ab133112 – NFkB p65 Transcription Factor Assay Kit

Instructions for Use

For the detection of specific transcription factor DNA binding activity in nuclear extracts.

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

Version 3a Last Updated 20 August 2024

Table of Contents

1. Overview

ab133112 is a non-radioactive, sensitive method for detecting specific transcription factor DNA binding activity in nuclear extracts. 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 NFkB response element is immobilized onto the bottom of wells of a 96-well plate (See Figure 1). NFkB contained in a nuclear extract, binds specifically to the NFkB response element. NFkB (p65) is detected by addition of specific primary antibody directed against NFkB (p65). A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. ab133112 detects Human, Mouse, and Rat NFkB (p65). It will not cross-react with NFkB (p50).

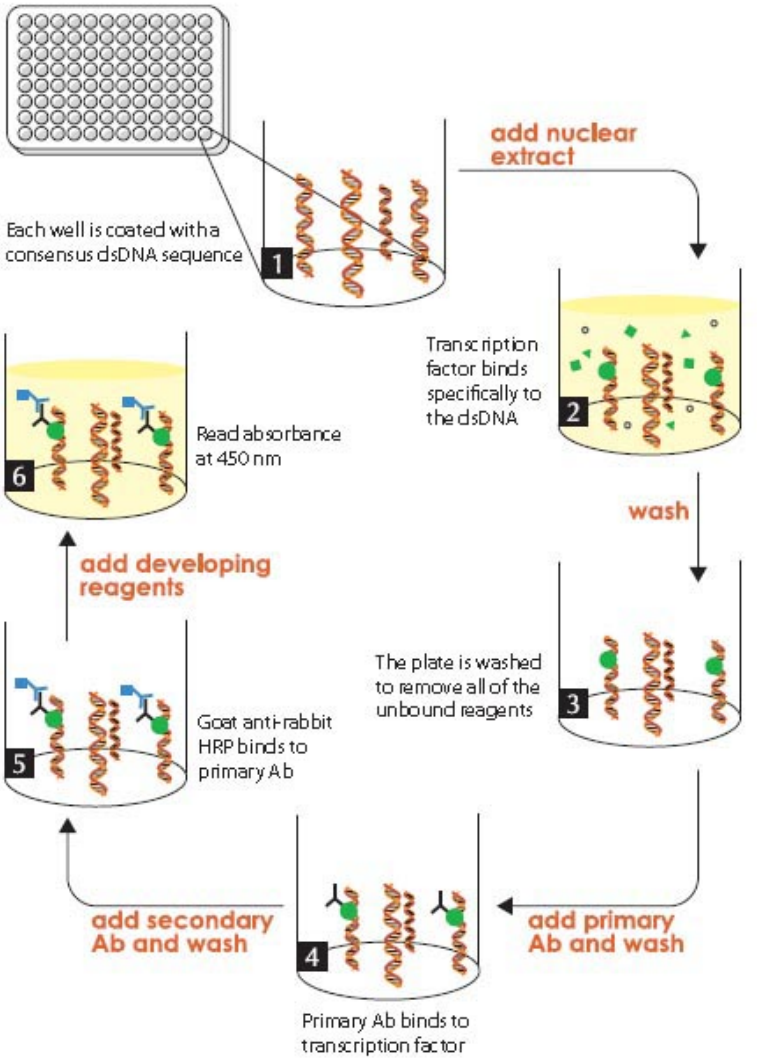


Figure 1. Schematic of the Transcription Factor Binding Assay

2. Background

The NFkB/Rel family of transcription factors is comprised of several structurally-related proteins that form homodimers and heterodimers and include p65/p105, p52/p100, RelA (p65), c-Rel/NFkB. Members of this family are responsible for regulating over 150 target genes, including the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules. Because of this, NFkB has often been called a central mediator of the Human immune response. Acting as dimers, these transcription factors bind to DNA sequences, collectively called κB sites, thereby regulating expression of target genes. In most cells, Rel/NFkB transcription complexes are present in an inactive form in the cytoplasm, bound to an inhibitor IκB. Certain stimuli result in the phosphorylation, ubiquitination and subsequent degradation of IκB proteins thereby enabling translocation of NFkB into the nucleus. The most common Rel/NFkB dimer in mammals contains p50-RelA (p50/p65) heterodimers and is specifically called NFkB. One of the target genes activated by NFkB is that encoding IκBα. This feedback mechanism allows newly synthesized IκBα to enter the nucleus, remove NFkB from DNA and transport it back to the cytoplasm thereby restoring its inactive state. The importance of Rel/NFkB transcription factors in human inflammation and certain diseases makes them attractive targets for potential therapeutics.

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)	3 mL	4°C
Transcription Factor Reagent A	120 µL	-20°C
Transcription Factor NFkB (Human p65) Positive Control	1 vial	-80°C
Transcription Factor Antibody Binding Buffer (10X)	3 mL	4°C
Transcription Factor NFkB (p65) Primary Antibody	1 vial	-20°C
Wash Buffer Concentrate (400X)	5 mL	4°C
Polysorbate 20	1 vial	RT
Transcription Factor NFkB Competitor dsDNA	1 vial	-20°C

Transcription Factor Goat Anti-Rabbit HRP Conjugate	120 µL	-20°C
Transcription Factor NFkB 96-Well Strip Plate	1	4°C
96-Well Cover Sheet	1	RT
Transcription Factor Developing Solution	12 mL	4°C
Transcription Factor Stop Solution	12 mL	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.
- 300 mM dithiothreitol (DTT).
- Nuclear Extraction Kit or buffers for preparation of nuclear extracts. We recommend Nuclear Extraction Kit (ab113474).

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.

4. Pre-Assay Preparation

A. Purification of Cellular Nuclear Extracts

Harvest cells following the procedure described in Nuclear Extraction Kit (ab113474).

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 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)

Once vial 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. *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/liter 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 µl	584 µl	7008 µl
Transcription Factor Binding Assay Buffer (4X)	25 µl	200 µl	2400 µl
Reagent A	1 µl	8 µl	96 µl
300 mM DTT	1 µl	8 µl	96 µl

Total Required	100 µl	800 µl	9600 µl

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

Transcription Factor NFkB (Human p65) Positive Control

Transcription Factor NF-κB (human p65) Positive Control contains 150 µl of clarified cell lysate. This lysate is provided as a positive control (PC) for NF-κB (p65) activation; it is not intended to be used as a standard for quantitative measurements. The positive control provided will produce a strong signal (>0.5 AU at 450 nm) when used at 10 µl/well. Serial two-fold dilutions of this PC can be used for monitoring the dynamic range of the assay. A decrease in signal may occur with repeated freeze/thaw cycles. It is recommended that the Transcription Factor NF-κB (human p65) Positive Control be aliquoted at 50 µl per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw cycles.

Positive Control Dilution Set Up

To prepare the PC for use in the ELISA: Obtain six clean test tubes and label them #PC1-PC6. Dilute 45 µl of Transcription Factor NF-κB (human p65) Positive Control with 405 µl of CTFB. This dilution is positive control 1 (PC1). Add 220 µl of CTFB to the tubes that correspond to PC2-PC6. Transfer 220 µl of the PC1 to tube PC2 and mix gently. Transfer 220 µl from PC2 to

PC3 and mix gently. Repeat this process for the remaining tubes.

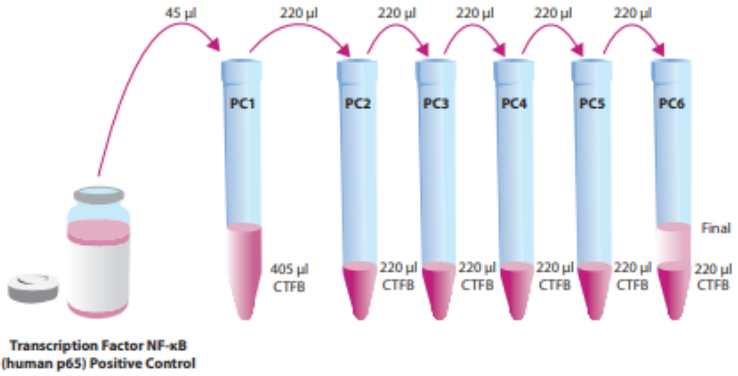


Figure 2. Preparation of the NF-κB (human p65) positive controls

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 sample and NSB wells.

⇩

Add Competitor dsDNA (optional) to appropriate wells.

⇩

Add positive control to appropriate wells.

Add sample containing NFkB to appropriate wells.

Incubate overnight at 4°C without shaking or 1 hour at room temperature on an orbital shaker

Wash each well 5 times with 1X wash buffer.

Add diluted NFkB primary antibody to each well

Incubate the plate for 1 hour at room temperature on an orbital shaker.

Wash each well 5 times with 1X Wash Buffer.

Add diluted Goat Anti-Rabbit HRP Conjugate

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

Wash each well 5 times with 1X Wash Buffer.

Add Developing Solution to wells.

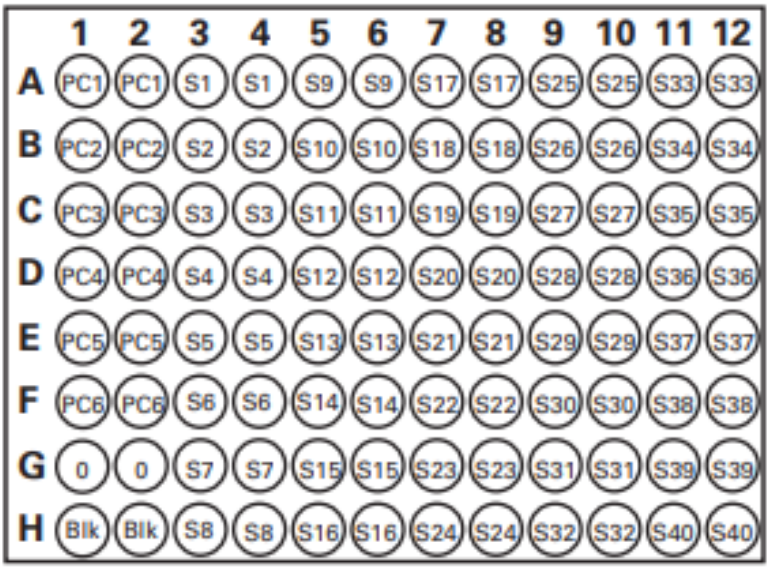
Incubate 15 to 45 minutes on an orbital shaker.

Add Stop Solution to wells.

Measure the absorbance at 450 nm.

B. Plate Setup

There is no specific pattern for using the wells on the plate. A typical layout for the PC1-PC6 serial dilutions and unknown samples of nuclear extracts (S1-S40) to be measured in duplicate is given below in Figure 3. We suggest you record the contents of each well on the template sheet provided. A suggested plate format is shown in Figure 3, below. The user may vary the location and type of wells present as necessary for each particular experiment.



Blk – Blank wells
0 – NBS wells
S1-S40 – Sample Wells
PC1-PC6 – Positive Control Wells

Figure 3. 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 Non-Specific Binding (NSB), two Blk and two PC wells be included.*

C. Performing the Assay

Binding of active NFkB (p65) 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 µl of CTFB to designated wells.

NSB - add 100 µl of CTFB to designated wells. Do not add NFkB (p65) to these wells.

C1 - Add 80 µl of CTFB prior to adding 10 µl of Transcription Factor NFkB Competitor dsDNA 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.

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

	Blk	**NSB**	**PC**	**S1-S40**	**C1**
CTFB	100 µl	100 µl	90 µl	90 µl	80 µl
Positive Control			10 µl		10 µl
Samples				10 µl	
Competitor dsDNA					10 µl

4. Use the cover provided to seal the plate. Incubate overnight at 4°C without shaking or one hour at room temperature on an orbital shaker. 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 NFkB (p65) Primary Antibody

1. Dilute the Transcription Factor NFkB (p65) Primary Antibody 1:100 in 1X ABB as outlined in Table 2 below. Add 100 µl of diluted NFkB (p65) Primary Antibody to each well except Blk wells.

Component	Volume/Well	Volume/Strip	Volume/96-well plate
1X ABB	99 µl	792 µl	9504 µl
NFkB (p65) 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 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.

Addition of 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 µl of diluted secondary antibody to each well except the Blk well.

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

Table 3. Dilution of Secondary Antibody

2. Use the adhesive cover provided 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, except the Blk wells being used add 100 µl of Transcription Factor Developing Solution, which has been equilibrated to room temperature.

2. Seal the plate with the cover sheet, and incubate the plate for 30 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 (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 Positive Control 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.

6. Data Analysis

A. Performance Characteristics

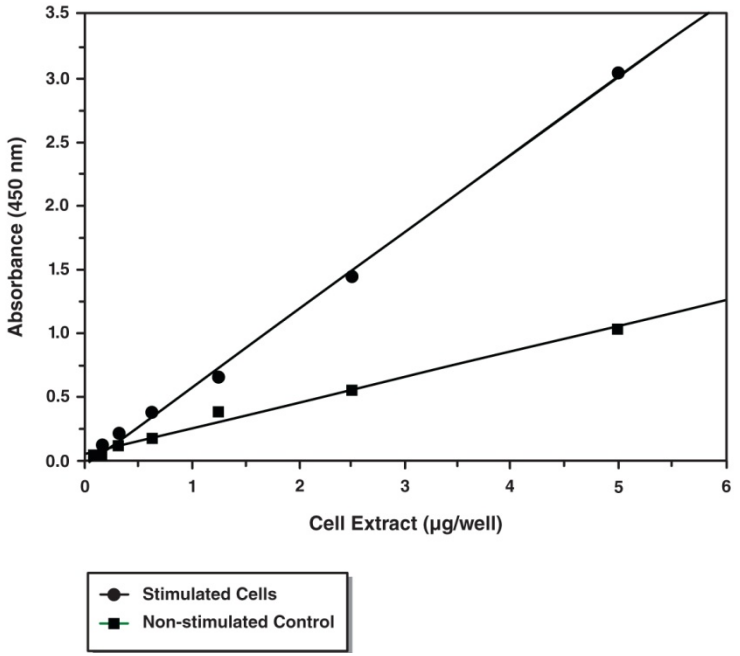


Figure 3. Assay of cell lysates isolated from stimulated (20 ng/ml TNFα for 30 minutes) and nonstimulated HeLa cells demonstrating NFkB (p65) activity.

B. Interferences

The following reagents were tested for interference in the assay.

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

7. Appendix – Sample Preparation

Sample Buffer Preparation

PBS (10X)

0.038 M NaH_2PO_4, 0.162 M Na_2HPO_4, 1.5 M NaCl, pH 7.5.

PBS (1X)

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

Nuclear Extraction Phophatase Inhibitor Cocktail (50X)

1 M NaF, 0.05 M β-glycerophosphate, 0.05 M Na_3OV_4 . 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 µM Na_2MoO_4, and 1 mM EDTA. Store at 4°C

Complete Extraction Hypotonic Buffer (1X)

Prepare as outlined in Table 4. The phosphatase and protease inhibitors lose activity shortly after dilution; therefore any unused 1X Complete Extraction 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	1000 µl

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 $MgCl_2$, 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 10^7 cells**
Nuclear Extraction	75 µl

Buffer (2X)	
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 ~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 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.

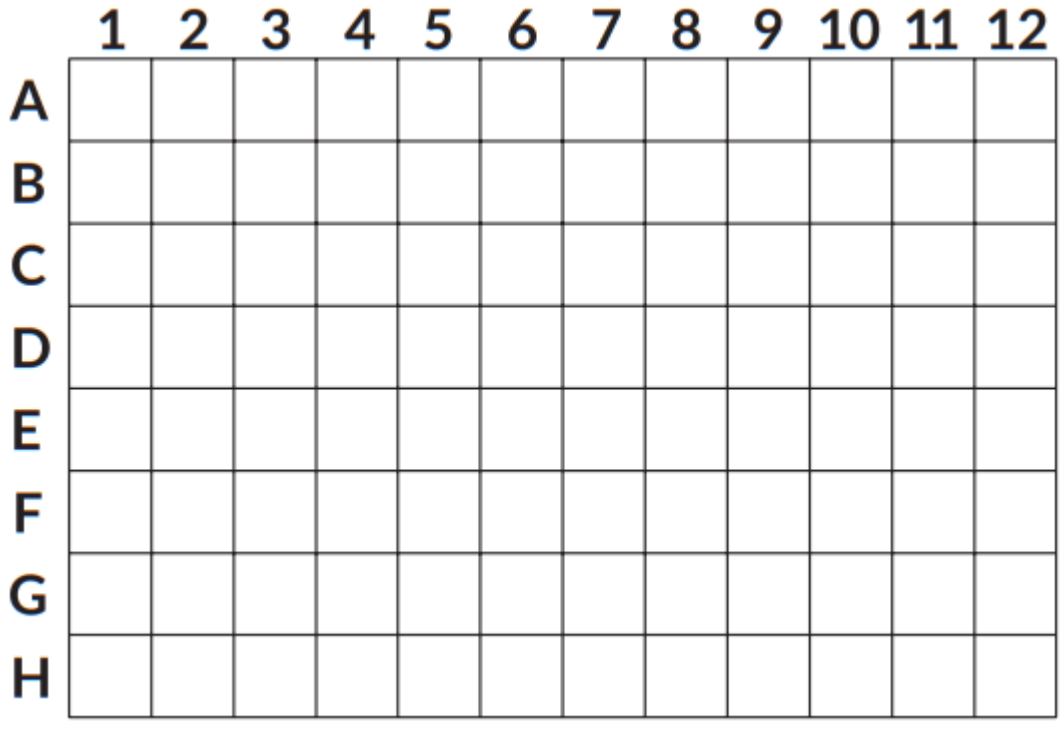
8. Troubleshooting

Problem	Possible Causes	Recommended Solutions

No signal or weak signal in all 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 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.	A. Check antibody dilutions and use amounts outlined in instructions. B. Follow the protocol for

	C. Over-developing.	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 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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