TransAM™

CREB / pCREB
Transcription Factor Assay Kits

(version D3)

Catalog Nos. 42096 & 42596 (CREB)
Catalog Nos. 43096 & 43596 (pCREB)

Revision History

Revision	Date	Description of Change
D2	Dec 2018	Updated Related Products available

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Overview

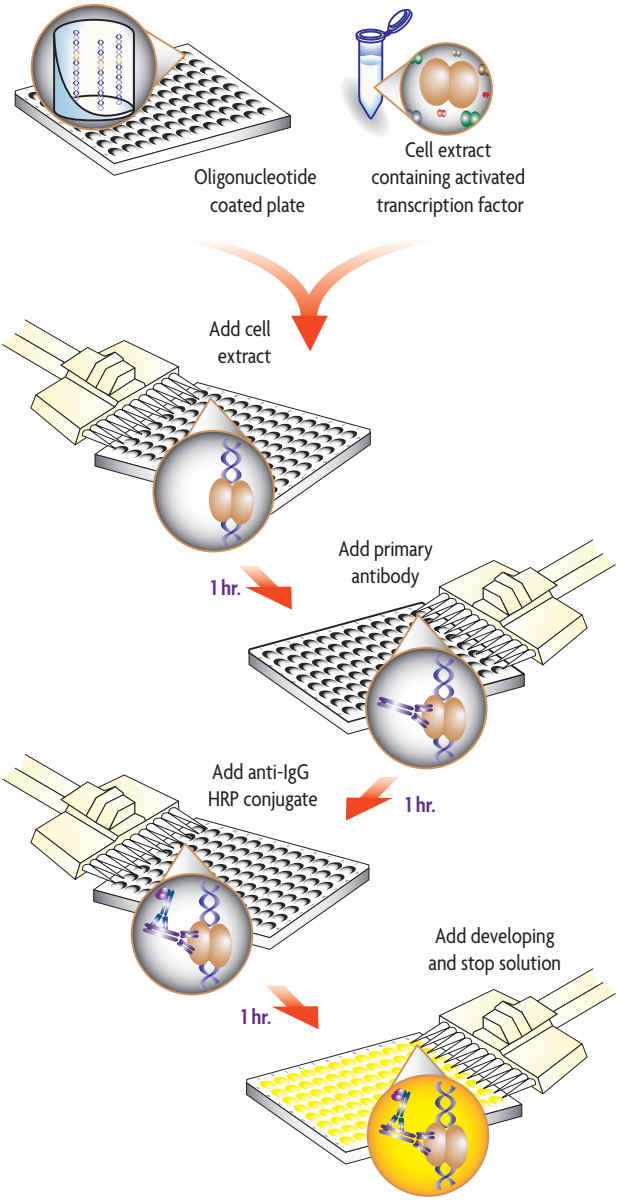
Cyclic AMP Response Element-Binding protein (CREB) is a transcription factor that is activated during changes in the cell environment to promote cell survival, differentiation and adaptive responses. Therefore, accurate monitoring of CREB activation in cells, tissues and animals is crucial for many biomedical 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 CREB and pCREB Kits are designed specifically to detect and quantify CREB activation. They contain a 96-well plate to which oligonucleotide containing a cAMP-responsive element (CRE) has been immobilized. The CREB contained in nuclear extracts specifically binds to this oligonucleotide and is detected through use of an antibody directed against either total CREB or phosphorylated CREB (pCREB). 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 CREB and pCREB Kits are available in two sizes:

product	format	catalog no.
TransAM CREB	1 x 96-well plate	42096
	5 x 96-well plates	42596
TransAM pCREB	1 x 96-well plate	43096
	5 x 96-well plates	43596

See Active Motif products related to the CREB transcription factor in Appendix, Section C.

Flow Chart of Process



Introduction

CREB Transcription Factor

Cyclic AMP Response Element-Binding protein (CREB) is a member of a large family of structurally related transcription factors that includes AFT1-4, c-Fos, c-Jun, c-Myc and C/EBP (see 1 for review). The members of this family, named bZIP, share a dimerization domain with a leucine zipper motif and a DNA-binding domain rich in basic residues (lysines and arginines). CREB proteins specifically recognize the cAMP-responsive element (CRE) promoter site (5´-TGACGTCA-3´)[2].

Alternative splicing of the CREB gene yields several forms of CREB protein. The three most abundant forms are CREBα (341 aa), also called CREB1, CREBΔ (327 aa) and CREBβ (301 and 387 aa), which are present in human, rat and mouse tissues[3, 4]. Two other gene products highly homologous to CREB-1 have been characterized: activating transcription factor 1 (ATF-1)[5] and cAMP response element modulator (CREM)[6].

The CREB family members bind to the CRE promoter site as homo- and heterodimers[7]. The ratio between these homo- and heterodimers, which depends on the cell type, regulates the CREB transcriptional activity as the homodimers have a longer half-life than CREB/ATF-1 heterodimers[8].

Whereas ATF2-4 are able to heterodimerize with other bZIP factors, such as c-Jun and c-Fos[5, 9], the CREB-1, CREM and ATF-1 proteins only heterodimerize between each other. Therefore, CREB-1, CREM and ATF-1 members may represent a distinctive group with the CREB family.

The CREB proteins activate transcription of target genes in response to a diverse array of stimuli, such as peptide hormones, growth factors and neuronal activity. Activation of CREB is mediated by a variety of protein kinases including protein kinase A (PKA), mitogen-activated protein kinases (MAPKs) and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) that phosphorylate CREB at the Ser-133 residue[10]. Phosphorylation of Ser 133 is required for CREB-mediated transcription but not for dimerization and DNA-binding activity. Phosphorylation does, however, increase CREB's affinity for its promoter site (see 1 for review). Upon cell stimulation with forskolin, an activator of adenylyl cyclase[11], the kinases are activated by cAMP production and translocate to the nucleus where they phosphorylate CREB at Ser 133[12]. Therefore, CREB is almost exclusively nuclear in both unstimulated and stimulated cells. A cofactor, CREB-binding protein (CPB), specifically binds to phosphorylated CREB to enhance transcriptional activity[13].

CREB is critical for a variety of cellular processes such as cell proliferation, differentiation and adaptive responses. CREB is implicated in the learning and memorization processes[14] and contributes to neuronal adaptation to drugs of abuse[15]. CREB activity is also important for hormonal control of metabolic pathways, such as gluconeogenesis, by the hormones glucagon and insulin[16].

Transcription Factor Assays

To date, three methods are widely used to measure CREB activity, either directly or indirectly:

1. CREB phosphorylation can be determined by Western Blot by using antibodies specific for Ser-133 phosphorylated CREB. 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 CREB 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 CREB binding. If CREB is active in the nuclear extract, it will bind to the probe. To detect phosphorylated CREB, a supershift step in the presence of phospho-specific antibodies is required. 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. Gel-shift assays also require special precautions and equipment for handling radioactivity.

3. Another method used to assay CREB activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing the CRE sequence. The promoter can be artificial, made of several CRE cis-elements and a TATA box, or natural, like the somatostatin 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 CREB

CREB is a key factor in various immune and neuronal processes, and therefore represents an excellent pharmacological target. However, pharmaceutical research in this field has been limited by the lack of convenient assays suitable for high sample volume.

To overcome this, Active Motif is introducing a high-throughput assay to quantify CREB activation[17]. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM CREB and pCREB Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains a CRE (5´-TGACGTCA-3´). CREB contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used in TransAM CREB Kits recognizes detects endogenous levels of total CREB (independent of the phosphorylation state of CREB). In contrast, the primary antibody used in TransAM pCREB Kits specifically recognizes the Ser-133 phosphorylated CREB for detection of activated CREB only. Utilization 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 5.5 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 either total CREB or activated CREB and has been shown to be 4-fold more sensitive and 20-fold faster than the gel-retardation technique. With the 5.5-hour TransAM procedure, we could detect CREB activation using as little as 0.5 µg of nuclear extract from forskolin-stimulated cells. A comparable assay using EMSA required 2 µg of nuclear extract and a 3-day autoradiography.

TransAM has many applications including the study of drug potency toward CREB-related kinases, CREB transcriptional activity regulation and protein structure/function studies of CREB and its cofactors in areas such as learning, memorization, mental retardation, drug adaptation in the brain, T-cell repertoire development, hormonal control of metabolic pathways, and many more.

Kit Performance and Benefits

The TransAM CREB Kits are for research use only. Not for use in diagnostic procedures.

Detection limit: <0.5 µg nuclear extract/well.

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

Cross-reactivity: TransAM pCREB Kits detect bound phosphorylated CREB-1 (p43) in human, mouse, rat and hamster extracts. It may also recognize phosphorylated CREM-1 and ATF-1. TransAM CREB Kits detect total CREB (independent of its phosphorylation state) in human, mouse, rat and monkey extracts.

	pCREB	CREB
Human	+	+
Mouse	+	+
Rat	+	+

Assay time: 3.5 hours.

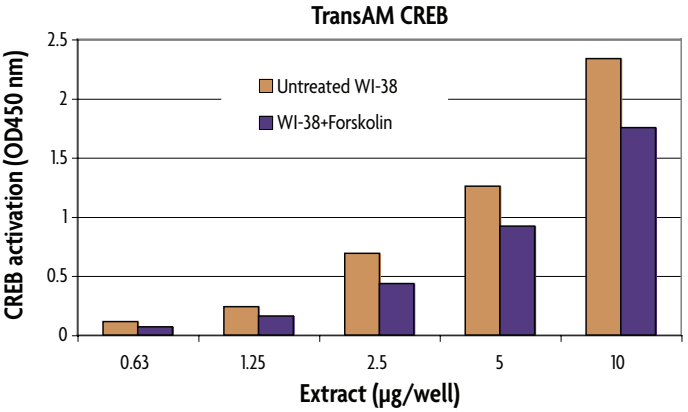


Figure 1: Monitoring CREB activity with the TransAM CREB Kit.

Nuclear extracts from WI-38 cells (Catalog No. 40310) and WI-38 cells stimulated with Forskolin (Catalog No. 40300) were assayed from 0.625-10 µg/well for CREB activation using the TransAM CREB Kit. Data shown are the results from wells assayed in duplicate. These results are provided for demonstration only.

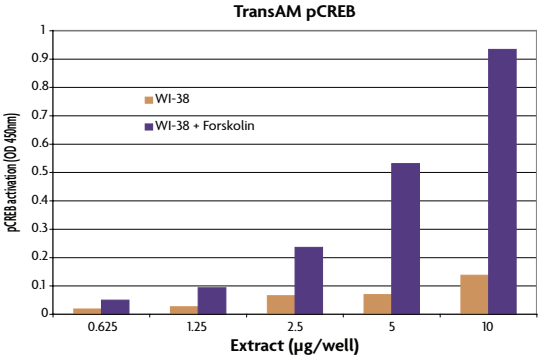


Figure 1: Monitoring pCREB activity with the TransAM pCREB Kit.

Nuclear extracts from WI-38 cells (Catalog No. 40310) and WI-38 cells stimulated with Forskolin (Catalog No. 40300) were assayed from 0.625-10 µg/well for pCREB activation using the TransAM pCREB Kit. Data shown are the results from wells assayed in duplicate. These results are provided for demonstration only.

Kit Components and Storage

TransAM CREB 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 when stored properly.

Reagents	Quantity 1 plate / 5 plates	Storage
CREB antibody, or pCREB antibody	10 µl / 50 µl 22 µl / 110 µl	-20°C for 6 months -20°C for 6 months
Anti-mouse HRP-conjugated IgG, or Anti-rabbit HRP-conjugated IgG	11 µl / 55 µl 11 µl / 55 µl	4°C for 6 months 4°C for 6 months
Wild-type oligonucleotide AM2	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM2	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
WI-38 (Forskolin) 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 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 CREB assay plate	1 / 5	4°C for 6 months
Plate sealer	1 / 5	Room temperature

Additional materials required

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform
- Nuclear extracts
- 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. Our Nuclear Extract Kit can also be purchased separately (Cat. Nos. 40010 & 40410). Lysis Buffer AM1 contains phosphatase inhibitors to prevent dephosphorylation of phosphorylated CREB during the extract preparation and the assay. The presence of these inhibitors gives a yellow coloration to Lysis Buffer AM1. 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 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 required for the assay by adding 1 µl of 1 M DTT and 10 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 any precipitates by warming to room temperature and vortexing for 1 minute prior to use.

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

Preparation of antibodies

Dilute the CREB antibody 1:1000 with 1X Antibody Binding Buffer. Use 100 µl per well. Dilute the anti-mouse HRP-conjugated secondary antibody 1:1000 with 1X Antibody Binding Buffer. Use 100 µl per well.

Dilute the pCREB antibody to 1:500 with the 1X Antibody Binding Buffer. Use 100 µl per well. Dilute the anti-rabbit HRP-conjugated secondary antibody 1:1000 with 1X Antibody Binding Buffer. Use 100 µl per well.

Developing Solution

100 µl are needed per well. 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

100 µl are needed per well. 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.

WI-38 nuclear extract

The WI-38 (Forskolin) nuclear extract is provided as a positive control for CREB activation. Sufficient extract is supplied for 20 reactions when used at 5 µg/well. We recommend aliquoting the extract in 5 µl fractions and storing at -80ºC to help avoid multiple freeze/thaw cycles of the extracts. Various nuclear extracts are available from Active Motif.

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for CREB binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the wild-type oligonucleotide will prevent CREB binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on CREB 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 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	0.02 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	0.03 µl	0.3 µl	1.8 µl	3.6 µl
	Herring sperm DNA	0.34 µl	2.7 µl	16.2 µl	32.4 µl
	Binding Buffer AM1	33.4 µl	267.0 µl	1.602 ml	3.2 ml
	TOTAL REQUIRED	**33.8 µl**	**270.0 µl**	**1.62 ml**	**3.24 ml**
Complete Binding Buffer with wild-type or mutated oligonucleotide	Wild-type or mutated oligo	2.0 µl	16.0 µl	96.0 µl	N/A
	Complete Binding Buffer	31.8 µl	254.0 µl	1.524 ml	N/A
	TOTAL REQUIRED	**33.8 µl**	**270.0 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 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**
Primary Antibody	CREB antibody	0.1 µl	0.9 µl	5.4 µl	10.8 µl
	1X Antibody Binding Buffer	112.4 µl	900 µl	5.4 ml	10.8 ml
	TOTAL REQUIRED	**112.5 µl**	**900 µl**	**5.4 ml**	**10.8 ml**
OR					
	pCREB antibody	0.2 µl	1.8 µl	10.8 µl	21.6 µl
	1X Antibody Binding Buffer	112.3 µl	900 µl	5.4 ml	10.8 ml
	TOTAL REQUIRED	**112.5 µl**	**900 µl**	**5.4 ml**	**10.8 ml**
Secondary Antibody	HRP-conjugated anti-mouse IgG	0.1 µl	0.9 µl	5.4 µl	10.8 µl
	1X Antibody Binding Buffer	112.4 µl	900 µl	5.4 ml	10.8 ml
	TOTAL REQUIRED	**112.5 µl**	**900 µl**	**5.4 ml**	**10.8 ml**
OR					
	HRP-conjugated anti-rabbit IgG	0.1 µl	0.9 µl	5.4 µl	10.8 µl
	1X Antibody Binding Buffer	112.4 µl	900 µl	5.4 ml	10.8 ml
	TOTAL REQUIRED	**112.5 µl**	**900 µl**	**5.4 ml**	**10.8 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.

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

Step 1: Binding of CREB 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 0.1-10 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts can be found on page 15.

   Positive control wells: For both CREB and pCREB we recommend adding 5 µg of the provided nuclear extract diluted in 20 µl of Complete Lysis Buffer per well (2 µl of nuclear extract in 18 µl of Complete Lysis Buffer per well).

   Blank wells: Add 20 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	30 µl	30 µl	30 µl	28 µl	28 µl
Wild-type oligonucleotide	–	–	–	2 µl	–
Mutated oligonucleotide	–	–	–	–	2 µl
Complete Lysis Buffer	20 µl	–	–	–	–
Sample in Complete Lysis Buffer	–	20 µl	20 µl	20 µl	20 µl

3. Use the provided adhesive cover to seal the plate. Incubate for 3 hours 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. **CREB:** Dilute the CREB antibody 1:1000 in 1X Antibody Binding Buffer and mix thoroughly.
   pCREB: Dilute the pCREB antibody 1:500 in 1X Antibody Binding Buffer and mix thoroughly.
2. Add 100 µl of diluted primary antibody to each well being used.
3. Cover the plate and incubate for 1 hour at room temperature without agitation.
4. 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. **CREB:** Dilute the HRP-conjugated anti-mouse IgG 1:1000 in 1X Antibody Binding Buffer and mix thoroughly.
   pCREB: Dilute the HRP-conjugated anti-rabbit IgG antibody 1:1000 in 1X Antibody Binding Buffer and mix thoroughly.
2. Add 100 µl diluted HRP-conjugated antibody to all wells being used.
3. Cover the plate and incubate for 1 hour at room temperature without agitation.
4. During this incubation, place the Developing Solution at room temperature.
5. 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 5-15 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 an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

References

1. Shaywitz A.J. and Greenberg M.E. (1999) *Annu. Rev. Biochem.* 68: 821-61.
2. Montminy M.R., Sevarino K.A., Wagner J.A., Mandel G. and Goodman R.H. (1986) *Proc. Natl. Acad. Sci. USA* 83: 6682-86.
3. Hoeffler J.P., Meyer T.E., Yun Y., Jameson J.L. and Habener J.F. (1988) *Science* 242: 1430-33.
4. Blendy J.A., Kaestner K.H., Schmid W., Gass P. and Schutz G. (1996) *EMBO J.* 15: 1098-106.
5. Hai T.W., Liu F., Coukos W.J. and Green M.R. (1989) *Genes Dev.* 3: 2083-90.
6. Foulkes N.S., Borelli E. and Sassone-Corsi P. (1991) *Cell* 64: 739-49.
7. Yamamoto K.K., Gonzalez G.A., Biggs W.H. and Montminy M.R. (1988) *Nature* 334: 494-98.
8. Kobayashi M. and Kawakami K. (1995) *Nucleic Acids Res.* 23: 2848-55.
9. Hai. T and Curran T. (1991) *Proc. Natl. Acad. Sci. USA* 88: 3720-24.
10. Gonzalez G.A. and Montminy M.R. (1989) *Cell* 59: 675-80.
11. Seamon K.B., Padgett W. and Daly J.W. (1981) *Proc. Natl. Acad. Sci. USA* 78: 3363-67.
12. Hagiwara M., Brindle P., Harootunian A., Armstrong R., Rivier J., Vale W., Tsien R. and Montminy M.R. (1993) *Mol. Cell. Biol.* 13: 4852-59.
13. Chrivia J.C., Kwok R.P., Lamb N., Hagiwara M., Montminy M.R. and Goodman R.H. (1993) *Nature* 365: 855-59.
14. Silva A.J., Kogan J.H., Frankland P.W. and Kida S. (1998) *Annu. Rev. Neurosci.* 21: 127-48.
15. Blendy J.A. and Maldonado R (1998) *J. Mol. Med.* 76: 104-10.
16. Hanson R.W. and Reshef L. (1997) *Annu. Rev. Biochem.* 66: 581-611.
17. 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
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 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 1:1,000-1:2,000 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
	CREB is poorly activated or inactivated in nuclear fractions	Perform a time course for CREB activation in the studied cell line
	Nuclear extracts are not from correct species	The CREB and pCREB antibodies work in human, mouse and rat species
	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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