., Lu J. and de Crombrugghe B. (1995) *PNAS USA.* 92(5):1624-8.
12. Zhao J.Q., Hoare S.F., McFarlane R., Muir S., Parkinson E.K., Black D.M. and Keith W.N. (1998) *Oncogene* 16(10):1345-50.

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
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.

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	Developing 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 1-2 µg/well
	Concentration of antibodies is too high	Perform antibody titration to determine optimal working concentration. Start using 1:1,000 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 10 µl aliquots and store at -80˚C to avoid multiple freeze/thaws
	NF-YA is poorly activated or inactivated in nuclear fractions	Perform a time course for NF-YA activation in the studied cell line
	Nuclear extracts are not from correct species	The NF-YA antibody works in human, mouse and rat samples
	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™ 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™ C/EBP α/β	1 x 96-well plate	44196
TransAM™ ER	1 x 96-well plate	41396
TransAM™ NFκB Family	2 x 96-well plates	43296
TransAM™ NFκB p50	1 x 96-well plate	41096
TransAM™ NFκB p52	1 x 96-well plate	48196
TransAM™ NFκB p65	1 x 96-well plate	40096
TransAM™ p53	1 x 96-well plate	41196
TransAM™ Sp1	1 x 96-well plate	41296
TransAM™ Sp1/Sp3	1 x 96-well plate	40496

For a complete list of the over 40 TransAM™ Kits available, please visit **www.activemotif.com/transam**

In-cell Phospho-specific ELISAs	Format	Colorimetric Kit Catalog No.	Chemiluminescent Kit Catalog No.
FACE™ AKT	1 x 96 rxns	48120	48220
FACE™ EGFR (Y845)	1 x 96 rxns	48340	48440
FACE™ EGFR (Y992)	1 x 96 rxns	48150	48250
FACE™ EGFR (Y1173)	1 x 96 rxns	48190	48290
FACE™ ERK1/2	1 x 96 rxns	48140	48240
FACE™ JNK	1 x 96 rxns	48110	48210
FACE™ MEK1/2	1 x 96 rxns	48180	48280
FACE™ p38	1 x 96 rxns	48100	48200

For a complete, up-to-date list of the over 20 FACE™ Kits available, please visit **www.activemotif.com/face**

Cell Extracts	Format	Catalog No.
3T3 nuclear extract (1 hr serum response)	200 µg	36001
3T3 nuclear extract	200 µg	36000
3T3-L1 nuclear extract	200 µg	36071
3T6 Swiss albino nuclear extract	200 µg	36002
Jurkat cytoplasmic extract	200 µg	36402
Jurkat cytoplasmic extract (TPA + CI)	200 µg	36401
Jurkat nuclear extract	200 µg	36014
Jurkat nuclear extract (CD3 activated)	200 µg	40850
Jurkat nuclear extract (Heat Shock)	200 µg	36069
Jurkat nuclear extract (IFNγ)	200 µg	36094
Jurkat nuclear extract (TPA + CI)	200 µg	36013

Nuclear, Cytoplasmic and Whole-cell Extraction	Format	Catalog No.
Nuclear Extract Kit	100 rxns	40010
	400 rxns	40410

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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Carlsbad, CA 92008
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