TransAM® STAT3

Transcription Factor Assay Kit

(version C3)

Catalog Nos. 45196 & 45696

Revision History

Revision	Date	Description of Change
C2	June 2019	Corrected recipes in Quick Chart for Prepaing Buffers

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Overview

Signal transducers and activators of transcription (STAT) proteins are latent transcription factors that are activated by phosphorylation via tyrosine kinases. Over 35 different extracellular polypeptides activate Janus kinase associated receptors, leading to phosphorylation of Janus kinases and the subsequent phosphorylation of STAT proteins. Upon phosphorylation, the STAT proteins dimerize and migrate to the nucleus where they exert transcriptional activation. Phosphorylation of a single tyrosine localized around residue 700 is crucial for activation of each STAT family member[1]. STAT proteins are involved in a wide variety of biological pathways. STAT1 is involved in the activation of IFNα and IFNγ genes, STAT2 in the activation of IFNα genes, STAT4 in T-helper cell development and STAT5 in milk production. Disruption of STAT functions in mouse leads to several defects such as immune deficiency (STAT1), embryonic lethality (STAT2), lack of gastrulation (STAT3), T-helper 1 cell dysfunction (STAT4), lack of lactation (STAT5A, 5B) and T-helper 2 cell dysfunction (STAT6). The disruption of STAT signaling blocks neoplastic transformation, thus making inhibitors of STAT proteins candidates for the treatment of cancer. Therefore, accurate monitoring of STAT activity in cells, tissues or animals is crucial for biomedical research and drug development.

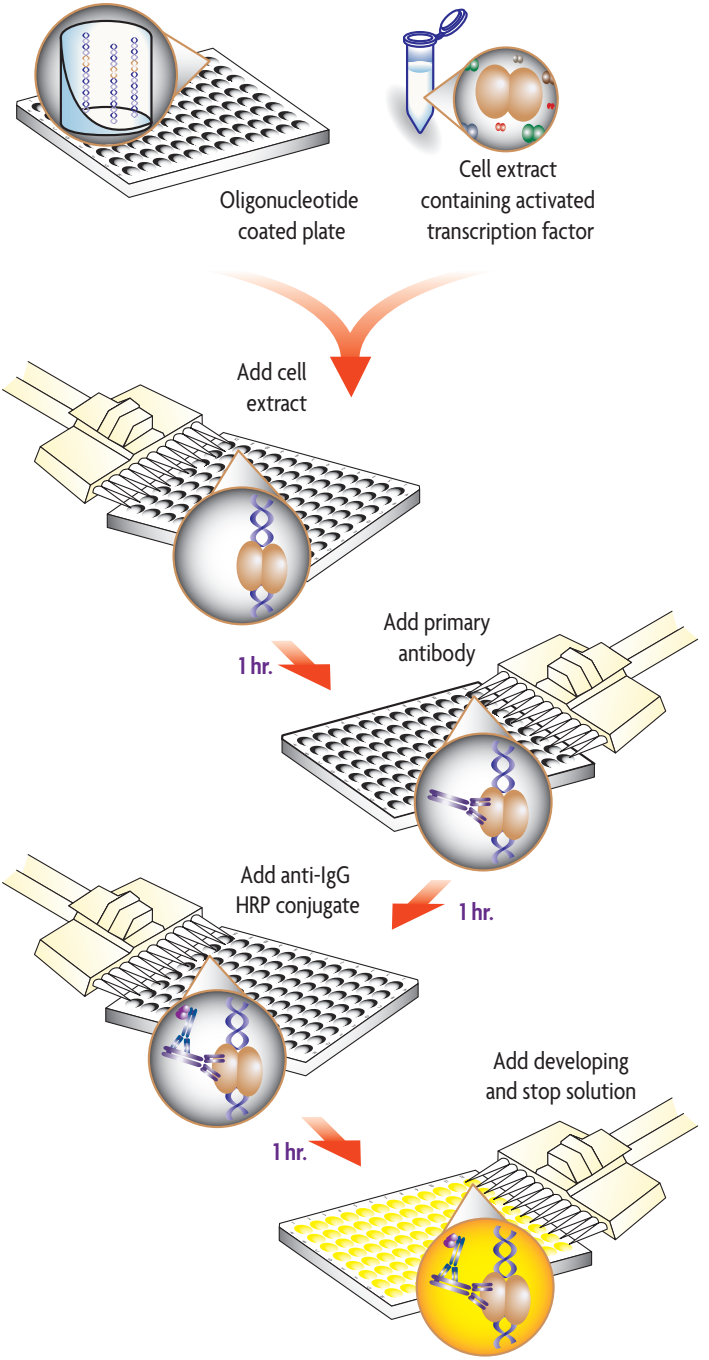
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM STAT3 Kits are designed specifically for the study of STAT pathways. They contain a 96-well plate to which oligonucleotide containing a STAT consensus binding site has been immobilized. STATs contained in nuclear extracts binds specifically to this oligonucleotide and are detected through use of an antibody directed against STAT3. 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 high-throughput screening applications:

product	format	catalog no.
TransAM STAT3	1 x 96-well plate	45196
	5 x 96-well plates	45696

See the Active Motif products related to the STAT signaling pathway, including the TransAM STAT Family Kit, in Appendix, Section B.

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

Flow Chart of Process



Introduction

STAT Transcription Factor

STAT (signal transducers and activators of transcription) transcription factors were discovered fourteen years ago as mediators of interferon-induced gene expression. They comprise a family of latent cytoplasmic proteins that are activated to participate in gene control when cells encounter various extracellular polypeptides. Their critical role in development and normal cell signaling has been largely determined through the analysis of transgenic mice lacking individual STAT genes. The STAT family consists of seven members that are activated by virtually every cytokine and growth factor (Table 1).

Factor	Mass (kDa)	Activating Cytokine	Reference No.
STAT1	91	IFNα, IFNγ, EGF, PDGF, FGF, ACRII,	1
STAT2	113	IFNα/β (with STAT1)	1
STAT3	92	IL-6, LIF, CNTF, OM, CT-1, EGF, G-CSF, IL-10, leptin	1, 2, 3
STAT4	89	IL-12	2, 4
STAT 5A, 5B	77, 80	IL-2, IL-3, IL-5, IL-7, IL-15, GM-CSF, Prolactin, Epo	5, 6
STAT6	94	IL-4, IL-13	7, 8

The STAT proteins are unique among transcription factors in containing an SH2 (src-homology 2), phosphotyrosine-binding domain, a common protein-protein interaction domain among signaling proteins[9]. Tyrosine phosphorylation around residue 700 is essential for the dimerization of STATs and the concomitant nuclear translocation of the dimer. Ligand-activated receptors that catalyze this phosphorylation include receptors with intrinsic tyrosine kinase activity (epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and colony-stimulating factor-1) as well as receptors that lack intrinsic tyrosine kinase activity but to which Janus kinases (JAKs) are noncovalently associated[10, 11]. Receptors to which JAKs are bound are often referred to as cytokine receptors. Their ligands include IFN-α, -β and -γ; interleukins (IL) 2 to 7, 10 to 13, and 15; and erythropoietin, growth hormone, prolactin, thrombopoietin and other polypeptides. STAT dimers and heterodimers, but not monomers, are competent to bind DNA. The known DNA binding heterodimers are STAT1:2 (strong binding requires the joint presence of another protein, p48) and STAT1:3[12]. STATs that form homodimers that bind DNA include STAT 1, 3, 4, 5 (STAT5A and 5B interact in a manner equivalent to a heterodimer) and 6[10, 11, 12].

In most cases, STAT activation is transient. Inactivation of STAT proteins is carried out by several mechanisms, including dephosphorylation of STAT proteins in the nucleus and degradation through the ubiquitin-proteosome pathway[13]. A novel family of negative feedback inhibitors of the JAK-STAT pathway has been identified, referred to as suppressor-of-cytokine-signaling (SOCS) proteins/JAK binding (JAB) proteins, and STAT-induced STAT inhibitors (SSIs)[14, 15, 16]. In addition, a family of protein inhibitors of activated STAT (PIAS) proteins has been identified[17, 18].

Transcription Factor Assays

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

1. STAT expression can be measured by Western blot, using antibodies raised against STAT subunits. 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 STAT 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 STAT binding. If STAT 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 STAT activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing the STAT consensus sequence. This promoter can be artificial, made of several STAT cis-elements and a TATA box, or natural, like the HIV long terminal repeat (LTR) sequence. 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 STAT3

STAT transcription factors convert extracellular stimuli into diverse biological responses such as the regulation of development, cell proliferation, differentiation and apoptosis. Aberrant STAT signaling can affect the outcome of these pathways and result in oncogenesis. Discovery and development of novel inhibitors of STAT signaling hold significant promise for providing more effective treatment for a wide variety of cancers at various stages of malignant progression.

To help achieve this, Active Motif offers a high-throughput assay to quantify STAT activation[7]. The TransAM STAT3 and STAT Family Kits combine a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM STAT3 and STAT Family Kits contain a 96-well plate on which has been immobilized oligonucleotide containing the STAT consensus binding site (5´-TTCCCGGAA-3´). The active form of STAT contained in nuclear extract specifically binds to this oligonucleotide. The primary antibodies used to detect STAT recognize an epitope on STAT 1α, 3, 5A or 5B that is accessible only when STAT is activated and bound to its target DNA. The anti-STAT1α antibody recognizes only the alpha subunit of human STAT1, the anti-STAT3 antibody recognizes both the alpha and beta forms of human STAT3, and the anti-STAT5A and 5B antibodies recognize both the alpha and beta forms of human STAT5A and STAT5B. An HRP-conjugated secondary antibody provides a sensitive colorimetric readout that is easily quantified by spectrophotometry. Once the nuclear extract is prepared, this assay is completed in less than 3.5

hours. As this assay is performed in a 96-well plate, a large number of samples can be handled simultaneously, allowing for high-throughput automation. This assay is specific for STAT activation and has been shown to be 10-fold more sensitive and 40-fold faster than the gel retardation technique. With the 3.5-hour procedure of TransAM, we could detect STAT activation with as little as 0.6 µg of nuclear extract. A comparable assay using EMSA required 5 µg of nuclear extract and a 5-day autoradiography.

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

Kit Performance and Benefits

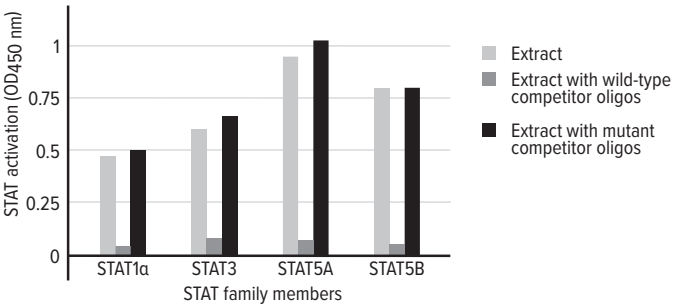
The TransAM STAT3 Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: < 0.6 µg nuclear extract/well. The TransAM STAT3 Kit is up to 10-fold more sensitive than EMSA.

Range of detection: TransAM provides quantitative results from 0.3 to 10 µg of nuclear extract/well.

Cross-reactivity: TransAM STAT3 detects STAT3 from human, mouse and rat origin. Reactivity with other species has not been determined.

Assay time: 3.5 hours. TransAM is 40-fold faster than EMSA.



Monitoring STAT Family member activation using the TransAM STAT Family Kit. STAT 1α, 3, 5A and 5B activation were assayed using the TransAM STAT Family Kit. 1:1000 dilutions of STAT1 and STAT5 antibodies and a 1:1000 dilution of STAT3 antibody were tested using 5 µg/well of nuclear extract prepared from a stimulated cell line: STAT1α was tested with COS-7 (IFNγ), STAT3 with Hep G2 (IL-6, 100 ng/ml), and STAT5A and STAT5B with Nb2 (prolactin). Assays were performed in the absence or presence of 20 pmol of competitor oligonucleotide that contains either a wild-type or mutated STAT consensus binding site. Note that the wild-type oligonucleotide reduces STAT binding by over 90%, while incubation with the mutant STAT competitor oligo has a limited effect on STAT binding to DNA. This data is provided for demonstration purposes only. .

Kit Components and Storage

TransAM STAT3 Kits are for research use only. Not for use in diagnostic procedures. 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. All components are guaranteed stable for 6 months from date of receipt if stored properly.

Reagents	Quantity 1 plate / 5 plates	Storage / Stability
STAT3 antibody	20 µl / 100 µl	-20°C for 6 months
Anti-rabbit HRP-conjugated IgG	11 µl / 55 µl (0.2 µg/µl)	4°C for 6 months
Wild-type oligonucleotide AM6	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM6	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Hep G2 (IL-6, 100 ng/ml) nuclear extract	40 µl / 200 µl (2.5 µg/µl)	-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
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 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 AM2	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 STAT assay plate	1 / 5	4°C for 6 months
Plate sealer	1 / 5	Room Termperature

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)

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.

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 10 µl of 1 µg/µl Herring sperm DNA per ml of Binding Buffer AM6 (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Complete Binding Buffer.

Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 100 ml of 1X Wash Buffer required, dilute 10 ml 10X Wash Buffer AM2 with 90 ml distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in the 10X Wash Buffer AM2 may form clumps, therefore homogenize the buffer by vortexing for 2 minutes prior to use.

Preparation of 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer AM2 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 AM2 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 1:1000 and the 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. 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 Hep G2 nuclear extract is provided as a positive control for STAT activation. Sufficient extract is supplied for 20 reactions. This extract is optimized to give a strong signal when used at 5 µg/well. We recommend aliquoting the extracts in fractions and storing at -80°C to help avoid multiple freeze/thaw cycles of the extracts. Various cell extracts are available from Active Motif.

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for STAT binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent STAT binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on STAT 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 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	0.02 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	0.07 µl	0.54 µl	3.2 µl	6.5 µl
	Herring sperm DNA	0.34 µl	2.7 µl	16.2 µl	32.4 µl
	Binding Buffer AM6	33.4 µl	267 µl	1.6 ml	3.2 ml
	TOTAL REQUIRED	**33.8 µl**	**270 µl**	**1.62 ml**	**3.24 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	31.8 µl	254 µl	1.524 ml	N/A
	TOTAL REQUIRED	33.8 µl	270 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 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 AM2	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.

STAT 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 STAT 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 provided nuclear extract diluted in 20 µl of Complete Lysis Buffer per well (2 µl of extract in 18 µl of Complete Lysis Buffer per well).

   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 diluted STAT3 antibody (1:1000 dilution in 1X Antibody Binding Buffer) 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 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 room-temperature Developing Solution to all wells being used.
2. Incubate 2-10 minutes at room temperature protected from direct light. Monitor the blue color development in the sample and positive control wells until it turns medium to dark blue. Do not overdevelop.
3. Add 100 µl Stop Solution. In presence of the acid, the blue color turns yellow.
4. Read absorbance on a spectrophotometer within 5 minutes at 450 nm with a reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

References

1. Bauerle P.A. and Baichwal V.R. (1997) *Adv. Immunol*. 65: 111-137.
2. Parry G.C. and Mackman N. (1994) J. *Biol. Chem.* 269: 20823-20825.
3. Verma I.M., Stevenson J.K., Schwarz E.M., Van Antwerp D. and Miyamoto S. (1995) *Genes & Dev.* 9: 2723-2735.
4. Gilmore T.D. and Morin P.J. (1993) *Trends Genetics* 9: 427-433.
5. Bauerle P.A. and Baltimore D. (1988) *Cell* 53: 211-217.
6. Chen Z., Hagler J., Palombella V.J., Melandri F., Scherer D., Ballard D. and Maniatis T. (1995) *Genes & Dev.* 9: 1586-1597.
7. 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. 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	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 1-2 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 50 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
	STAT3 is poorly activated or inactivated in nuclear fractions	Perform a time course for STAT3 activation in the studied cell line
	Nuclear extracts are not from correct species	Refer to cross-reactivity table on page 5
	Salt concentration too high in binding reaction	Reduce amount of extract per well or dialyze extract before use

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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