TransAM™

Sp1 & Sp1/Sp3
Transcription Factor Assay Kits

(version C4)

Catalog Nos. 41296 & 41796 (Sp1)
Catalog Nos. 40496 & 40996 (Sp1/Sp3)

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Overview

Sp1 is one of the first cellular transcription factors to be identified, cloned and characterized for its ability to bind to GC-boxes in regulatory promoter elements. Sp1 has always been described as a ubiquitous transcription factor that is required for the constitutive and inducible expression of a variety of genes, such as in cell cycle or mammalian development. Identification of new members of the Sp family, *e.g.* Sp2, Sp3, Sp4 and BTEB, have redefined the understanding of Sp1-regulated genes. Therefore, accurate monitoring of Sp family 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.

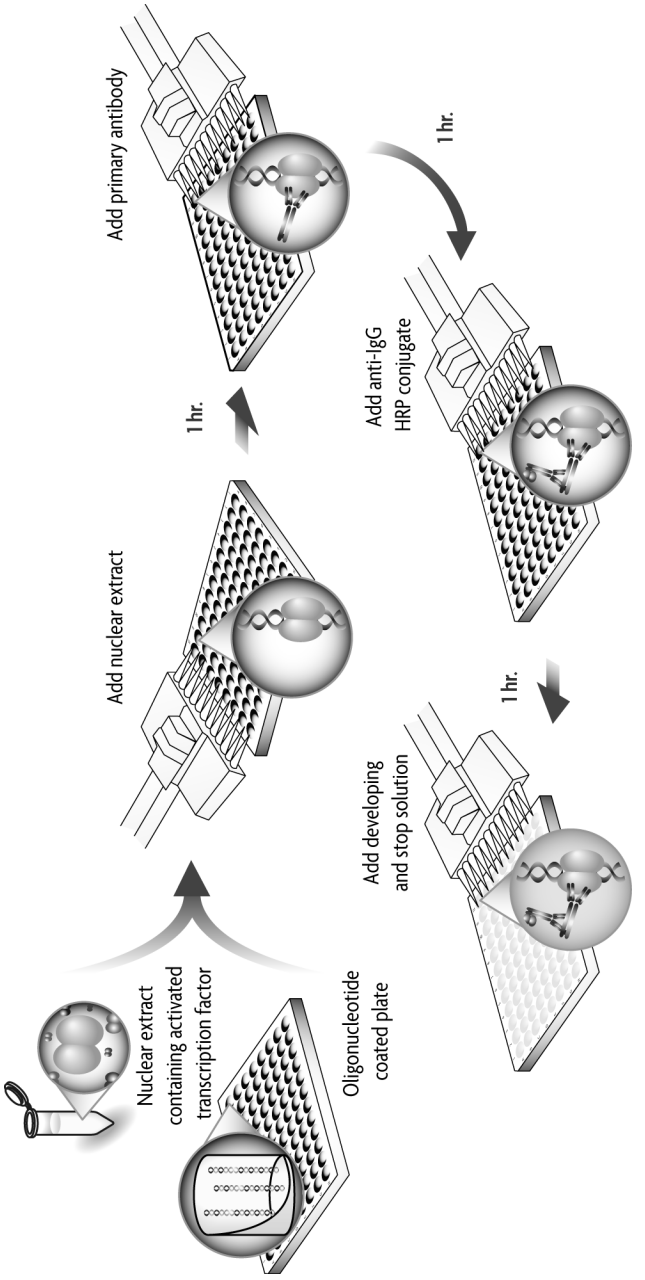
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM Sp1 and Sp1/Sp3 Kits are designed specifically for the study of Sp1-regulated genes. They contain a 96-well plate to which an oligonucleotide containing the Sp1 consensus binding site has been immobilized. Sp1 and Sp3 contained in nuclear extracts bind specifically to this oligonucleotide and are detected through use of an antibody directed against Sp1 or Sp3. 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 Sp1 and Sp1/Sp3 Kits are available in two sizes:

product	format	catalog no.
TransAM Sp1	1 x 96-well plate	41296
	5 x 96 well plates	41796
TransAM Sp1/Sp3	1 x 96-well plate	40496
	5 x 96 well plates	40996

The procedure outlined in this manual can be used for either TransAM Sp1 or TransAM Sp1/Sp3 Kits. See Active Motif products related to the Sp1 and Sp3 transcription factors in Appendix, Section B.

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

Flow Chart of Process



Sp1 and Sp3 Transcription Factors

The Sp1 transcription factor is a 105 kDa protein that can activate a wide subset of mammalian genes containing upstream promoter elements called a GC box (GGGGCGGGG) and the related GT/CACCC box (GGTGTGGGG)[1, 2]. The C-terminal domain of Sp1 harbors three contiguous Cys-X_4-Cys-X_{12}-His-X_3-His repeats, which are typical of the Cys_2His_2-type zinc-finger DNA-binding domain that was first found in the TFIIIA transcription factor. Specific variations in the ubiquitous expression of Sp1 suggest its involvement in gene regulation of cell cycle, hormonal activation and development patterning[3]. Sp1 knock-out embryos show a broad range of abnormalities and usually die around day 11 of gestation. Recent studies suggest that Sp1 is an important regulator of expression of the methyl-CpG-binding protein MeCP2[4].

The Sp zinc finger transcription factor family is composed of four members (Sp1, Sp2, Sp3 and Sp4) that share similarity within their DNA-binding domains, transactivation domains and tissue expression patterns[2]. Sp1, Sp3 and Sp4 are more closely related to each other than to Sp2, which does not bind to a GC-box but to a GT-rich element[5]. Sp1 contains two glutamine-rich transcriptional activation domains that mediate direct interactions with the TATA box-binding protein (TBP) involved in the TFIID-RNA polymerase II complex. These activation domains can also interact directly with TAF110 transcription factor[6]. A cooperative interaction between Sp1 and NFκB p65 is required for the efficient stimulation of HIV-1 transcription[7]. Sp1 has been shown to interact with YY1, Oct-1, E2F-1, E2F-3 and p74[8, 9]. The role of Sp3 alone is not well defined as an activator or repressor. However, gene transcription has been shown to be regulated by the ratio of Sp1 and Sp3 in different cell models[10-12]. These two Sp-family members compete with each other to bind to Sp1 DNA binding sites. Sp4 expression appears to be restricted to a few tissues. Sp4 is highly expressed during the development of the mouse embryo central nervous system (CNS) and seems to be required for normal male reproductive behavior[13]. Recently, the role of Sp1 in breast cancer and aging has been reassessed due to its effect on estrogen and progesterone receptor transcription levels[14]. Sp1 phosphorylation has also been the focus of investigations on proapoptotic and angiogenic gene transcription regulation in vascular smooth muscle and vascular endothelial cells[15, 16].

Transcription Factor Assays

To date, three methods are widely used to measure Sp1/Sp3 expression, either directly or indirectly:

1. Sp1/Sp3 expression can be determined by Western blot by using antibodies specific for Sp1 or Sp3 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 Sp1 and Sp3 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 Sp1 binding. If Sp1 and Sp3 are 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 Sp1 and Sp3 activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing a Sp1 consensus binding site. The promoter can be artificial, made of a GC box and a TATA box, or natural, like promoter sequences from viral regulator elements, such as the HIV-1 LTR promoter. 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 Sp1 & Sp1/Sp3

Sp1-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 suitable for discriminating the Sp family members and performing high numbers of experiments.

To overcome this, Active Motif is introducing a high-throughput assay to quantify Sp1 and Sp3 activation[17]. TransAM Kits combine a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM Sp1 and Sp1/Sp3 Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains a Sp1 consensus binding site (5´-GGGGCGGGG-3´). Sp1 and Sp3 contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used in TransAM Sp1 and Sp1/Sp3 Kits recognizes an accessible epitope on Sp1 or Sp3 protein upon DNA binding. Addition of a secondary HRP-conjugated antibody provides a sensitive colorimetric readout that is 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 Sp1 and Sp3 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 Sp1 and Sp3 activation using as little as 0.6 µg of nuclear extract from MCF-7 cells. A comparable assay using EMSA required 2.5 µg of nuclear extract and a 3-day autoradiography.

TransAM Sp1 and Sp1/Sp3 have many applications including the study of Sp1 transcriptional activity regulation and protein structure/function studies of Sp1/Sp3 and their mutated variants in areas such as angiogenesis, metastasis, tumorigenesis and many more.

Kit Performance and Benefits

The TransAM Sp1 and Sp1/Sp3 Kits are for research use only. Not for use in diagnostic procedures.

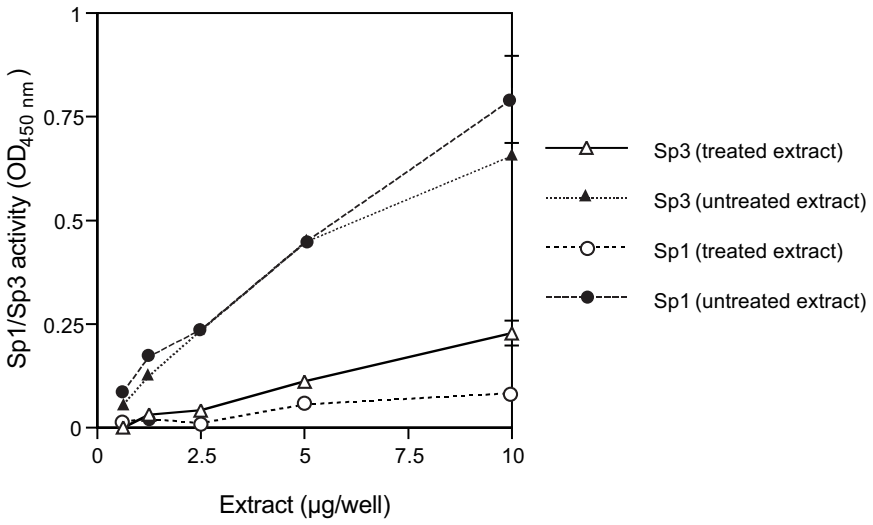
Detection limit: < 0.6 µg nuclear extract/well. TransAM Sp1/Sp3 is 5-fold more sensitive than EMSA.

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

Cross-reactivity: TransAM Sp1/Sp3 detects Sp1 and Sp3 from human, mouse and rat origin.

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

Kit Components and Storage



Monitoring Sp1 and Sp3 activity with the TransAM Sp1/Sp3 Kit: Different amounts of untreated and H_2O_2-post treated nuclear extracts from MCF-7 cells are tested for Sp1 and Sp3 activity by using the TransAM Sp1/Sp3 Kit. These curves are provided for demonstration only.

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
Sp1 antibody and/or Sp3 antibody	11 µl / 55 µl 11 µl / 55 µl	-20°C for 6 months -20°C for 6 months
Anti-rabbit HRP-conjugated IgG	11 µl / 55 µl (0.4 µg/µl)	4°C for 6 months
Wild-type oligonucleotide AM22	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM22	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
MCF-7 nuclear extract	40 µl / 200 µl (2.5 µg/µl)	-80°C for 6 month
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
Poly [d(I-C)]	100 µl / 500 µl (17 µg/ml)	-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 month
Developing Solution	11 ml / 55 ml	4°C for 6 months
Stop Solution	11 ml / 55 ml	4°C for 6 months
96-well Sp1 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.

Preparation of Complete Binding Buffer

Prepare the amount of Complete Binding Buffer required for the assay by adding 2 µl of DTT and 10 µl of 17 µg/ml poly[d(I-C)] 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 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 both primary and HRP-conjugated secondary antibodies to 1:1000 with the 1X Antibody Binding Buffer. Depending on the particular assay, the signal:noise ratio may be optimized by using higher dilutions of both antibodies. This may decrease the sensitivity of the assay.

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

Developing Solution

The Developing Solution should be warmed to room temperature before use. The Developing Solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. A blue color present in the Developing Solution indicates that it has been contaminated and must be discarded. Prior to use, place the Developing Solution at room temperature for at least 1 hour. Transfer the amount of Developing Solution required for the assay into a secondary container before aliquoting into the wells (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Developing Solution.

Stop Solution

Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Stop Solution.

WARNING: The Stop Solution is corrosive. Wear personal protective equipment when handling, *i.e.* safety glasses, gloves and labcoat.

Nuclear extract

The MCF-7 nuclear extract is provided as a positive control for Sp1 and Sp3 activation. Sufficient extract is supplied for 25 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 B. Related Products).

Wild-type and mutated consensus oligonucleotides

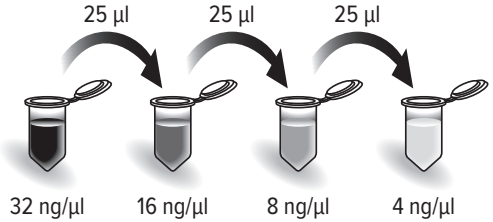
The wild-type consensus oligonucleotide is provided as a competitor for Sp1 and Sp3 binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent Sp1 and Sp3 binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on Sp1 and Sp3 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.

Optional- Preparation of standard curve

For those who wish to quantify the amount of Sp1 in their samples, Active Motif offers recombinant Sp1 for use as a protein standard (see Appendix, Section B. Related Products).

1. Begin with a 100 ng/µl working stock of recombinant protein (use the TransAM Complete Lysis Buffer to dilute the protein). Set up a standard curve in duplicate using the following concentrations: 32, 16, 8, 4 and 0 ng/µl. Note: The preceding range is provided as guidance, a broader range of values may be used.

2. Make up a 32 ng/µl solution by adding 16 µl of the 100 ng/µl working stock to 34 µl of Complete Lysis Buffer. Next, pipette 25 µl of Complete Lysis Buffer into the 3 remaining tubes. Use the 32 ng/µl solution to prepare a dilution series as indicated below. Be sure to mix each tube thoroughly before each transfer. The 32 ng/µl standard serves as the high standard, while Complete Lysis Buffer alone serves as the 0.0 ng/µl.



3. 10 µl from each tube will be aliquoted to the wells in Step 1, No. 2 of the protocol and will correspond to the following quantities of Sp1: 320, 160, 80, 40 and 0.0 ng/well.

Quick Chart for Preparing Buffers

Reagents to prepare	Components	1 well	1 strip (8 wells)	6 strips (48 wells)	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
	Poly [d(I-C)]	0.45 µl	3.6 µl	21.6 µl	43.2 µl
	Binding Buffer AM1	44.5 µl	356 µl	2.134 ml	4.268 ml
	TOTAL REQUIRED	**45 µl**	**360 µl**	**2.16 ml**	**4.32 ml**
Complete Binding Buffer with wild-type or mutated oligonucleotide	Wild-type or mutated oligo	2 µl	16 µl	96 µl	N/A
	Complete Binding Buffer	43 µl	344 µl	2.064 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 m
	10X Wash Buffer AM2	225 µl	1.8 ml	10.8 ml	21.6 m
	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.

Sp1 & Sp1/Sp3 Transcription Factor Assays

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 Sp1 and/or Sp3 to its consensus sequence

1. Add 40 µl Complete Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 40 µl Complete Binding Buffer that contains 20 pmol (2 µl) of the wild-type or mutated oligonucleotide (see the Buffer Preparation section above for a description of competitive binding).
2. **Sample wells:** Add 10 µl of sample diluted in Complete Lysis Buffer per well. We recommend using 2-10 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 MCF-7 nuclear extract diluted in 10 µl of Complete Lysis Buffer per well (2 µl of extract in 8 µl of Complete Lysis Buffer per well).

   Blank wells: Add 10 µl Complete Lysis Buffer only per well.

   OPTIONAL – Protein standard wells: Add 10 µl of the appropriate protein standard diluted in Complete Lysis Buffer to each well being used (see page 8, Preparation of standard curve).
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 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. 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-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 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 2-5 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.

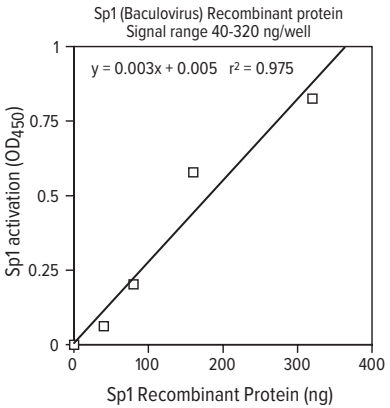
OPTIONAL – Calculation of results using the standard curve

If you have generated a standard curve using Active Motif's recombinant Sp1 protein, average the duplicate readings for each standard, control, and sample and subtract the optical density (OD) obtained from the zero standard.

Plot the OD for the standards against the quantity (ng/well) of the standards and draw the best fit curve. The data can be linearized using log/log paper and regression analysis may also be applied.

To quantify the amount of Sp1 in the samples, find the absorbance value for the samples on the y-axis and extend a horizontal line to the standard curve. At the intersection point extend a vertical line to the x-axis and read the corresponding standard value. Note: If the samples have been diluted, the value read from the standard curve must be multiplied by the dilution factor.

Example curve:
The following standard curve is provided for demonstration only. A standard curve should be made every time an experiment is performed.



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 0.5 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 Bradford-based assay.

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.

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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
	Sp1 or Sp3 is poorly expressed or inactivated in nuclear fractions	Perform a time course for Sp1 or Sp3 expression in the studied cell line
	Extracts are not from human, mouse or rat origin	Perform study with a human, mouse or rat 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.

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