

ab133113 – PPAR (alpha, delta, gamma) Transcription Factor Assay Kit

Instructions for Use

For the detection of specific transcription factor DNA binding activity in nuclear extracts and whole cell lysates.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

ab133113 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 PPAR response element is immobilized onto the bottom of wells of a 96-well plate (see Figure 1). PPARs contained in a nuclear extract, bind specifically to the PPAR response element. PPAR alpha, delta, or gamma are detected by addition of specific primary antibodies directed against the individual PPARs. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. ab133113 comes with a single plate that measures all three isoforms of PPAR alpha, delta, and gamma. There are enough reagents for one-third of a plate for each isoform.

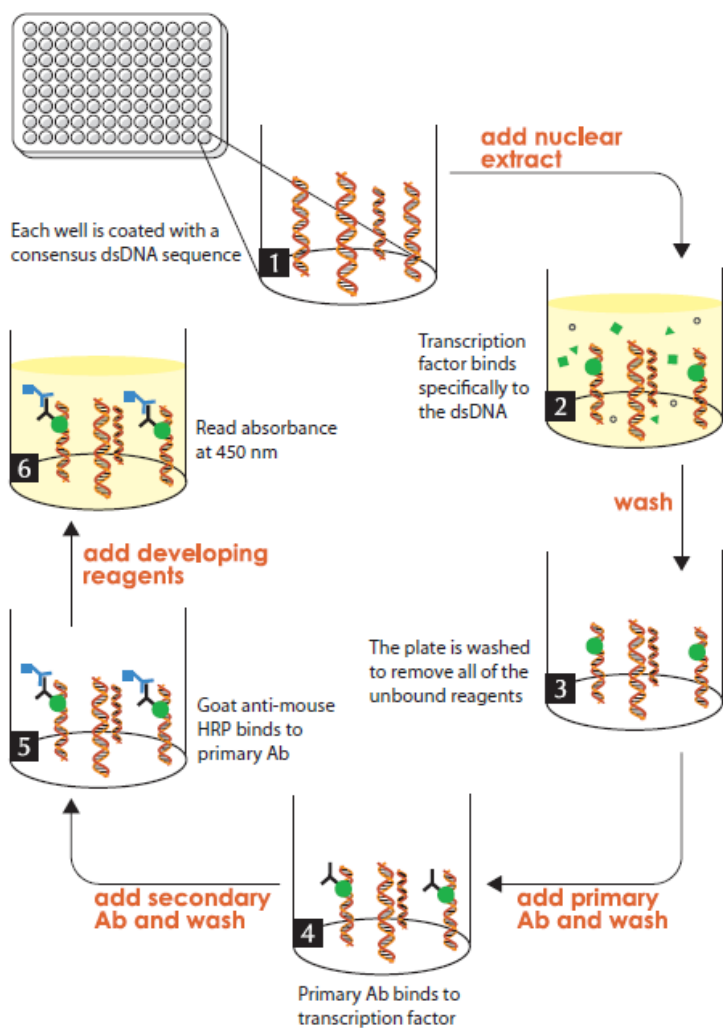


Figure 1. Schematic of the Transcription Factor Binding Assay

2. Background

Peroxisome Proliferator-activated Receptors (PPARs) are ligand-activated transcription factors belonging to the large superfamily of nuclear receptors. They are activated by a variety of fatty acids and fatty acid derivatives such as prostaglandins and leukotrienes. PPARs play pivotal roles in the regulation of lipid metabolism and homeostasis and are important indirect as well as direct regulators of cellular insulin sensitivity. There are three major PPAR isotypes; PPAR alpha, PPAR gamma, and PPAR delta/beta which all bind to PPAR responsive elements (PPRE's) as heterodimers with RXR, another member of the nuclear receptor superfamily. PPAR alpha primarily activates genes encoding proteins involved in fatty acid oxidation, while PPAR gamma primarily activates genes directly involved in lipogenic pathway and insulin signaling. Members of the PPAR family are important direct targets of many antidiabetic and hypolipidemic drugs.

3. Components and Storage

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 20 µL/vial, and stored at -80°C. After use we recommend each kit component be stored according to the temperature listed below.

Item	Quantity	Storage
Transcription Factor Binding Assay Buffer (4X)	1 vial	4°C
Transcription Factor Reagent A	1 vial	-20°C
Transcription Factor Complete PPAR alpha Positive Control	1 vial	-80°C
Transcription Factor Complete PPAR delta Positive Control	1 vial	-80°C
Transcription Factor Complete PPAR gamma Positive Control	1 vial	-80°C
Transcription Factor Antibody Binding Buffer (10X)	1 vial	4°C
Transcription Factor Complete PPAR alpha Primary Antibody	1 vial	-20°C

Item	Quantity	Storage
Transcription Factor Complete PPAR delta Primary Antibody	1 vial	-20°C
Transcription Factor Complete PPAR gamma Primary Antibody	1 vial	-20°C
Wash Buffer Concentrate (400X)	1 vial	4°C
Polysorbate 20	1 vial	RT
Transcription Factor PPAR Competitor dsDNA	1 vial	-20°C
Transcription Factor Goat Anti-Rabbit HRP Conjugate	1 vial	-20°C
Transcription Factor PPAR 96-Well Strip Plate	1 plate	4°C
96-Well Cover Sheet	1 cover	RT
Transcription Factor Developing Solution	1 vial	4°C
Transcription Factor Stop Solution	1 vial	4°C

Materials Needed But Not Supplied

- A plate reader capable of measuring absorbance at 450 nm.
- Adjustable pipettes and a repeat pipettor.
- A source of UltraPure water or HPLC-grade water are acceptable.
- 300 mM dithiothreitol (DTT).
- Nuclear Extraction Kit or buffers for preparation of nuclear extracts. We recommend Nuclear Extraction Kit (ab221978).

Note: The components in each kit lot have been quality tested.

4. Pre-Assay Preparation

A. Purification of Cellular Nuclear Extracts

Harvest cells following the procedure described in Nuclear Extraction Kit (ab221978).

Alternatively, follow the procedure described in Appendix (Section 7).

Keep a small aliquot of the nuclear extract to quantitate the protein concentration.

B. Reagent Preparation

Transcription Factor Antibody Binding Buffer (10X)

One vial 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 six months.

Wash Buffer Concentrate (400X)

One vial contains 5 mL of 400X Wash Buffer. Dilute the contents of the vial to a total volume of 2 L with UltraPure water and add 1 mL of Polysorbate 20. *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a pipette. 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/L of Wash Buffer). Store at 4°C for up to two months.

Transcription Factor Binding Assay Buffer (4X)

One vial 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 in Table 1. 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 μ L	584 μ L	7008 μ L
Transcription Factor Binding Assay Buffer (4X)	25 μ L	200 μ L	2400 μ L
Reagent A	1 μ L	8 μ L	96 μ L
300 mM DTT	1 μ L	8 μ L	96 μ L
Total Required	100 μ L	800 μ L	9600 μ L

Table 1. Preparation of Complete Transcription Factor Binding Assay Buffer.

Transcription Factor PPAR alpha, delta, gamma Positive Controls

One vial contains 50 μ l of clarified cell lysate. These lysates serve as a positive control for PPAR alpha, delta, and gamma, respectively; it is not intended for plate to plate comparisons. The Positive Control provided is sufficient for 5 reactions and will provide a strong signal (>0.5 AU at 450 nm) when used at 10 μ l/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 μ l per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw cycles.

5. Assay Protocol

A. Summary

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

Prepare CTFB as directed in the Pre-Assay Preparation section.



Add CTFB to Blk and NSB wells.



Add Competitor dsDNA (optional) to appropriate wells.



Add positive control to appropriate wells.



Add sample containing PPAR alpha, delta, and/or gamma to appropriate wells.



Incubate overnight at 4°C without agitation.



Wash each well 5 times with 1X wash buffer.



Add diluted PPAR alpha, delta, and/or gamma Primary Antibody to each well (except blk wells).



Incubate at RT without agitation.



Wash each well 5 times with 1X Wash Buffer.



Add diluted Goat Anti-Mouse HRP Conjugate (except Blk wells).



Incubate at RT without agitation.



Wash each well 5 times with 1X Wash Buffer.



Add Developing Solution to wells.



Incubate 15 to 45 minutes with gentle agitation.



Add Stop Solution to wells.



Measure the absorbance.

B. Plate Setup

There is no specific pattern for using the wells on the plate. A typical layout of PPAR Positive Control (PC), Competitor dsDNA (C1), and samples of nuclear extracts (S1-S44) to be measured in duplicate is given below in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44
E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37	NSB	NSB
F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38	PC	PC
G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39	Blk	Blk
H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40	C1	C1

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 (PC) should be run every time.*
- *For each plate or set of strips it is recommended that two Blk, two Non-Specific Binding (NSB), and two PC wells be included*

C. Performing the Assay

Binding of active PPAR alpha, delta, and/or gamma 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 1.
3. Add appropriate amount of reagent(s) listed below to the designated wells as follows:

Blk - add 100 μ l of CTFB to designated wells.

NSB - add 100 μ l of CTFB to designated wells. Do not add samples or Positive Control to these wells.

C1 - Add 80 μ l of CTFB prior to adding 10 μ l of Transcription Factor PPAR Competitor dsDNA to designated wells. Add 10 μ l 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 μ l of CTFB followed by 10 μ l of Nuclear Extract to designated wells. A protocol for isolation of nuclear extracts is given in Section 4.

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

4. Use the cover provided to seal the plate. Incubate overnight at 4°C or one hour at room temperature without agitation (incubation for one hour will result in a less sensitive assay).
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 PPAR Primary Antibody

1. Dilute the Transcription Factor PPAR Primary Antibody 1:100 in 1X ABB as outlined in Table 2. Add 100 µl of diluted PPAR Primary Antibodies to each well except the Blk wells.

Component	Volume/Well	Volume/Strip	Volume/ 96-well plate
1X ABB	99 µL	792 µL	9504 µL
PPAR Primary Antibody	1 µL	8 µL	96 µL
Total Required	100 µL	800 µL	9600 µL

Table 2. Dilution of Primary Antibody.

2. Use the adhesive cover provided to seal the plate.
3. Incubate the plate for one hour at room temperature without agitation.
4. Empty the wells and wash each well five times with 200 μ L 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 1:100 in 1X ABB as outlined in Table 3 below. Add 100 μ L of diluted secondary antibody to each well except the Blk wells.

Component	Volume/Well	Volume/Strip	Volume/ 96-well plate
1X ABB	99 μ L	792 μ L	9504 μ L
Goat Anti-Mouse HRP Conjugate	1 μ L	8 μ L	96 μ L
Total Required	100 μ L	800 μ L	9600 μ L

Table 3. Dilution of Secondary Antibody

2. Use the adhesive cover provided to seal the plate.
3. Incubate for one hour at room temperature without agitation.
4. Empty the wells and wash five times with 200 μ L 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.

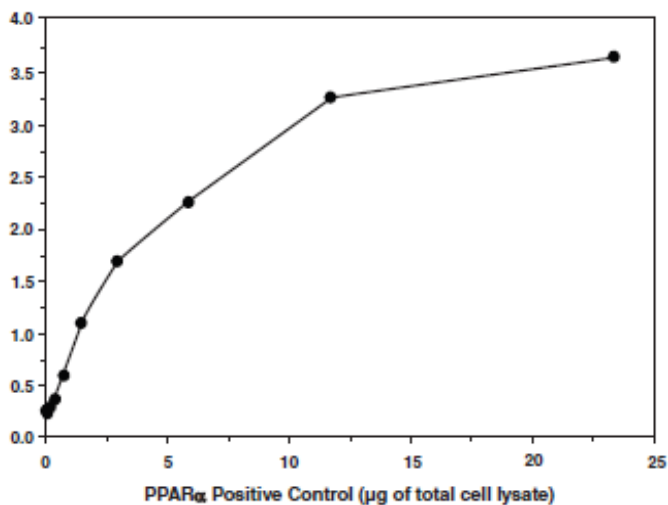
Develop and Read the Plate:

1. To each well being used add 100 μ l of Transcription Factor Developing Solution which has been equilibrated to room temperature.
2. Incubate the plate for 15 to 45 minutes at room temperature with gentle agitation protected from light. Allow the wells to turn medium to dark blue prior to adding Transcription Factor Stop Solution. (This reaction can be monitored by taking absorbance measurements at 655 nm prior to stopping the reactions; An OD₆₅₅ of 0.4-0.5 yields an OD₄₅₀ of approximately 1). Monitor development of sample wells to ensure adequate color development prior to stopping the reaction. *NOTE: Do not overdevelop; however Positive Control wells may need to overdevelop to allow adequate color development in sample wells.*

3. Add 100 μ l 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.

6. Data Analysis

A. Performance Characteristics



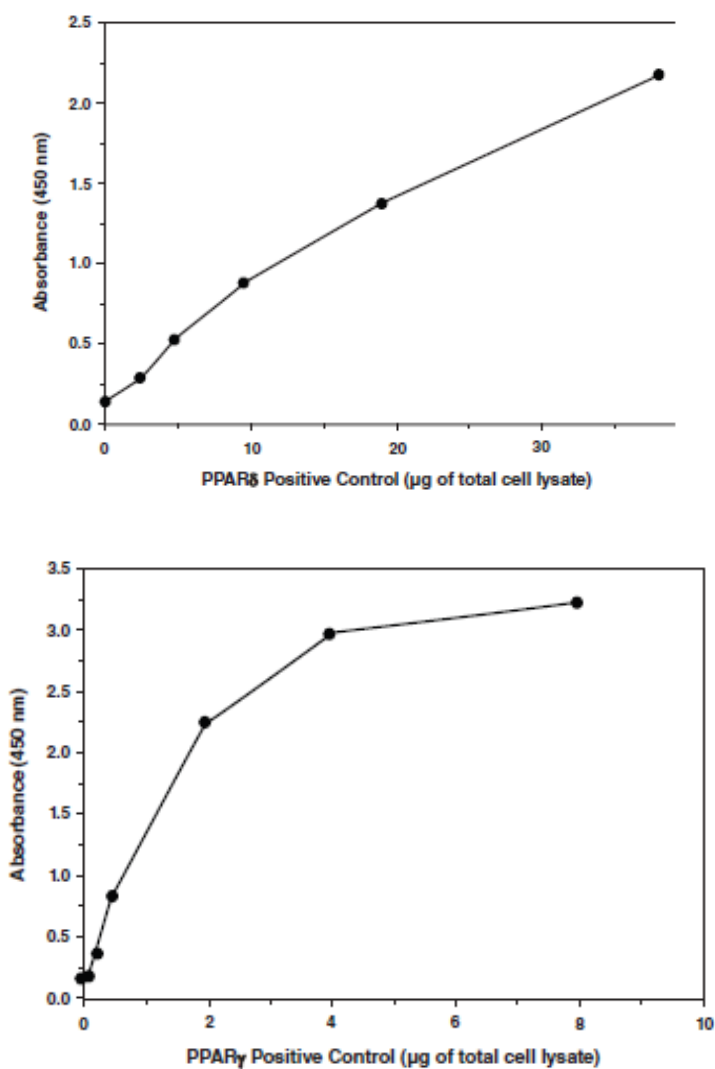


Figure 3. Schematic of the Transcription Factor Binding Assay. Increasing amounts of positive control (total lysate) are assayed for

PPAR alpha, delta, and gamma DNA-binding activity using ab133113.

Interferences

The following reagents were tested for interference in the assay.

Reagent	Will Interfere (Yes or No)
EGTA (\leq mM)	No
EDTA (\leq0.5 mM)	No
ZnCl (any concentration)	Yes
DTT (between 1 and 5 mM)	No
Dimethylsulfoxide (\leq1.5%)	No

7. Appendix – Sample Preparation

Sample Buffer Preparation

PBS (10X)

Dissolve 80 g NaCl, 2.0 g KCl, 14.4 g Na₂HPO₄, and 2.4 g KH₂PO₄ in 800 mL distilled H₂O. Adjust pH to 7.4 with HCl. Adjust volume to 1 L with H₂O

PBS (1X)

Dilute 100 mL of 10X stock with 900 mL distilled H₂O

Nuclear Extraction Phosphatase Inhibitor Cocktail (50X)

1 M NaF, 0.05 M β-glycerophosphate, and 0.05 M Na₃OV₄. Store at -80°C

PBS/Phosphatase Inhibitor Solution

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

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 -80°C.

Nuclear Extraction Hypotonic Buffer (10X)

100 mM HEPES, pH 7.5, containing 40 mM NaF, 100 μ M Na₂MoO₄, and 1 mM EDTA. Store at 4°C.

Complete Extraction Hypotonic Buffer (1X)

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

Complete Extraction Hypotonic Buffer (1X)

Prepare as outlined in Table 4.

Reagent	150 mm plate $\sim 1.5 \times 10^7$ cells
Hypotonic Buffer (10X)	100 μ L
Phosphatase Inhibitors (50X)	20 μ L
Protease Inhibitors (100X)	10 μ L
Distilled Water	870 μ L
Total Volume	1000 μ L

Table 4. Preparation of Complete Extraction Hypotonic Buffer.

Nonidet P-40 Assay Reagent (10%)

Nonidet P-40 or suitable substitute at a concentration of 10% (v/v) in H₂O. Store at room temperature

Nuclear Extraction Buffer (2X)

20 mM HEPES, pH 7.9, containing, 0.2 mM EDTA, 3 mM MgCl₂, 840 mM NaCl, and 20% glycerol (v/v). Store at 4°C

Complete Nuclear Extraction Buffer (1X)

Prepare as outlined in Table 5. 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 10⁷ 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 5. Preparation of Complete Nuclear Extraction Buffer

Purification of Cellular Nuclear Extracts

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 $\sim 10^7$ 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 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 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 μ L 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.

8. Troubleshooting

Problem	Possible Causes	Recommended Solutions
<p>No signal or weak signal in all wells</p>	<ul style="list-style-type: none"> 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 not added to correct volume 	<ul style="list-style-type: none"> 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. Pre-warm the Developing Solution to room temperature prior to use F. Check pipettes to ensure correct amount of developing solution was added to wells

<p>High signal in all wells</p>	<p>A. Incorrect dilution of antibody (too high)</p> <p>B. Improper/inadequate washing of wells</p> <p>C. Over-developing</p>	<p>A. Check antibody dilutions and use amounts outlined in instructions</p> <p>B. Follow the protocol for washing wells using the correct number of times and volumes</p> <p>C. Decrease the incubation time when using the developing reagent.</p>
<p>High background (NSB)</p>	<p>Incorrect dilution of antibody (too high)</p>	<p>Check antibody dilutions and use amounts outlined in the instructions</p>

<p>Weak signal in sample wells</p>	<ul style="list-style-type: none"> A. Sample concentration is too low B. Incorrect dilution of antibody C. Salt concentrations affecting binding between DNA and protein 	<ul style="list-style-type: none"> 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)
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