TransAM™
C/EBP α/β
Transcription Factor Assay Kits

Catalog Nos. 44196 & 44696

(version D3)

Revision History

Revision	Date	Description of Change
D1	June 2019	Corrected recipes in Quick Chart for Preparing Buffers
D3	May 2023	Dilution of secondary antibody (HRP-conjugate anti-mouse IgG) has changed from 1:1,000 to 1:500. Twice as much material has been provided to account for the change.

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Overview

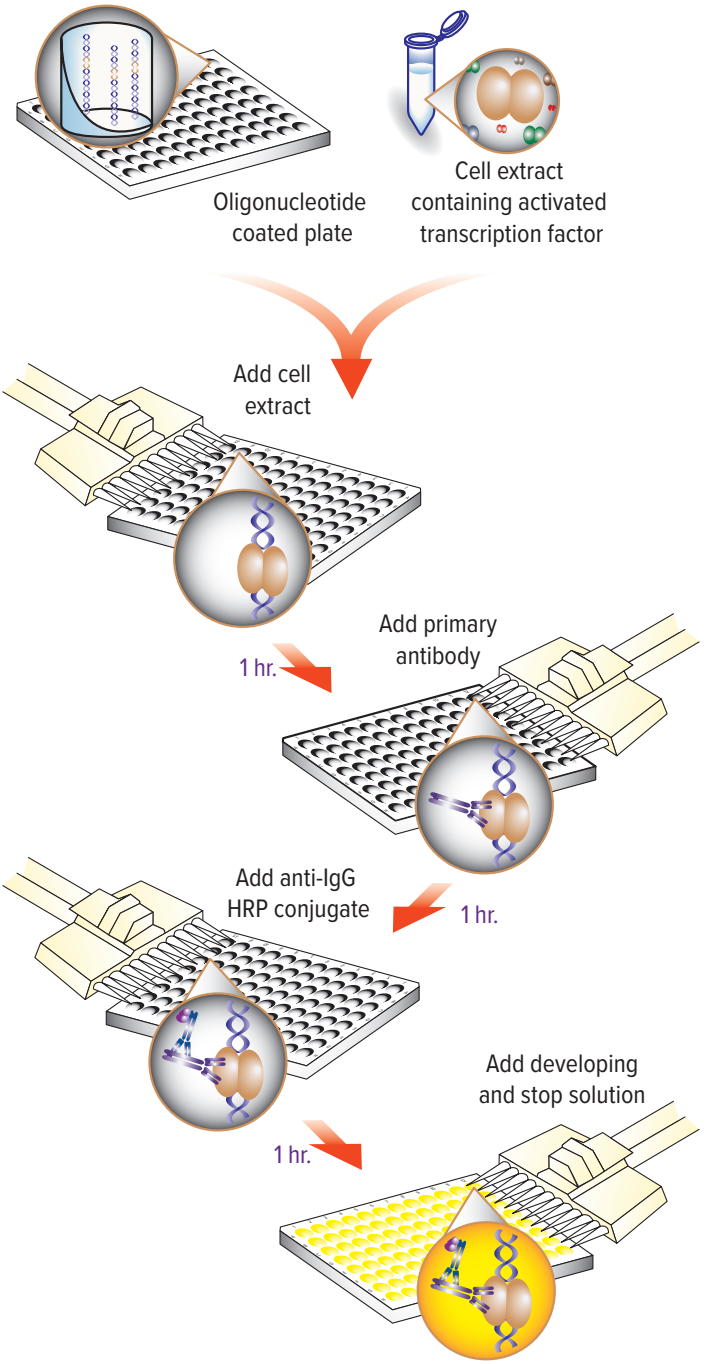
The family of C/EBP (CCAAT-ehancer binding protein) basic leucine zipper transcription factors includes C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ and C/EBPε, all of which exhibit similar DNA-binding specificities and contain a leucine zipper dimerization region. C/EBP's play an important role in regulating the balance between cell growth and differentiation. The expression of C/EBP is a function of tissue type as well as temporal period of development. Therefore, accurate monitoring of C/EBP 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 C/EBP α/β Kits are designed specifically for the study of C/EBP regulated genes. They contain a 96-well plate to which an oligonucleotide containing the C/EBP consensus binding site has been immobilized. The C/EBPα and C/EBPβ contained in nuclear extracts specifically binds to this oligonucleotide. By using an antibody that is directed against either the C/EBPα or C/EBPβ, the C/EBP bound to the oligonucleotide is detected. 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 detachable wells is suitable for manual use or high-throughput screening applications. TransAM C/EBPα/β Kits are available in two sizes:

product	format	catalog no.
TransAM C/EBP α/β	1 x 96-well plate	44196
	5 x 96-well plates	44696

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

Flow Chart of Process



Introduction

C/EBP Transcription Factor

C/EBPs play an important role in regulating the balance between cell growth and differentiation[17]. Expression of C/EBP is, therefore, a function of tissue type as well as temporal period of development[1,13].

C/EBPβ is an important transcriptional activator that is involved in the regulation of a number of genes related to the immune and inflammatory response and lipid storage. In addition, C/EBPβ is required for macrophage and B-cell development[14]. The C/EBPβ regulator also plays a role in the IFN-signaling pathways and is a mediator of IL-6 signaling[11, 15]. C/EBPβ expression is enhanced by cytokines during acute phase reactions and may mediate the increase of acute phase proteins. In other systems, expression of C/EBPβ has been reported to be modulated by growth factor and C/EBPβ participates in growth hormone-regulated gene expression[2]. Physiological and functional interactions of C/EBP with NFκB, Sp1 and other proteins have also been observed[7, 8, 12, 16].

There is a temporal order of expression for C/EBPα and C/EBPβ in differentiating cells, and C/EBPβ is often required for expression of C/EBPα[2]. While C/EBPβ is induced early during adipocyte differentiation, C/EBPα is required in the late phase of adipocyte differentiation and up-regulates the battery of fat-specific genes required for the synthesis, uptake and storage of long chain fatty acids[18].

C/EBPα is a master regulator of myeloid progenitors[5], cooperates with other transcription factors and plays a critical role in early granulocyte development[9, 10]. Furthermore, C/EBPα is an inhibitor of cell proliferation, and is down-regulated in a large proportion of lung cancers[6].

Transcription Factor Assays

To date, three methods are widely used to measure C/EBP expression, either directly or indirectly.

1. C/EBP expression can be determined by Western blot by using antibodies specific for C/EBP proteins. This method is time consuming (up to 2 days once the nuclear nuclear extract are prepared), and is not suitable for processing large numbers of samples.
2. The DNA-binding capacity of C/EBP 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/EBP binding. If C/EBP 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 C/EBP activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing a C/EBP consensus binding site. The promoter can be artificial, made of a CCAAT box and a TATA box, or natural, like the promoter sequences from adipocyte-specific genes, such as the stearoyl-CoA desaturase 1 (SCD1) gene promoter[3] or the β_3-adrenergic receptor (β_3AR) gene promoter[4]. However, the procedure is limited by the following issues: (i) reporter gene assays have to be repeated several times to obtain statistically reliable data; and (ii) reporter gene assays are sensitive to confounding factors that may influence the expression level of the reporter gene; therefore, assays 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/EBP α/β

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

To overcome this, Active Motif is introducing a high-throughput assay to quantify C/EBP activation. TransAM Kits combine a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM C/EBP α/β Kits contain a 96-well plate to which an oligonucleotide that contains a C/EBP consensus binding site (5´-GCAAT-3´) has been immobilized. C/EBP α and β contained in the nuclear extract specifically binds to this oligonucleotide. The primary antibodies used in the TransAM C/EBP α/β Kit recognize an accessible epitope on C/EBPα or C/EBPβ protein upon DNA binding. An HRP-conjugated secondary 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/EBP α and β 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 C/EBP α and β using as little as 0.5 µg of nuclear extract from rat liver or U-937 cells. A comparable assay using EMSA would require 2.5 µg of nuclear extract and a 3-day autoradiography.

TransAM C/EBP α/β Kits can be used to examine cell differentiation and development as well as the regulation of the balance between cell growth and differentiation. Applications include the study of C/EBP transcriptional activity regulation, the regulation of lipid storage, Th1 immune response and macrophage function, the role of growth hormone on C/EBP-regulated gene expression, the regulation and significance of changes in the composition of the C/EBP-containing protein complex and the study of protein structure/function of C/EBP and its mutated variants.

Kit Performance and Benefits

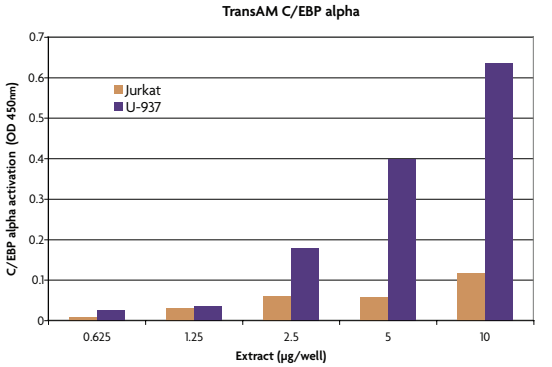
Detection limit: > 0.5 µg nuclear extract per well. TransAM C/EBP α/β is 5-fold more sensitive than EMSA.

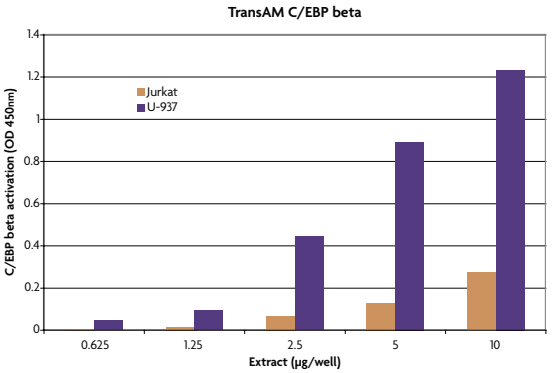
Range of detection: TransAM provides quantitative results from 0.5 to 10 µg of nuclear extract per well.

Cross-reactivity: Detects C/EBPα from human origin and C/EBPβ from human, mouse, and rat origin.

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

TransAM C/EBP α/β





Monitoring C/EBP activation with TransAM C/EBP α/β Kit.

Nuclear extracts from Jurkat cells (Catalog No. 36014) and U-937 cells (Catalog No. 36030) were assayed from 0.625-10 µg per well for C/EBP α or C/EBP β detection using the TransAM C/EBP α/β Kit. Data shown are the results from wells assayed in duplicate. These results are provided for demonstration only.

Kit Components and Storage

TransAM C/EBP α/β Kits are for research use only. Not for use in diagnostic procedures. Except for the nuclear extract that must be kept at -80°C, kit components can be stored at -20°C prior to first use. Then, we recommend storing each component at the temperature indicated in the table below. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
C/EBPα and C/EBPβ antibody	22 µl / 110 µl	4°C for 6 months
HRP-conjugated anti-mouse IgG	24 µl / 120 µl	4°C for 6 months
Wild-type oligonucleotide AM10	100 µl / 500 µl (20 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM10	100 µl / 500 µl (20 pmol/µl)	-20°C for 6 months
Positive control nuclear extract	40 µl / 200 µl (2.5 µg/µl)	-80°C for 6 months
Dithiothreitol (DTT) (1 M)	100 µl / 500 µl	-20°C for 6 months
Protease Inhibitor Cocktail	100 µl / 500 µl	-20°C for 6 months
Herring sperm DNA	100 µl / 500 µl (1 µg/µl)	-20°C for 6 months
Lysis Buffer AM1	10 ml / 50 ml	4°C for 6 months
Binding Buffer AM1	10 ml / 50 ml	4°C for 6 months
10X Wash Buffer AM1	22 ml / 110 ml	4°C for 6 months
10X Antibody Binding Buffer AM1	2.2 ml / 11 ml	4°C for 6 months
Developing Solution	11 ml / 55 ml	4°C for 6 months
Stop Solution	11 ml / 55 ml	4°C for 6 months
96-well C/EBP assay plate	1 / 5	4°C for 6 months
Plate sealer	1 / 5	Room temperature

Additional materials required

- Nuclear extracts
- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform/orbital shaker
- Microplate spectrophotometer capable of reading at 450 nm (655 nm as 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. Please refer to the Appendix Section A for a protocol to prepare a nuclear extract. Our Nuclear Extract Kit can also be purchased separately (Cat. Nos. 40010 & 40410). When preparing extracts, it is suggested to perform the final lysis step using the Lysis Buffer AM1 provided in the TransAM Kit. All subsequent dilutions should also be performed using Lysis Buffer AM1 from the TransAM Kit. Prepare the amount of Complete Lysis Buffer required for the assay by adding 1 µl of 1 M DTT and 10 µl Protease Inhibitor Cocktail per ml of Lysis Buffer AM1 (see the Quick Chart for Preparing Buffers in this section). Some of the protease inhibitors lose their activity after a few hours once diluted. Therefore, we recommend adding protease inhibitors immediately prior to use. Any remaining Complete Lysis Buffer should be discarded if not used in the same day.

Preparation of Complete Binding Buffer

Prepare the amount of Complete Binding Buffer AM1 required for the assay by adding 2 µl of DTT and 20 µl of 1 µg/µl Herring sperm DNA per ml of Binding Buffer AM1 (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Complete Binding Buffer.

Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 100 ml of 1X Wash Buffer required, dilute 10 ml 10X Wash Buffer AM1 with 90 ml distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in the 10X Wash Buffer AM1 may form clumps, therefore, it is necessary to completely resuspend any precipitates by incubating at 50°C for 2 minutes and mixing prior to use.

Preparation of 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer AM1 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section)*. Mix gently to avoid foaming. Discard remaining 1X Antibody Binding Buffer after use. The BSA contained in the 10X Antibody Binding Buffer AM1 may form clumps, therefore it is necessary to completely resuspend the buffer by warming to room temperature and vortexing for 1 minute prior to use.

Dilute primary antibodies 1:500 and HRP-conjugated secondary antibody to 1:500 with the 1X Antibody Binding buffer and mix thoroughly.

* 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 U-937 nuclear extract is provided as a positive control for C/EBPα and C/EBPβ detection. This extract is optimized to give a strong signal when used at 10 µg/well. We recommend aliquoting the extract in 5 µl fractions and storing at -80ºC. Avoid multiple freeze/thaw cycles of the extract. Sufficient extract is supplied for 10 reactions per plate. Various cell extracts are available from Active Motif (see Appendix, Section C. Related Products).

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for C/EBP binding in order to monitor the specificity of the assay. This competition assay will confirm that the protein subunits binding to the plate are specific for the C/EBP consensus binding sequence. Used at 40 pmol/well, the oligonucleotide will prevent C/EBP binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on C/EBP 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 first, then add the cell extract. It is not necessary to perform an incubation step of the oligonucleotide in the well prior to addition of the cell extract. The oligonucleotide competition only needs to be performed as a control. It is suggested to test the oligonucleotide competition each new cell type used.

Quick Chart for Preparing Buffers

Reagents to prepare	Components	For 1 well	For 1 strip (8 wells)	For 6 strips (48 wells)	For 12 strips (96 wells)
Complete Lysis Buffer	DTT	0.01 µl	0.1 µl	0.6 µl	1.2 µl
	Protease Inhibitor Cocktail	0.12 µl	0.9 µl	5.4 µl	10.8 µl
	Lysis Buffer AM1	11.12 µl	89 µl	534 µl	1.068 ml
	TOTAL REQUIRED	11.25 µl	90 µl	540 µl	1.08 ml
Complete Binding Buffer	DTT	0.09 µl	0.7 µl	4.3 µl	8.6 µl
	Herring sperm DNA	0.89 µl	7 µl	42.7 µl	85.5 µl
	Binding Buffer AM1	44 µl	352 µl	2.112 ml	4.224 ml
	TOTAL REQUIRED	45 µl	360 µl	2.16 ml	4.32 ml
Complete Binding Buffer with C/EBP wild-type or mutated oligonucleotide	C/EBP wild-type or mutated oligo	2 µl	16 µl	96 µl	N/A
	Complete Binding Buffer	43 µl	334 µl	2.064 ml	N/A
	TOTAL REQUIRED	45 µl	360 µl	2.16 ml	N/A
1X Wash Buffer	Distilled water	2.025 ml	16.2 ml	97.2 ml	194.4 ml
	10X Wash Buffer AM1	225 µl	1.8 ml	10.8 ml	21.6 ml
	TOTAL REQUIRED	2.25 ml	18 ml	108 ml	216 ml
1X Antibody Binding Buffer*	Distilled water	202.5 µl	1.62 ml	9.72 ml	19.44 ml
	10X Ab Binding Buffer AM1	22.5 µl	180 µl	1.08 ml	2.16 ml
	TOTAL REQUIRED	225 µl	1.8 ml	10.8 ml	21.6 ml
Developing Solution	TOTAL REQUIRED	112.5 µl	900 µl	5.4 ml	10.8 ml
Stop Solution	TOTAL REQUIRED	112.5 µl	900 µl	5.4 ml	10.8 ml

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

Read the entire protocol before use.

Determine the appropriate number of microwell strips required for testing samples, controls and blanks in duplicate. Store the unused strips in the aluminum pouch at 4°C. 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 unused wells are stable at room temperature for the duration of the assay if kept dry. Once the assay is finished, unused strips should be returned to the aluminum pouch and stored at 4°C for a separate assay. Use the strip holder while performing 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/EPB to its Consensus Sequence

1. Add 40 µl Complete Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 40 µl Complete Binding Buffer that contains 40 pmol (2 µl) of the wild-type or mutated 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 2-20 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts is provided on page 13.

   Positive control wells: Add 10 µg of the provided U-937 nuclear extract (4 µl of nuclear extract in 6 µl of Complete Lysis Buffer per well).

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

Reagents	Blank wells	Positive Control no competition	Sample no competition	(Optional) wild-type	(Optional) mutated
Complete Binding Buffer	40 µl	40 µl	40 µl	38 µl	38 µl
Wild-type oligonucleotide	–	–	–	2 µl	–
Mutated oligonucleotide	–	–	–	–	2 µl
Complete Lysis Buffer	10 µl	–	–	–	–
Sample in Complete Lysis Buffer	–	10 µl	10 µl	10 µl	10 µl
Recombinant protein sample	–	–	–	–	–

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). Keep any unused wells covered during the remaining steps in order to preserve those wells for future assays. Any unused strips from the stripwell plate can be placed in the foil bag, sealed with tape and stored at 4°C.
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 of diluted C/EBPα or C/EBPβ antibody (1:500 dilution in 1X Antibody Binding Buffer) to each well being used, including blank wells.
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 (1:500 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. 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

13. Remove as much of the final wash as possible by blotting the plate on paper towels.
14. Add 100 µl of room temperature Developing Solution to all wells being used.
15. Incubate under low light conditions from 30 seconds to 5 minutes at room temperature protected from direct light. Please read the Certificate of Analysis supplied with this kit for optimal development time associated with this lot number. Monitor the blue color development in the sample wells until they turn medium to dark blue. Do not overdevelop.
16. Add 100 µl of Stop Solution to all the wells. In presence of the acid, the blue color turns yellow.
17. Read absorbance on a spectrophotometer within 5 minutes at 450nm with an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

    Reading the reference wavelength is optional. Most microtiter plate readers are equipped to perform dual wavelength analysis and with the appropriate software, will automatically subtract the reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, you may read the plate twice, once at 450 nm and once at 655 nm then manually subtract the 655 nm OD from the 450 nm OD values.

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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
0 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 a small aliquot of 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 1:1,000 for primary antibody and 1:5,000 for the secondary antibody. The sensitivity of the assay will be decreased
No signal or weak signal in sample wells	Not enough nuclear extract per well	Increase amount of nuclear extract. Not to exceed 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/EBP is poorly activated or inactivated in nuclear fractions	Perform a time course for C/EBP activation in the studied cell line
	Nuclear extracts are not from correct species	The C/EBPα antibody works in human samples, and C/EBPβ antibodies work in human, mouse and rat samples.
	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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