CREB (Phospho-Ser133) Transcription Factor Assay Kit

Item No. 10009846

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TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Kit components may be stored at -20°C prior to use. For long term storage, the Positive Control should be thawed on ice, aliquoted at 25 µl/vial, and stored at -80°C. After opening kit, we recommend each kit component be stored according to the temperature listed below.

Item Number	Item	Quantity	Storage
10006880	Transcription Factor Binding Assay Buffer (4X)	1 vial/3 ml	4°C
10007472	Transcription Factor Reagent A	1 vial/120 µl	-20°C
10009849	TF CREB (Phospho-Ser^{133}) Positive Control	1 vial/150 µl	-80°C
10006882	Transcription Factor Antibody Binding Buffer (10X)	1 vial/3 ml	4°C
10009848	TF CREB (Phospho-Ser^{133}) Primary Antibody	1 vial/120 µl	-20°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
10009488	TF CREB (Phospho-Ser^{133}) Competitor dsDNA	1 vial/120 µl	-20°C
10006884	Transcription Factor Goat Anti-Rabbit HRP Conjugate	1 vial/120 µl	-20°C
10009489	TF CREB (Phospho-Ser^{133}) 96-Well Strip Plate	1 plate	4°C
400012	96-Well Cover Sheet	1 cover	RT
10006888	Transcription Factor Developing Solution	1 vial/12 ml	4°C
10006889	Transcription Factor Stop Solution	1 vial/12 ml	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

Kit components may be stored at -20°C prior to use. For long term storage, the Positive Control should be thawed on ice, aliquoted at 25 µl/vial and stored at -80°C. If the assay will be used on multiple days, we recommend each kit component be stored according to the temperatures listed in the booklet.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

E-Mail: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. 300 mM dithiothreitol (DTT)
5. Nuclear Extraction Kit available from Cayman (Item No. 10009277) or buffers for preparation of nuclear extracts (see pages 9-13)

NOTE: The components in each kit lot have been quality assured and warranted in this specific combination only; please do not mix them with components from other lots.

INTRODUCTION

Background

Cyclic AMP response element-binding protein (CREB) belongs to a large family of structurally related transcription factors called bZIP and includes AFT1-4, c-Fos, c-Myc, c-Jun, and C/EBP.[1,2] These basic region leucine zipper (bZIP) transcription factors have a DNA-binding domain and a dimerization domain that contains the leucine zipper motif. CREB proteins recognize and bind to the cAMP-responsive element promoter (CRE) site and regulate transcription of many downstream genes that play important roles in metabolic regulation, depression, and in signaling pathways that enable long term memory.[3]

The best characterized activator of CREB is cAMP-dependent protein kinase A (PKA)-mediated phosphorylation of CREB on Serine133 (Ser^{133}) following G protein-coupled receptor (GPCR) stimulation by growth factors, neurotransmitters, or peptide hormones.[4] For example, binding of ligands to their cognate GPCR ultimately leads to an immediate increase in intracellular cAMP levels resulting in liberation of the catalytic subunits of PKA. The free catalytic subunits enter the cell nucleus where they phosphorylate CREB at Ser^{133} activating a cascade of events that includes recruitment of CREB-binding protein (CBP) and p300 along with other cofactors in the assembly of a large transcriptional complex.[6]

CBP and p300 contain intrinsic histone deacetylation (HDAC) activity that assist in the activation of transcription and ultimately enable the synthesis of RNA by RNA polymerase II. Additional diverse stimuli such as hypoxia, growth factors, UV light, survival signals, and stress signals are also known activators of CREB.[4] These various stimuli act through a variety of complex signaling pathways that converge to phosphorylate CREB and induce transcription or inactivate the CREB transcription complex. The activated kinases able to phosphorylate CREB include (but are not limited to) PKA, AKT, MAPK-activated ribosomal S6 kinases (RSKs), and MSK1. Many of these kinases phosphorylate CREB at residues other than Ser^{133} to initiate binding to DNA and transcription of downstream targets. Other post-translational modifications of CREB include acetylation, sumoylation, and glycosylation which add to the complexity and diversity of its transcriptional regulation in the cell.[5] CREB is involved in long term memory and could potentially be utilized for targeted drug therapy for memory loss in Alzheimer's disease, stroke, drug abuse, and head trauma.

About This Assay

Cayman's CREB (Phospho-Ser^{133}) Transcription Factor Assay is a non-radioactive, sensitive method for detecting CREB DNA binding activity in nuclear extracts and whole cell lysates. A 96-well enzyme-linked immunosorbent assay (ELISA) replaces the cumbersome radioactive electrophoretic mobility shift assay (EMSA). A specific double stranded DNA (dsDNA) consensus sequence containing the cAMP response element (CRE) is immobilized to the wells of a 96-well plate (see Figure 1, on page 8). CREB contained in a nuclear extract or whole cell lysate binds specifically to the CRE. The activated CREB transcription factor complex is detected by addition of a specific primary antibody directed against Phospho-Ser^{133} on CREB. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm.

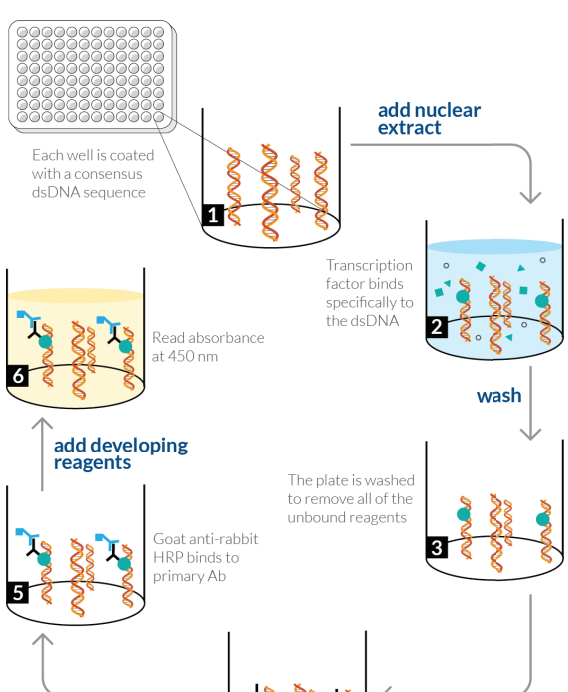


Figure 1. Schematic of the Transcription Factor Binding Assay

PRE-ASSAY PREPARATION

Sample Buffer Preparation

All buffers and reagents below required for preparation of Nuclear Extracts can be purchased directly from Cayman. Alternatively, Cayman's Nuclear Extraction Kit (Item No. 10009277) can be used to isolate Nuclear Proteins.

1. **Nuclear Extraction PBS (10X)**

   1.71 M NaCl, 33.53 mM KCl, 126.8 mM Na_2HPO_4, 22.04 mM KH_2PO_4, pH 7.4

2. **Nuclear Exraction PBS (1X)**

   Dilute 100 ml of 10X stock with 900 ml distilled H_2O

3. **Nuclear Extraction Phosphatase Inhibitor Cocktail (50X)**

   0.5 M NaF

   0.05 M β-glycerophosphate

   0.05 M Na_3OV_4

   Store at -80°C

4. **Nuclear Extraction PBS/Phosphatase Inhibitor Solution (1X)**

   Add 200 µl of 50X Phosphatase Inhibitor Solution to 10 ml of 1X Nuclear Extraction PBS, mix well, and keep on ice. Make fresh daily.

5. **Nuclear Extraction Protease Inhibitor Cocktail (100X)**

   10 mM AEBSF

   0.5 mM Bestatin

   0.2 mM Leupeptin Hemisulfate Salt

   0.15 mM E-64

   0.1 mM Pepstatin A

   0.008 mM Aprotinin from Bovine Lung

   Made in DMSO, store at -20°C

6. **Nuclear Extraction Hypotonic Buffer (10X)**

   100 mM HEPES, pH 7.5, containing 40 mM NaF, 100 µM Na_2MoO_4, and 1 mM EDTA

   Store at 4°C

7. **Complete Hypotonic Buffer (1X)**

   Prepare as outlined in Table 1. The phosphatase and protease inhibitors lose activity shortly after dilution; therefore any unused 1X Complete Hypotonic Buffer should be discarded.

Reagent	150 mm plate ~1.5 x 10^7 cells
Hypotonic Buffer (10X)	100 µl
Phosphatase Inhibitors (50X)	20 µl
Protease Inhibitors (100X)	10 µl
Distilled Water	870 µl
Total Volume	1,000 µl

Table 1. Preparation of Complete Hypotonic Buffer

8. **Nonidet P-40 Assay Reagent (10%)**

   Nonidet P-40 or suitable substitute at a concentration of 10% (v/v) in H_2O

   Store at room temperature

9. **Nuclear Extraction Buffer (2X)**

   20 mM HEPES, pH 7.9, containing, 0.2 mM EDTA, 3 mM $MgCl_2$, 840 mM NaCl, and 20% glycerol (v/v)

   Store at 4°C

10. **Complete Nuclear Extraction Buffer (1X)**

    Prepare as outlined in Table 2. Some of the phosphatase and protease inhibitors lose activity shortly after dilution; therefore any remaining 1X Extraction Buffer should be discarded.

Reagent	150 mm plate ~1.5 x 10^7 cells
Nuclear Extraction Buffer (2X)	75 µl
Protease Inhibitors (100X)	1.5 µl
Phosphatase Inhibitors (50X)	3.0 µl
DTT (10 mM)	15 µl
Distilled Water	55.5 µl
Total Volume	150 µl

Table 2. Preparation of Complete Nuclear Extraction Buffer

Purification of Cellular Nuclear Extracts

Cayman's Nuclear Extraction Kit (Item No. 10009277) can be used to isolate nuclear proteins. Alternatively, the procedure described below can be used for a 15 ml cell suspension grown in a T75 flask or adherent cells (100 mm dish 80-90% confluent) where 10^7 cells yields approximately 50 µg of nuclear protein.

1. Collect 10^7 cells in pre-chilled 15 ml tubes.
2. Centrifuge suspended cells at 300 x g for five minutes at 4°C.
3. Discard the supernatant. Resuspend cell pellet in 5 ml of ice-cold 1X Nuclear Extraction PBS/Phosphatase Inhibitor Solution and centrifuge at 300 x g for five minutes at 4°C. Repeat one time.
4. Discard the supernatant. Add 500 µl ice-cold 1X Complete Hypotonic Buffer. Mix gently by pipetting and transfer resuspended pellet to pre-chilled 1.5 ml microcentrifuge tube.
5. Incubate cells on ice for 15 minutes allowing cells to swell.
6. Add 100 µl of 10% Nonidet P-40. Mix gently by pipetting.
7. Centrifuge for 30 seconds (pulse spin) at 4°C in a microcentrifuge. Transfer the supernatant which contains the cytosolic fraction to a new tube and store at -80°C.
8. Resuspend the pellet in 100 µl ice-cold Extraction Buffer (with protease and phosphatase inhibitors). Vortex 15 seconds at the highest setting and then gently rock the tube on ice for 15 minutes using a shaking platform. Vortex sample for 30 seconds at the highest setting and gently rock for an additional 15 minutes.
9. Centrifuge at 14,000 x g for 10 minutes at 4°C. The supernatant contains the nuclear fraction. Aliquot to clean chilled tubes, flash freeze, and store at -80°C. Avoid freeze/thaw cycles. The extracts are ready to use in the assay.
10. Keep a small aliquot of the nuclear extract to quantitate the protein concentration.

Reagent Preparation

1. **Transcription Factor Antibody Binding Buffer (10X)**

   One vial (Item No. 10006882) contains 3 ml of a 10X stock of Transcription Factor Antibody Binding Buffer (ABB) to be used for diluting the primary and secondary antibodies. To prepare 1X ABB, dilute 1:10 by adding 27 ml of UltraPure water. Store at 4°C for up to two months.

2. **Wash Buffer Concentrate (400X)**

   One vial (Item No. 400062) contains 5 ml of 400X Wash Buffer. Dilute the contents of the vial to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035). *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a pipet. A positive displacement device such as a syringe should be used to deliver small quantities accurately.* A smaller volume of Wash Buffer Concentrate can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer). Store at 4°C for up to two months.

3. **Transcription Factor Binding Assay Buffer (4X)**

One vial (Item No. 10006880) contains 3 ml of a 4X stock of Transcription Factor Binding Assay Buffer (TFB). Prepare Complete Transcription Factor Binding Assay Buffer (CTFB) immediately prior to use in 1.5 ml centrifuge tubes or 15 ml conical tubes as outlined below in Table 3. This buffer is now referred to as CTFB. *NOTE: It is recommended that the CTFB be used the same day it is prepared.*

Component	Volume/ Well	Volume/ Strip	Volume/ 96-well plate
UltraPure water	73 µl	584 µl	7,008 µl
Transcription Factor Binding Assay Buffer (4X)	25 µl	200 µl	2,400 µl
Reagent A (Item No. 10007472)	1 µl	8 µl	96 µl
300 mM DTT	1 µl	8 µl	96 µl
Total Required	100 µl	800 µl	9,600 µl

Table 3. Preparation of Complete Transcription Factor Binding Assay Buffer.

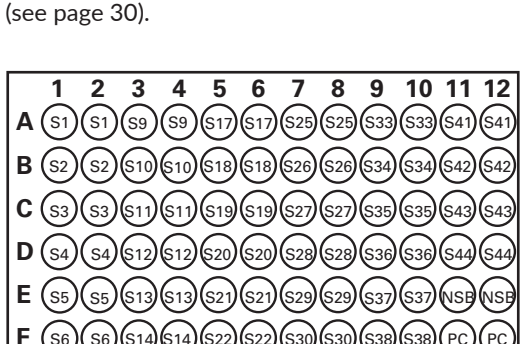
4. **Transcription Factor CREB (Phospho-Ser^{133}) Positive Control**

One vial (Item No. 10009849) contains 150 µl of unstimulated HeLa nuclear extract. This lysate is provided as a positive control for (Phospho-Ser^{133}) CREB; it is not intended for plate to plate comparisons. The Positive Control provided is sufficient for 15 reactions and will provide a strong signal (>0.5 AU at 450 nm) when used at 10 µl/well. When using this Positive Control, a decrease in signal may occur with repeated freeze/thaw cycles. It is recommended that the Positive Control be aliquoted at 25 µl per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw cycles.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of CREB (Phospho-Ser^{133}) positive control (PC), competitor dsDNA (C1), and samples of nuclear extracts (S1-S44) to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 30).



S1-S44 - Sample Wells
NSB - Non-specific Binding Wells
PC - Positive Control Wells
Blk - Blank Wells
C1 - Competitor dsDNA Wells

Figure 2. Sample plate format

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- It is not necessary to use all the wells on the plate at one time; however a positive control should be run every time.
- For each plate or set of strips it is recommended that two Blk, two NSB, and two PC wells be included.

Performing the Assay

Binding of active CREB (Phospho-Ser^{133}) to the consensus sequence:

1. Equilibrate the plate and buffers to room temperature prior to opening. Remove the plate from the foil and select the number of strips needed. The 96-well plate supplied with this kit is ready to use.

   NOTE: If you are not using all of the strips at once, place the unused strips back in the plate packet and store at 2-4°C. Be sure that the packet is sealed with the desiccant inside.

2. Prepare the CTFB as outlined in Table 3, on page 16.

3. Add appropriate amount of reagent(s) listed below to the designated wells as follows:

   Blk - add 100 µl of CTFB to designated wells.

   NSB - add 100 µl of CTFB to designated wells. Do not add samples or positive control to these wells.

   C1 - Add 80 µl of CTFB prior to adding 10 µl of TF CREB (Phospho-Ser^{133}) Competitor dsDNA (Item No. 10009488) to designated wells. Add 10 µl of control cell lysate or unknown sample.

   NOTE: Competitor dsDNA must be added prior to adding the Positive Control or nuclear extracts.

   S1-S44 - Add 90 µl of CTFB followed by 10 µl of Nuclear Extract to designated wells. A protocol for isolation of nuclear extracts is given on page 14.

   PC - Add 90 µl of CTFB followed by 10 µl of Positive Control to appropriate wells.

4. Use the 96-Well Cover Sheet (Item No. 400012) provided to seal the plate. Incubate overnight at 4°C without shaking or one hour at room temperature on an orbital shaker.

5. Empty the wells and wash five times with 200 µl of 1X Wash Buffer. After each wash empty the wells in the sink. After the final wash (wash #5), tap the plate on a paper towel to remove any residual Wash Buffer.

Addition of TF CREB (Phospho-Ser^{133}) Primary Antibody

1. Dilute the TF CREB (Phospho-Ser^{133}) Primary Antibody (Item No. 10009848) 1:100 in 1X ABB as outlined in Table 4 below. Add 100 µl of diluted CREB (Phospho-Ser^{133}) Primary Antibody to each well except the Blk wells.

Component	Volume/ Well	Volume/ Strip	Volume/ 96-well plate
1X ABB	99 µl	792 µl	9,504 µl
CREB (Phospho-Ser^{133}) Primary Antibody	1 µl	8 µl	96 µl
Total required	100 µl	800 µl	9,600 µl

Table 4. Dilution of Primary Antibody

2. Use an adhesive cover to seal the plate.

3. Incubate for one hour at room temperature on an orbital shaker.

4. Empty the wells and wash each well five times with 200 µl of 1X Wash Buffer. After each wash, empty the contents of the plate into the sink. After the final wash (wash #5), tap the plate three to five times on a paper towel to remove any residual Wash Buffer.

Addition of the Transcription Factor Goat Anti-Rabbit HRP Conjugate

1. Dilute the Transcription Factor Goat Anti-Rabbit HRP Conjugate (Item No. 10006884) 1:100 in 1X ABB as outlined in Table 5 below. Add 100 µl of diluted Secondary Antibody to each well except the Blk wells.

Component	Volume/ Well	Volume/ Strip	Volume/ 96-well plate
1X ABB	99 µl	792 µl	9,504 µl
Goat Anti-Rabbit HRP Conjugate	1 µl	8 µl	96 µl
Total required	100 µl	800 µl	9,600 µl

Table 5. Dilution of Secondary Antibody

2. Use an adhesive cover to seal the plate.
3. Incubate for one hour at room temperature on an orbital shaker.
4. Empty the wells and wash five times with 200 µl of 1X Wash Buffer. After each wash, empty the contents of the plate into the sink. After the final wash (wash #5), tap the plate three to five times on a paper towel to remove any residual Wash Buffer.

Develop and Read the Plate

1. To each well being used add 100 µl of Transcription Factor Developing Solution (Item No. 10006888) which has been equilibrated to room temperature.
2. Incubate the plate for 15 to 45 minutes at room temperature on an orbital shaker protected from light. Allow the wells to turn medium to dark blue prior to adding Transcription Factor Stop Solution (Item No. 10006889). (This reaction can be monitored by taking absorbance measurements at 655 nm prior to stopping the reactions; An OD_{655} of 0.4-0.5 yields an OD_{450} of approximately 1). Monitor development of sample wells to ensure adequate color development prior to stopping the reaction. *NOTE: Do not overdevelop; however PC wells may need to overdevelop to allow adequate color development in sample wells.*
3. Add 100 µl of Stop Solution per well being used. The solution within the wells will change from blue to yellow after adding the Stop Solution.
4. Read absorbance at 450 nm within five minutes of adding the Stop Solution. Blank the plate reader according to the manufacturer's requirements using the blank wells.

Assay Procedure Summary

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when initially performing the assay.

1. Add appropriate amounts of CTFB, competitor dsDNA, positive control, or sample to wells as indicated in Table 6 (see page 25).
2. Incubate overnight at 4°C without shaking or one hour at room temperature on an orbital shaker.
3. Wash each well five times with 200 µl of 1X Wash Buffer.
4. Add 100 µl of diluted CREB (Phospho-Ser^{133}) Primary Antibody per well (except Blk wells).
5. Incubate one hour at room temperature on an orbital shaker.
6. Wash each well five times with 200 µl of 1X Wash Buffer.
7. Add 100 µl of diluted Secondary Antibody per well (except Blk wells).
8. Incubate one hour at room temperature on an orbital shaker.
9. Wash each well five times with 200 µl of 1X Wash Buffer.
10. Add 100 µl of Developing Solution per well.
11. Incubate 15 to 45 minutes with at room temperature on an orbital shaker.
12. Add 100 µl of Stop Solution per well.
13. Measure the absorbance at 450 nm.

Steps	Reagent	Blk	NSB	PC	C1	S1-S44
1. Add reagents	CTFB	100 µl	100 µl	90 µl	80 µl	90 µl
	Competitor dsDNA				10 µl	
	Positive Control			10 µl	10 µl	
	Samples					10 µl
2. Incubate	Cover plate and incubate overnight at 4°C without shaking or one hour at RT on an orbital shaker					
3. Wash	Wash all wells five times					
4. Add reagents	Primary Antibody		100 µl	100 µl	100 µl	100 µl
5. Incubate	Cover plate and incubate one hour at RT without agitation					
6. Wash	Wash all wells five times					
7. Add reagents	Secondary Antibody		100 µl	100 µl	100 µl	100 µl
8. Incubate	Cover plate and incubate one hour at RT on an orbital shaker					
9. Wash	Wash all wells five times for 15-45 minutes at RT on an orbital shaker					
10. Add reagents	Developer Solution	100 µl	100 µl	100 µl	100 µl	100 µl
11. Incubate	Monitor development in wells					
12. Add reagents	Stop Solution	100 µl	100 µl	100 µl	100 µl	100 µl
13. Read	Read plate at wavelength of 450 nm					

Table 6. Quick Protocol Guide

Performance Characteristics

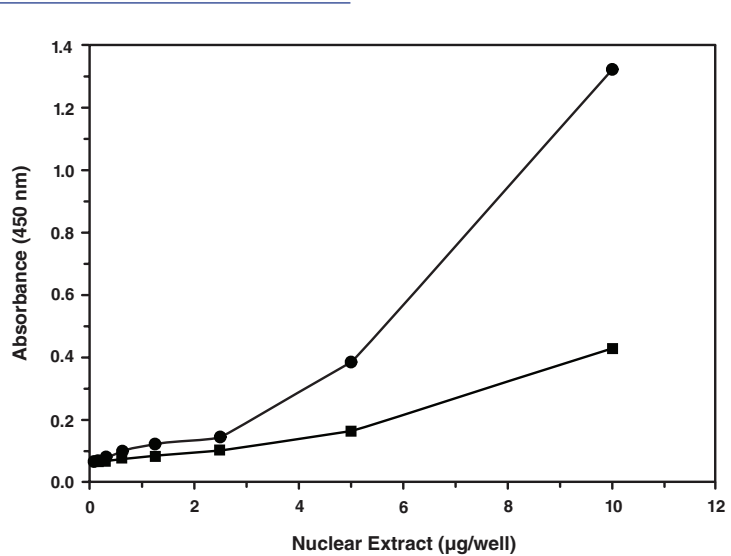


Figure 3. Assay of nuclear fractions isolated from stimulated (10 µM Forskolin for 30 minutes) and nonstimulated HepG2 cells demonstrating activated CREB (Phospho-Ser^{133}).

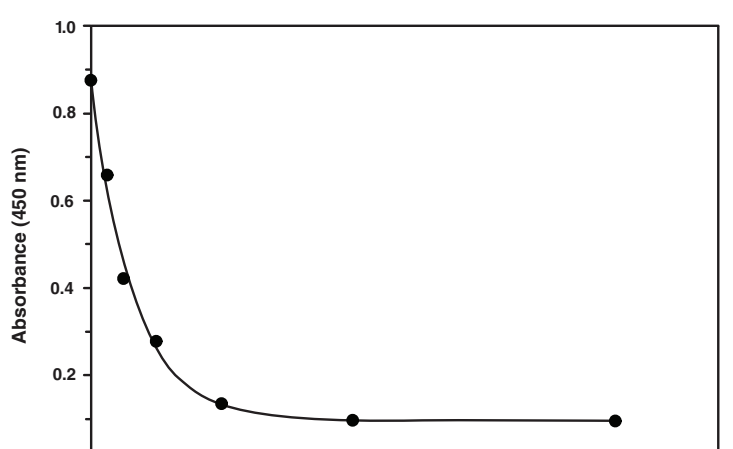


Figure 4. Assay of stimulated PC-12 nuclear extracts following treatment with PP2A. This experiment demonstrates the specificity of the CREB (Phospho-Ser^{133}) Primary Antibody.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No signal or weak signal in control wells	A. Omission of key reagent B. Plate reader settings not correct C. Reagent/reagents expired D. Salt concentrations affected binding between DNA and protein E. Developing reagent used cold F. Developing reagent not added at correct volume	A. Check that all reagents have been added and in the correct order; perform the assay using the Positive Control B. Check wavelength setting on plate reader and change to 450 nm C. Check expiration date on reagents D. Reduce the amount of nuclear extract used in the assay, or reduce the amount of salt in the nuclear extracts (alternatively can perform buffer exchange) E. Prewarm the Developing Solution to room temperature prior to use F. Check pipettes to ensure correct amount of Developing Solution was added to wells
High signal in all wells	A. Incorrect dilution of antibody (too high) B. Improper/inadequate washing of wells C. Overdeveloping	A. Check antibody dilutions and use amounts outlined in instructions B. Follow the protocol for washing wells using the correct number of times and volumes C. Decrease the incubation time when using the developing reagent
High background (NSB)	Incorrect dilution of antibody (too high)	Check antibody dilutions and use amounts outlined in the instructions
Weak signal in sample wells	A. Sample concentration is too low B. Incorrect dilution of antibody C. Salt concentrations affecting binding between DNA and protein	A. Increase the amount of nuclear extract used; loss of signal can occur with multiple freeze/thaw cycles of the sample; prepare fresh nuclear extracts and aliquot as outlined in product insert B. Check antibody dilutions and use amounts outlined in the instructions C. Reduce the amount of nuclear extract used in the assay or reduce the amount of salt in the nuclear extracts (alternatively can perform buffer exchange)

References

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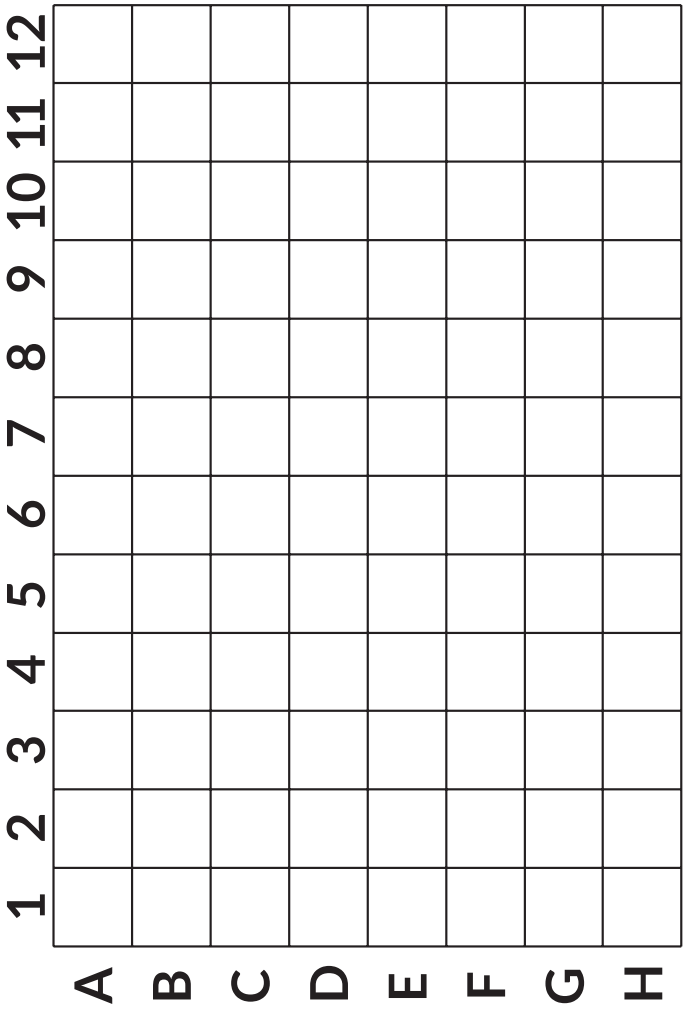

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.