TransAM™
NFATc1 Transcription Factor Assay Kits

(version D2)

Catalog Nos. 40296 & 40796

TABLE OF CONTENTS Page

Overview

The transcription factor known as Nuclear Factor of Activated T cells (NFAT) binds to several sites within the regulatory region of the IL-2 gene and other genes induced during the immune response. Because its activation is inhibited by the main immunosuppressive drugs in current clinical use, cyclosporin A and tacrolimus (FK506), accurate monitoring of NFAT activation in cells, tissues and 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.

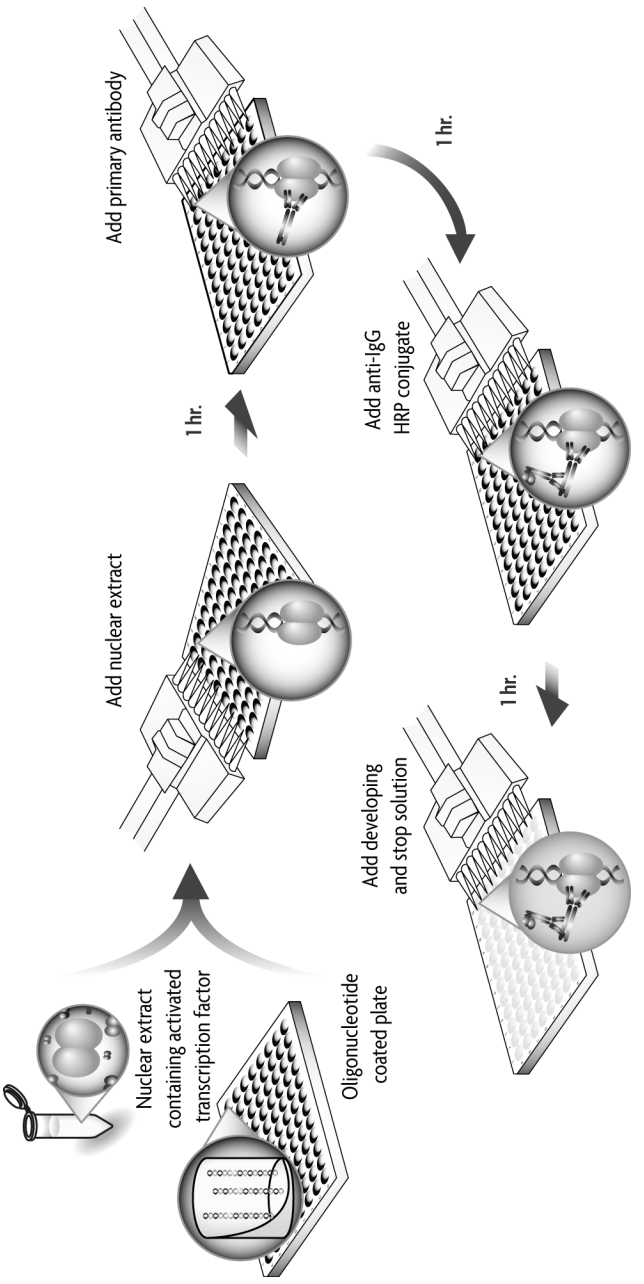
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM NFAT Kits are designed specifically for the study of the NFAT activation pathway. They contain a 96-well plate to which an oligonucleotide containing the NFAT consensus binding site has been immobilized. NFAT contained in nuclear extracts binds specifically to this oligonucleotide and is detected through use of an antibody directed against NFATc1. 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 NFATc1 Kits are available in two sizes:

product	format	catalog no.
TransAM NFATc1	1 x 96-well plate	40296
	5 x 96 well plates	40796

See Active Motif products related to the NFAT transcription factor in Appendix, Section B.

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

Flow Chart of Process



Introduction

NFAT Transcription Factors

NFAT proteins are transcription factors that were first identified as inducers of the immune response[1]. As demonstrated later, these proteins also play varied roles in cell differentiation and adaptation for vascular endothelial cells or skeletal muscle cells (see 2 for review). There are four NFAT family members (NFAT1-4, NFAT2 is also called NFATc or NFATc1) from which numerous isoforms are generated by alternative splicing[1]. NFAT mRNAs are found in peripheral blood lymphocytes, spleen (NFAT1 and 2) and thymus (NFAT4). NFATs modulate the expression of numerous cytokines such as IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, GM-CSF, IFNα, IFNγ and CD40L[1]. In resting cells, NFATc1 is confined to the cytoplasm, where it is maintained in a phosphorylated state by the action of constitutive kinases. Upon stimulation, NFATc1 is dephosphorylated by calcineurin, a Ca^{2+}-dependent phosphatase (see 3 for review), and migrates to the nucleus. NFATc1 dephosphorylation is stimulated by Ca^{2+}-coupled membrane receptors, such as T cell and B cell receptors, and the CD40, FceRI, CD16 and G protein-associated receptors (thrombin or H1 histamine receptors). NFATc1 can also be activated by calcium ionophores. Cyclosporin A and FK506 immunosuppressor drugs inhibit calcineurin activity on NFAT. Receptors not associated with calcium movement are not expected to stimulate NFAT[1]. When calcium levels drop, calcineurin becomes inactive, and NFATc1 is rephosphorylated by kinases and exported back into the cytoplasm.

NFAT phosphoproteins share two conserved domains: a DNA-binding domain (DBD) displaying limited similarity to the Rel protein family DBD[4], and modulating interactions with AP-1 dimers; and a NFAT homology region (NHR), upstream of the DBD, that regulates translocation and DNA-binding activity. The regulatory domain is dephosphorylated by the calcium- and calmodulin-dependent phosphatase calcineurin, which controls NFAT nuclear translocation. Transactivation domains can be found at the N- and C-terminal ends of the NFAT proteins[5]. NFATs bind to the DNA consensus motif 5´-T/AGGAAA-3´ as monomers[6]. NFATs can cooperatively interact with AP-1 and GATA proteins for DNA binding[7, 8]. NFAT can also bind to certain kB-like sites[9]. Vitamin D3 receptor heterodimers (RXR:VDR) can abrogate NFAT modulation of IL-2 by binding to a site which overlaps the NFAT distal site[10]. Sites in IL-2 and GM-CSF promoters can accommodate both NFAT and the Ets-family member, Elf-1[11, 12].

Transcription Factor Assays

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

1. NFAT activation can be determined by Western Blot by using antibodies specific for NFAT proteins. This method is time consuming (up to 2 days once the nuclear extracts are prepared) and is not suitable for processing large numbers of samples.
2. The DNA-binding capacity of NFAT 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 NFAT binding. If NFATs are active in the nuclear extract, they 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 NFAT activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing a NFAT consensus binding site. The promoter can be artificial, made of several NFAT sites and a TATA box, or natural, like promoter sequences from cytokine genes. 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 NFATc1

NFAT-regulated genes are mainly involved in immune response. Recent studies have shown that NFATs also regulate cell development and cellular adaptation. However, this field has been hampered by the lack of convenient assays suitable for discriminating the NFAT family members and for performing high sample number experiments.

To overcome this, Active Motif is introducing a high-throughput assay to quantify NFATc1 activation[13]. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM NFAT Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains a NFAT consensus binding site (5´-AGGAAA-3´). NFATs contained in nuclear extract specifically bind to this oligonucleotide. The primary antibody used in TransAM NFATc1 Kit recognizes an accessible epitope on NFATc1 protein upon DNA binding. 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 NFATc1 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 NFATc1 activation using as little as 0.6 µg of nuclear extract. A comparable assay using EMSA required 5 µg of nuclear extract and a 2-day autoradiography.

TransAM NFATc1 has many applications including the study of NFATc1-mediated transcriptional regulation, protein structure/function studies of NFATc1, NFATc1 cytoplasmic/nuclear shuttling and NFATc1 antagonist drug validation in areas such as immune response, angiogenesis, myogenesis, and many more.

Kit Performance and Benefits

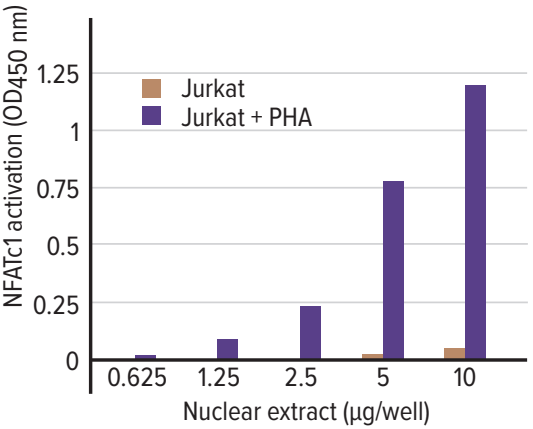
The TransAM NFATc1 Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: < 0.6 µg nuclear extract/well. TransAM NFATc1 is 10-fold more sensitive than EMSA.

Range of detection: TransAM provides quantitative results from 0.6 to 10 µg of nuclear extract/well (see graph below).

Cross-reactivity: TransAM NFATc1 detects NFATc1 from human and mouse origin.

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



Monitoring NFATc1 activation with the TransAM NFATc1 Kit: Different amounts of nuclear extract from untreated and PHA treated Jurkat cells are tested for NFATc1 activation by using the TransAM NFATc1 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 1 plate / 5 plates	Storage / Stability
NFATc1 antibody	22 µl / 110 µl (0.2 mg/ml)	4°C for 6 months
Anti-mouse HRP-conjugated IgG	11 µl / 55 µl (0.4 mg/ml)	4°C for 6 months
Wild-type oligonucleotide AM9	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM9	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Jurkat nuclear extract (PHA treated)	40 µl / 200 µl (2.5 mg/ml)	-80°C for 6 months
Dithiothreitol (DTT)	100 µl / 500 µl (1 M)	-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 AM1	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 NFAT assay plate	1 / 5	4°C for 6 months
Plate sealer	1 / 5	

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 cell 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.

Binding Buffer

This is supplied ready to use.

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 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 primary and HRP-conjugated secondary antibodies to 1:500 and 1:1000, respectively, 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 Jurkat nuclear extract (PHA treated) is provided as a positive control for NFAT 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 5 µl fractions and storing at -80ºC. Avoid multiple freeze/thaw cycles of the extract. Various cell extracts are available from Active Motif (see Appendix, Section B. Related Products).

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for NFAT binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent NFAT binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on NFAT binding. Prepare the required amount of wild-type and/or mutated consensus oligonucleotide by adding 2 µl of appropriate oligonucleotide to 43 µl of Binding Buffer AM1 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**
Binding Buffer AM1	**TOTAL REQUIRED**	**45 µl**	**360 µl**	**2.16 ml**	**4.32 ml**
Binding Buffer with wild-type or mutated oligonucleotide	Wild-type or mutated oligo	2 µl	16 µl	96 µl	N/A
	Binding Buffer AM1	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 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.

NFATc1 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, 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 Binding Buffer, Wash Buffer, Antibody Binding Buffer, Developing Solution and Stop Solution into the wells being used.

Step 1: Binding of NFAT to its consensus sequence

1. Add 40 µl Binding Buffer AM1 to each well to be used. If you wish to perform competitive binding experiments, add 40 µl Binding Buffer AM1 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 2-10 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts can be found on page 11.

   Positive control wells: Add 5 µg of the provided 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 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 diluted NFATc1 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 Wash Buffer (as described in Step 1, No. 4).

Step 3: Binding of secondary antibody

1. Add 100 µl of diluted anti-mouse 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. 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 5-10 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.

Preparation of Nuclear Extract

For your convenience, Active Motif offers a Nuclear Extract Kit (Cat. Nos. 40010 & 40410). This kit contains buffers optiimized 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 protein assay, such as Active Motif's ProStain™ Protein Quantification Kit (Cat. No. 15001). .

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

1. Rao A., Luo C. and Hogan P.G. (1997) *Annu. Rev. Immunol.* 15: 707-47.
2. Horsley V. and Pavlath G. K. (2002) J. *Cell Biol.* 156: 771-4.
3. Kiani A., Rao A. and Arambvuru J. (2000) *Immunity* 12: 359-72.
4. Jain J., Burgeon E., Badalian T.M., Hogan P.G. and Rao A. (1995) *J. Biol Chem.* 270: 4138-45.
5. Luo C., Burgeon E., Carew J.A., McCaffrey P.G., Badalian T.M., Lane W.S., Hogan P.G. and Rao A. (1996) *Mol. Cell Biol.* 16: 3955-66.
6. Hoey T., Sun Y.L., Williamson K. and Xu X. (1995) *Immunity* 2: 461-72.
7. Chen L., Oakley M.G., Glover J.N., Jain J., Dervan P.B., Hogan P.G., Rao A. and Verdine G.L. (1995) *Curr. Biol.* 5: 882-9.
8. Musaro A., McCullagh K.J., Naya F.J., Olson E.N. and Rosenthal N. (1999) *Nature* 400: 581-5.
9. McCaffrey P.G., Goldfeld A.E. and Rao A. (1994) *J. Biol. Chem.* 269: 30445-50.
10. Alroy I., Towers T.L. and Freedman L.P. (1995) *Mol. Cell Biol.* 15: 5789-99.
11. Thompson C.B., Wang C.Y., Ho I.C., Bohjanen P.R., Petryniak B., June C.H., Miesfeldt S., Zhang L., Nabel G.J., Karpinski B., et al. (1992) *Mol. Cell Biol.* 12: 1043-53.
12. Wang C.Y., Bassuk A.G., Boise L.H., Thompson C.B., Bravo R., Leiden J.M. (1994) *Mol. Cell Biol.* 14: 1153-9.
13. Renard P., Ernest I., Houbion A., Art M., Le Calvez H., Raes M. and Remacle J. (2001) *Nucleic Acids Res.* 29: (4) e21.

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 and follow washing recommendations	Ensure all wells are filled with Wash Buffer
	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 1:1000 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 not to exceed 40 µg/well
	NFAT is poorly activated or inactivated in nuclear fractions	Perform a time course for NFAT activation in the studied cell line
	Extracts are not from human or mouse origin	Perform study with a human or mouse model

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

Active Motif North America

Toll free: 877.222.9543
Direct: 760.431.1263
Fax: 760.431.1351
E-mail: tech_service@activemotif.com

Active Motif Europe

UK Free Phone: 0800/169 31 47
France Free Phone: 0800/90 99 79
Germany Free Phone: 0800/181 99 10
Direct: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050
E-mail: eurotech@activemotif.com

Active Motif Japan

Direct: +81 (0)3 5225 3638
Fax: +81 (0)3 5261 8733
E-mail: japantech@activemotif.com

Active Motif China

Direct: (86)-21-20926090
Cell Phone: 18521362870
E-mail: techchina@activemotif.com

Visit Active Motif online at **activemotif.com**