SREBP-2 Transcription Factor Assay Kit

Item No. 10007819

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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 use 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
10008857	Transcription Factor SREBP-2 Positive Control	1 vial/150 µl	-80°C
10006882	Transcription Factor Antibody Binding Buffer (10X)	1 vial/3 ml	4°C
10008859	Transcription Factor SREBP-2 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
10008860	Transcription Factor SREBP Competitor dsDNA	1 vial/120 µl	-20°C
10006884	Transcription Factor Goat Anti-Rabbit HRP Conjugate	1 vial120 µl	-20°C
10008858	Transcription Factor SREBP 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 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

Lipid homeostasis in vertebrate cells is regulated by a family of transcription factors called sterol regulatory element-binding proteins (SREBPs).[1,2] SREBP's directly activate the expression of over 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids.[1] Three different SREBP isoforms designated SREBP-1a, SREBP-1c, and SREBP-2 are encoded by two different genes and belong to the basic helix-loop-helix-leucine zipper (bHLH-ZIP) family of transcription factors.[2] SREBP-2 activates cholesterol synthesis by upregulating expression of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene sythase. It is also involved in activating genes required to generate NADPH, which is consumed at multiple stages of cholesterol biosynthesis.[2] Regulation of SREBP-2 activity is controlled by cholesterol levels in the endoplasmic reticulum (ER) membrane.[3-5] When cholesterol levels are high, SREBP exists as an ER membrane-bound precursor and SREBP cleavage-activating protein (SCAP) is bound to cholesterol. Upon depletion of cholesterol, SCAP becomes activated and escorts SREBP to the Golgi where it is proteolytically cleaved by site 1 protease (S1P), then by S2P. The active transcription factor consisting of the NH_2-terminal domain, designated as nuclear SREBP (nSREBP), translocates into the nucleus. In the nucleus, SREBP binds to sterol regulatory elements (SREs), thereby activating genes involved in lipid homeostasis. Reducing circulating cholesterol and modulation of lipid biosynthesis has important clinical implications for many diseases, including obesity, type 2 diabetes, and atherosclerosis.

About This Assay

Cayman's SREBP-2 Transcription Factor Assay is a non-radioactive, sensitive method for detecting specific transcription factor 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) sequence containing the SREBP response element is immobilized to the wells of a 96-well plate (see Figure 1, on page 8). SREBP contained in a nuclear extract, binds specifically to the SREBP response element. SREBP is detected by addition of a specific primary antibody directed against SREBP. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm.

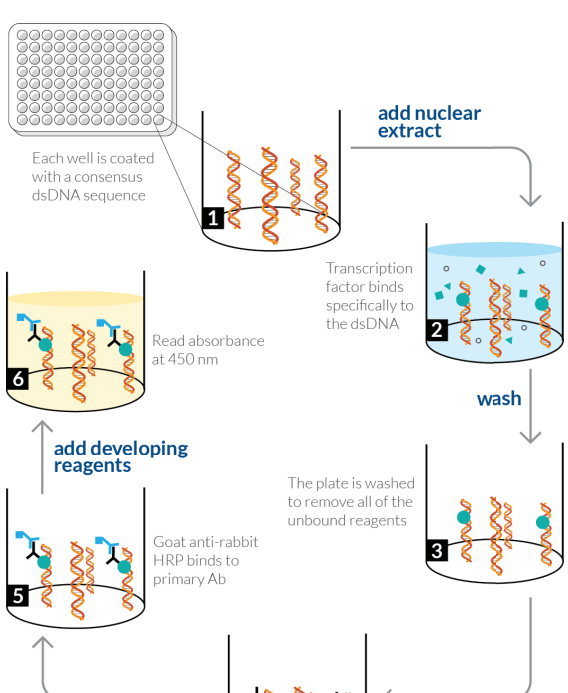


Figure 1. Schematic of the Transcription Factor Binding Assay

PRE-ASSAY PREPARATION

Sample Buffer Preparation

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

1. **Nulcear 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 Extraction PBS (1X)**

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

3. **Nuclear Extraction Phosphatase Inhibitor Solution (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 Hypontonic 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 a 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 (or suitable substitute). 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 Complete Nuclear Extraction Buffer (1X) (with protease and phosphatase inhibitors). Vortex 15 seconds at highest setting then gently rock the tube on ice for 15 minutes using a shaking platform. Vortex sample for 30 seconds at 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 one year.

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 TFB Assay Buffer (CTFB) immediately prior to use in 1.5 ml centrifuge tubes or 15 ml conical tubes as outlined in Table 3. This buffer is now referred to as CTFB. *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
4X Transcription Factor Binding Assay Buffer	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

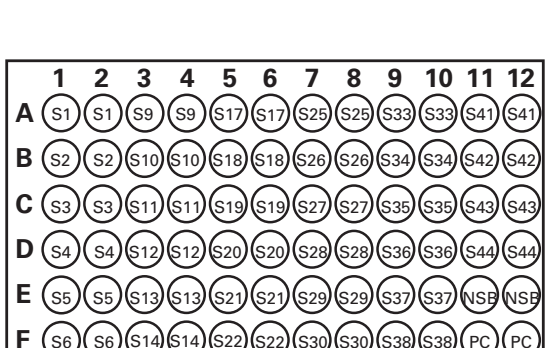
4. **Transcription Factor SREBP-2 Positive Control**

One vial (Item No. 10008857) contains 150 µl of clarified cell lysate. This lysate is provided as a positive control for SREBP-2 activation; 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 SREBP-2 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 SREBP-2 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 15.

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 Transcription Factor SREBP Competitor dsDNA (Item No. 10008860) to designated wells. Add 10 µl of control cell lysate or 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.

   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 Transcription Factor SREBP-2 Primary Antibody

1. Dilute the Transcription Factor SREBP-2 Primary Antibody (Item No. 10008859) 1:100 in 1X ABB as outlined in Table 2 below. Add 100 µl of diluted SREBP-2 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
SREBP-2 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 3 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 coverto 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 SREBP-2 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 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 on an orbital shaker					
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					
10. Add reagents	Developer	100 µl	100 µl	100 µl	100 µl	100 µl
11. Incubate	Monitor development in wells for 15-45 minutes at RT on an orbital shaker					
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

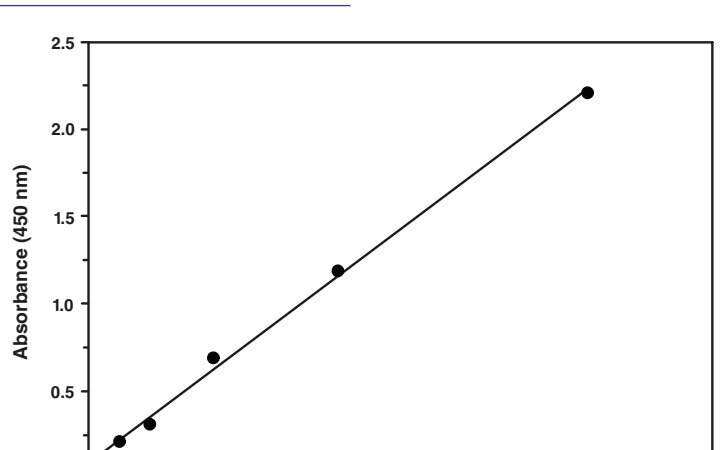


Figure 3. Assay of recombinant SREBP-2 from *E. coli* cell lysates

Interferences

The following reagents were tested for interference in the assay.

Reagent	Will Interfere (Yes or No)
EGTA (≤1 mM)	No
EDTA (≤0.5 mM)	No
ZnCl (any concentration)	Yes
DTT (between 1 and 5 mM)	No
Dimethylsulfoxide (≤1.5%)	No

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 to 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. Warm 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 (see page 21) B. Follow the protocol for washing wells using the correct number of times and volumes (see page 24) 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 (see page 21)

Problem cont.	Possible Causes cont.	Recommended Solutions cont.
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 booklet (see page 14) B. Check antibody dilutions and use amounts outlined in the instructions (see page 21) 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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2. Osborne, T.F. Sterol regulatory element-binding proteins (SREBP's): Key regulators of nutritional homeostasis and insulin action. *J. Biol. Chem.* **275(42)**, 32379-32382 (2000).
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4. Moon, Y.-A. The SCAP/SREBP pathway: A mediator of hepatic steatosis. *Endocrinol. Metab.* **32(1),** 6-10 (2017).
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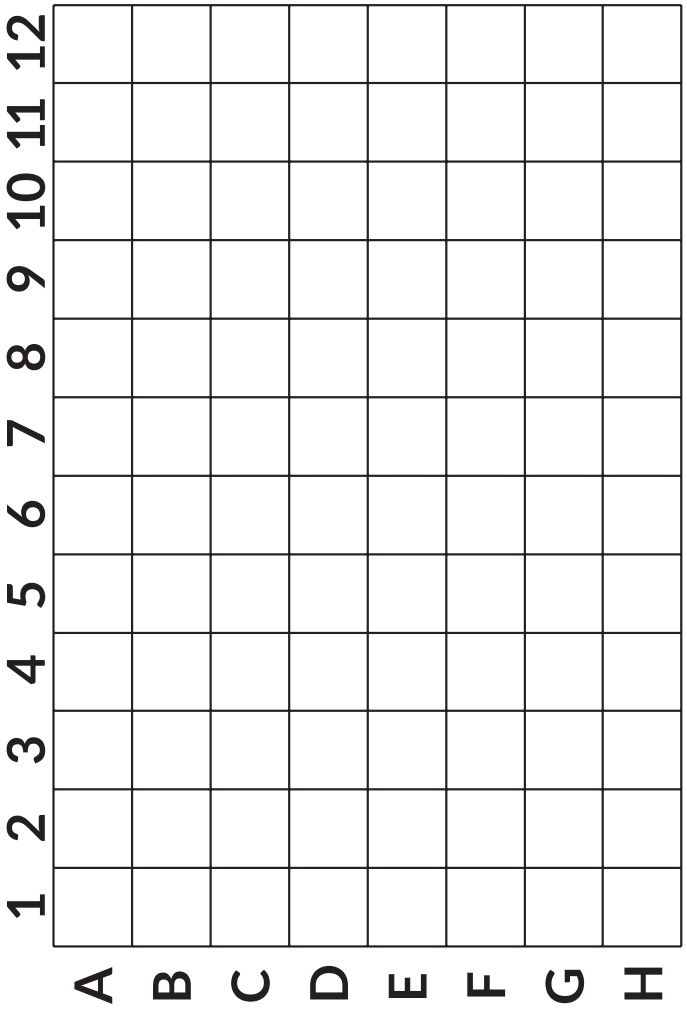

NOTES

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.