TransAM™ c-Myc

Transcription Factor Assay Kits

(version A5)

Catalog Nos. 43396 & 43896

Revision	Date	Description of Change
A5	July 17, 2023	c-Myc antibody has changed to new monoclonal antibody. However, dilution used in protocol and species reactivity are the same as previous.

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Overview

c-Myc is a transcription factor that regulates cell growth and differentiation, glycolysis and apoptosis.[1] Deregulation of c-Myc has been implicated in the origin of diverse human cancers, and may contribute to as much as one-seventh of U.S. cancer deaths.[2] Because of this link between c-Myc and cancer, accurate monitoring of c-Myc in cells, tissues and animals is crucial for many biomedical research and drug development projects. To date, such research projects are tedious and time consuming, and lack high-throughput screening methods.

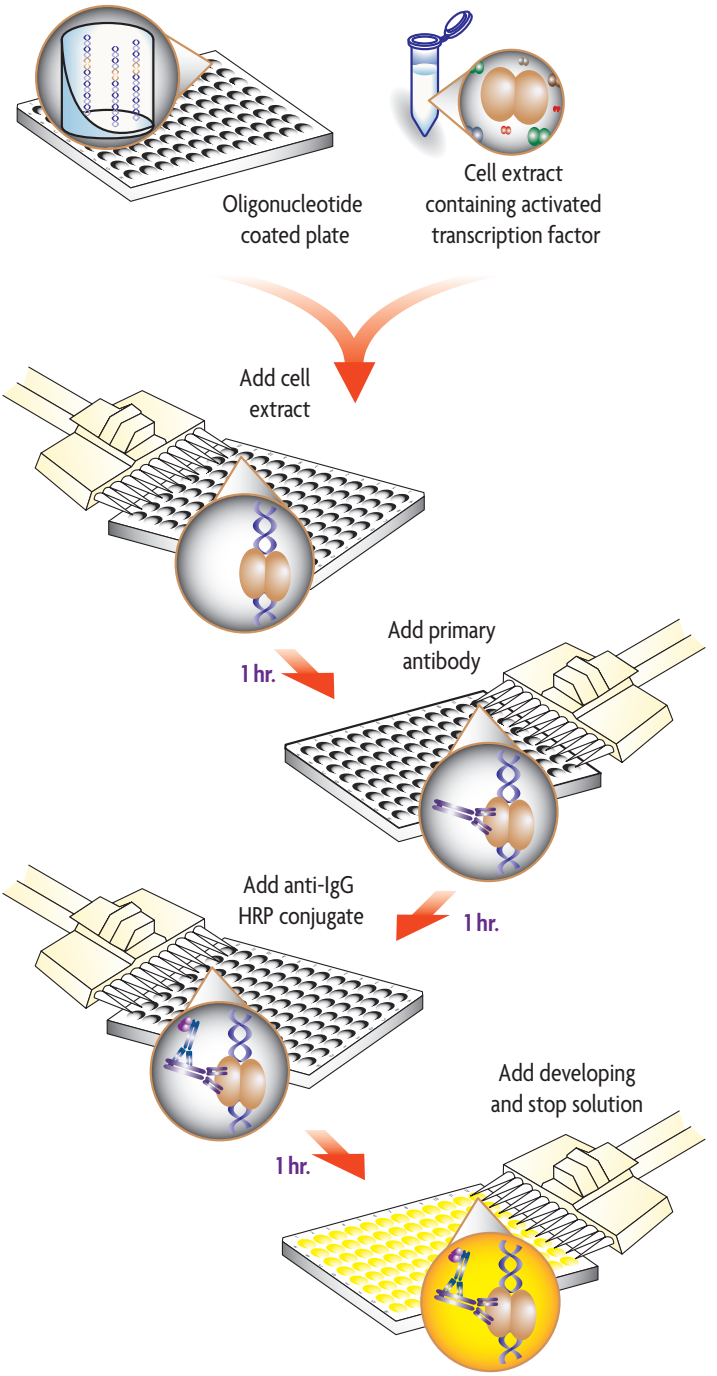
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM c-Myc Kits are designed specifically to detect and quantify c-Myc activation. They contain a 96-well plate to which oligonucleotide containing a c-Myc consensus sequence has been immobilized. c-Myc dimers contained in nuclear extracts bind specifically to this oligonucleotide and are detected through use of an antibody directed against c-Myc. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provides a 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 c-Myc Kits are available in two sizes:

product	format	catalog no.
TransAM c-Myc	1 x 96-well plate	43396
	5 x 96 well plates	43896

Active Motif also offers the TransAM MAPK Family Kit that directly assays the transcription factors ATF-2, c-Jun, c-Myc, MEF2 and STAT1, all of which are regulated by MAP kinase cascades. See this and other Active Motif products related to c-Myc in Appendix, Section B.

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

Flow Chart of Process



Introduction

c-Myc Transcription Factor

c-Myc was originally discovered as the cellular homolog of the retroviral v-myc oncogene[2], and is a transcription factor involved in a wide variety of cellular processes, including cell proliferation, replicative potential, growth, differentiation and apoptosis.[1] Expression of c-Myc is induced by mitogenic signals and suppressed by growth-inhibitory signals.[3] c-Myc is a member of the basic helix-loop-helix leucine zipper (bHLHzip) family, along with B-Myc, N-Myc, L-Myc and s-Myc.[2] Upon dimerization with the bHLHzip protein Max, c-Myc can bind to the E box motif CACGTG and activate transcription. The gene for c-Myc is located on human chromosome 8 and is comprised of three exons.[2] c-Myc is a 64 kDa protein[4] that has an N-terminal transactivation/repression domain and a C-terminal bHLH-LZ DNA binding domain.[3] c-Myc is phosphorylated at Ser62, which has been shown to be a regulatory site of phosphorylation. The phosphorylation of c-Myc causes increased function of the NH2-terminal transactivation domain, and studies have indicated that the expression of MAP kinase is responsible for increased c-Myc phosphorylation at Ser62.[5]

Because of the central role of c-Myc as an activator of diverse cellular processes, regulation of this transcription factor is crucial for proper cell function and ultimate survival. The main regulation of c-Myc occurs through its binding with the bHLHzip protein Max, which can also form heterodimers with members of the Mad family (Mad 1, 3, 4 and Mxi1). As c-Myc cannot bind to DNA without forming a heterodimer with Max, competition between c-Myc and Mad for the common partner Max is used to regulate c-Myc activity. While Max is a relatively stable protein, c-Myc degrades rapidly, with a half-life of 20-30 minutes.[3]

c-Myc regulates a wide variety of genes involved in the cell cycle, glycolysis and apoptosis, including cell cycle regulators CDC2-L1 and cyclin B1, and glycolysis regulator lactate dehydrogenase A.[1] c-Myc also mediates induction of transcription cofactors and components of DNA pol II, facilitating an overall increase in transcriptional activity during the G1/S transition. Genes associated with terminal differentiation such as NCAM, gadd45, neu and adrenomedullin can be repressed by c-Myc. Activation of c-Myc by proviral insertion, gene amplification and chromosomal translocation has been described in a variety of tumors.[3] The *c-myc* gene is amplified in various human cancers, with an elevated expression found in almost one-third of breast and colon cancers.[2] It has been estimated that approximately 70,000 U.S. cancer deaths per year are associated with changes in the *c-myc* gene or its expression.[2]

Transcription Factor Assays

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

1. c-Myc activation can be determined by Western blot by using antibodies specific for c-Myc 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 c-Myc 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 c-Myc binding. If c-Myc 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 cannot be applied to high-throughput screening. Gelshift assays also require special precautions and equipment for handling radioactivity.

3. Another method used to assay c-Myc activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing the c-Myc recognition site. The promoter can be artificial, made of several c-Myc elements or natural, like the LDHA promoter sequence. However, the procedure is limited by the following issues: (i) reporter gene assays have to be repeated several times to obtain statistically reliable data; and (ii) reporter gene assays are sensitive to confounding factors that may influence the expression level of the reporter gene, and therefore assays have to be carefully standardized. Reporter gene assays are sensitive and easy to perform with a large number of samples, but require efficient cell transfection with the reporter plasmid.

TransAM c-Myc

c-Myc is a central transcription factor that regulates cell growth and differentiation, glycolysis and apoptosis. Aberrant c-Myc signaling can affect the outcome of these pathways and result in oncogenesis, which makes it an excellent pharmacological target. However, this field has been hampered by the lack of convenient assays suitable for detecting c-Myc and for performing high sample number experiments.

To help achieve this, Active Motif offers a high-throughput assay to quantify c-Myc activation. The TransAM c-Myc Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM c-Myc Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains the c-Myc consensus binding site (5´-CACGTG-3´). The active form of c-Myc contained in nuclear extract binds specifically to this oligonucleotide. The primary antibody used in the TransAM c-Myc Kit recognizes an accessible epitope on c-Myc 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 c-Myc activation and has been shown to be 20-fold more sensitive and 20-fold faster than the gel-retardation technique. With the 3.5-hour TransAM procedure, we could detect c-Myc activation using as little as 0.25 µg of nuclear extract from Jurkat cells. A comparable assay using EMSA required 5 µg of nuclear extract and a 3-day autoradiography.

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

Kit Performance and Benefits

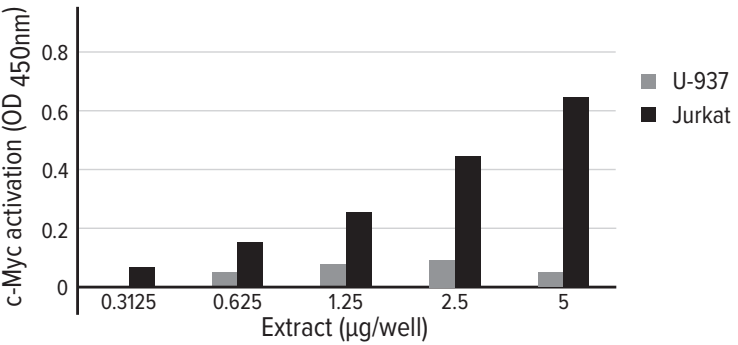
TransAM c-Myc Kits are for research use only. Not for use in diagnostic procedures.

Detection limit: < 0.25 µg nuclear extract/well. TransAM c-Myc is 20-fold more sensitive than EMSA.

Range of detection: TransAM provides quantitative results from 0.25 to 5 µg of nuclear extract/well (see graph below).

Cross-reactivity: The TransAM c-Myc Kit detects c-Myc from human and mouse origin.

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



Monitoring c-Myc activity with the TransAM c-Myc Kit: Different amounts of nuclear extracts from untreated Jurkat (1 day growth) and U-937 cells are tested for c-Myc activity by using the TransAM c-Myc 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
c-Myc antibody	11 µl / 55 µl	-20°C for 6 months
Anti-rabbit HRP-conjugated antibody	11 µl / 55 µl (0.2 µg/µl)	4°C for 6 months
Wild-type oligonucleotide AM3	100 µl / 500 µl (10 pmol/µl)	-20ºC for 6 months
Mutated oligonucleotide AM3	100 µl / 500 µl (10 pmol/µl)	-20ºC for 6 months
Jurkat (1 day growth) nuclear extract	40 µl / 200 µl (2.5 µg/µl)	-80ºC for 6 months
Dithiothreitol (DTT)	100 µl / 500 µl (1 M)	-20ºC for 6 months
Protease Inhibitor Cocktail	100 µl / 500 µl	-20ºC 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 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 c-Myc 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
- 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 nuclear 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 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 per ml of Binding Buffer AM6 (see the Quick Chart for Preparing Buffers in this section). After use, discard the 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, which makes it necessary to homogenize the buffer by incubating at 50°C for 2 minutes and mixing prior to use.

Preparation of 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer AM3 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section)*. Mix gently to avoid foaming. Discard remaining 1X Antibody Binding Buffer after use. The BSA contained in 10X Antibody Binding Buffer AM3 may form clumps, therefore you should homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use. Dilute the primary and secondary antibodies with the 1X Antibody Binding Buffer to 1:1000. 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).

Jurkat nuclear extract

The Jurkat (1 day growth) nuclear extract is provided as a positive control for c-Myc activation. Nuclear extract was made from unstimulated Jurkat cells which were seeded and grown for 24 hours prior to harvesting. 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 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

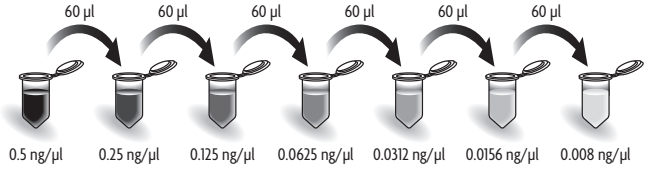
The wild-type consensus oligonucleotide is provided as a competitor for c-Myc binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent c-Myc binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should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

Optional- Preparation of standard curve

For those who wish to quantify the amount of c-Myc in their samples, Active Motif offers recombinant c-Myc 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: 0.5, 0.25, 0.125, 0.0625 and 0 ng/µl. Note: The preceding range is provided as guidance, a broader range of values may be used.
2. Make up a 0.5 ng/µl solution by adding 1.0 µl of the 100 ng/µl working stock to 199 µl of Complete Lysis Buffer. Next, pipette 25 µl of Complete Lysis Buffer into the 3 remaining

tubes. Use the 0.5 ng/µl solution to prepare a dilution series as indicated below. Be sure to mix each tube thoroughly before each transfer. The 0.5 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 c-Myc: 5, 2.5, 1.25, 0.625 and 0.0 ng/well.

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	89.0 µl	534.0 µl	1.068 ml
	TOTAL REQUIRED	**11.25 µl**	**90.0 µl**	**540.0 µl**	**1.08 ml**
Complete Binding Buffer	DTT	0.09 µl	0.72 µl	4.32 µl	8.64 µl
	Binding Buffer AM6	44.9 µl	359.3 µl	2.15 ml	4.31 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	2.158 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	225.0 µl	1.8 ml	10.8 ml	21.6 ml
	TOTAL REQUIRED	**2.25 ml**	**18.0 ml**	**108.0 ml**	**216.0 ml**
1X Antibody Binding Buffer*	Distilled water	202.5 µl	1.62 ml	9.72 ml	19.44 ml
	10X Ab Binding Buffer AM3	22.5 µl	180 µl	1.08 ml	2.16 ml
	TOTAL REQUIRED	**225.0 µl**	**1.8 ml**	**10.8 ml**	**21.6 ml**
Developing Solution	**TOTAL REQUIRED**	**112.5 µl**	**900.0 µl**	**5.4 ml**	**10.8 ml**
Stop Solution	**TOTAL REQUIRED**	**112.5 µl**	**900.0 µl**	**5.4 ml**	**10.8 ml**

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

c-Myc 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, 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 c-Myc 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 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. A protocol for preparing nuclear extracts can be found 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 only 10 µl Complete Lysis Buffer 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 c-Myc 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 HRP-conjugated antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. During this incubation, place the Developing Solution at room temperature.
4. Wash the wells 4 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric reaction

1. Add 100 µl Developing Solution to all wells being used.
2. Incubate 2-10 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 the 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

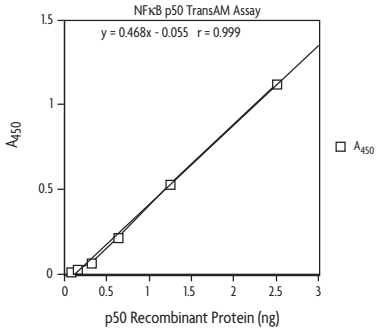
If you have generated a standard curve using Active Motif's recombinant c-Myc protein, average the duplicate readings for each standard, control, and sample and subtract the optical density (OD) obtained from the zero standard.

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 c-Myc 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 0.15 mg of nuclear proteins for 9 x 10^6 cells.

1. Wash cells with 10 ml ice-cold PBS/PIB. Discard PBS/PIB.
2. Add 10 ml ice-cold PBS/PIB and scrape the cells off the dish with a cell lifter. Transfer cells into a pre-chilled 15 ml tube and spin at 300 x *g* for 5 minutes at 4°C.
3. Resuspend the pellet in 1 ml ice-cold HB buffer by gentle pipetting and transfer the cells into a pre-chilled 1.5 ml tube.
4. Allow the cells to swell on ice for 15 minutes.
5. Add 50 µl 10% Nonidet P-40 (0.5 % final) and vortex the tube vigorously for 10 seconds.
6. Centrifuge the homogenate for 30 seconds at 4°C in a microcentrifuge. Remove the supernatant (cytoplasmic fraction) and, if you wish to save this for other uses, transfer it into a pre-chilled microcentrifuge tube. (Store the cytoplasmic fraction at –80ºC.)
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 –80ºC. 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 50ºC for 5 minutes. Mix again. Store at –20ºC.

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.

References

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2. Dang C.V. (1999) *Molecular & Cellular Biology* 19: 1-11.
3. Gregory M.A. *et al.* (2000) *Molecular & Cellular Biology* 20: 2423-2435.
4. Semmes O.J. *et al.* (1996) *J. Biol. Chem.* 271(16): 9730-9738.
5. Davis R.J. (1993) *J. Biol. Chem.* 268(20): 14553-14556.

Appendix

Section A. 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
	c-Myc is poorly activated or inactivated in nuclear fractions	Perform a time course for c-Myc activation in the studied cell line
	Nuclear extracts are not from correct species	Perform study with a human or mouse model.
	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.

Active Motif North America

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