

ab133104 – HIF-1 alpha Transcription Factor Assay Kit

Instructions for Use

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

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

Table of Contents

1. Overview	3
2. Background	5
3. Components and Storage	6
4. Pre-Assay Preparation	8
5. Assay Protocol	12
6. Data Analysis	22
7. Appendix – Sample Preparation	24
8. Troubleshooting	29

1. Overview

ab133104 is a non-radioactive, sensitive method for detecting specific transcription factor DNA binding activity in nuclear extracts and whole cell lysate. 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 HIF-1 alpha (HIF-1 α) response element (5'-ACGTG-3') is immobilized to the wells of a 96-well plate (see Figure 1). HIF-1 alpha contained in a nuclear extract, binds specifically to the HIF-1 alpha response element. The HIF transcription factor complex is detected by addition of a specific primary antibody directed against HIF-1 alpha. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. ab133104 detects Human, Murine, and Rat HIF-1 alpha.

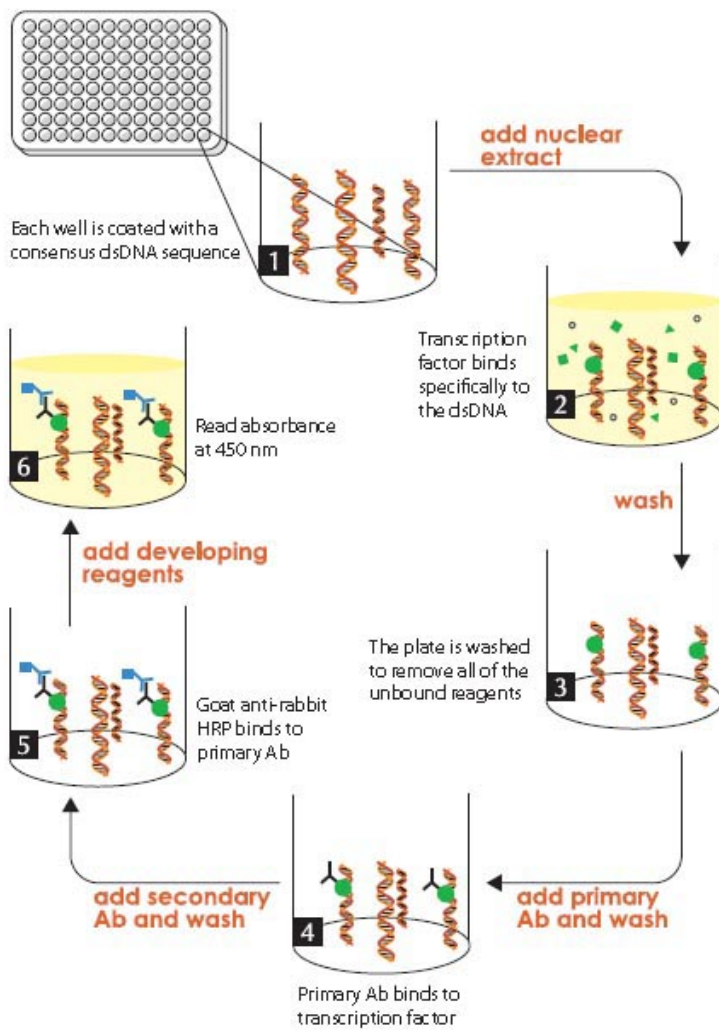


Figure 1. Schematic of the Transcription Factor Binding Assay

2. Background

The HIF (hypoxia-inducible factor) transcription factor complex is a member of the basic-helix-loop-helix (bHLH) family of transcription factors and plays an important role in maintaining oxygen homeostasis. Regulation of oxygen levels in mammalian cells is very important for cell survival and proper cell development. Low levels of oxygen (hypoxia) occur during tissue ischemia, infection, and in rapidly growing tissues, such as developing embryos or solid tumors. Under hypoxic conditions mammalian cells activate a large number of genes involved in glycolysis, angiogenesis, and hematopoiesis. These include erythropoietin (EPO), transferrin, transferrin receptor, vascular endothelial growth factor (VEGF), Flk-1, Flt-1, platelet-derived growth factor- β (PDGF- β), basic fibroblast growth factor (bFGF), and other genes affecting glycolysis. This hypoxic transcriptional response is mediated primarily by the HIF transcription complex, comprised of HIF-1 α , and HIF-1 β subunits. HIF-1 β , also called the aryl hydrocarbon receptor and nuclear translocator (ARNT), is constitutively expressed, whereas HIF-1 α is tightly regulated. HIF-1 α is stabilized under low oxygen (<5% O₂) leading to the formation of a functional heterodimer with ARNT and upregulation of hypoxic genes. When oxygen levels are normal, HIF-1 α becomes hydroxylated at the proline residues 402 and 577 and subsequently recognized by pVHL, a member of the E3 ubiquitination complex, thus targeting it for immediate ubiquitin-mediated degradation by the 26S proteasome. HIF-1 α has emerged

as an important drug target in breast and prostate cancer, cardiovascular disease, and ischemia.

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 x 3mL	4°C
Transcription Factor Reagent A	1 x 120µl	-20°C
Transcription Factor HIF-1 alpha Positive Control	1 vial	-80°C
Transcription Factor Antibody Binding Buffer (10X)	1 x 3mL	4°C
Transcription Factor HIF-1 alpha Primary Antibody	1 vial	-20°C
Wash Buffer Concentrate (400X)	1 x 5mL	RT
Polysorbate 20	1 vial	RT

Transcription Factor HIF-1 alpha Competitor dsDNA	1 vial	-20°C
Transcription Factor Goat Anti-Rabbit HRP Conjugate	1 x 100µl	-20°C
Transcription Factor HIF-1 alpha 96-Well Strip Plate	1 unit	4°C
96-Well Cover Sheet	1 unit	RT
Transcription Factor Developing Solution	1 x 12mL	4°C
Transcription Factor Stop Solution	1 x 12mL	RT

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/	Volume/
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		Strip	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 HIF-1 alpha Positive Control

One vial contains 150 μ l of NiCl₂ stimulated HeLa cell nuclear extract. This extract is provided as a positive control for HIF-1 alpha 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 μ l/well. When using this control, a decrease in signal may occur with repeated freeze/ thaw cycles. It is recommended that the positive control be aliquoted at 20 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 sample, Blk and NSB wells.



Add Competitor dsDNA (optional) to appropriate wells.



Add positive control to appropriate wells.



Add sample containing HIF-1 alpha to appropriate wells.



Incubate overnight at 4°C without agitation.



Wash each well 5 times with 1X wash buffer.



Add diluted HIF-1 alpha primary antibody to each well
(except blk wells).



Incubate 1 hour at RT without agitation.



Wash each well 5 times with 1X Wash Buffer.



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



Incubate 1 hour 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 at 450 nm.

B. Plate Setup

There is no specific pattern for using the wells on the plate. A typical layout of HIF-1 alpha 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 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 HIF-1 alpha 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 HIF-1 alpha samples or Positive Control to these wells.

C1 - Add 80 μ l of CTFB prior to adding 10 μ l of Transcription Factor HIF-1 alpha 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.

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 μ l 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 HIF-1 alpha Primary Antibody

1. Dilute the appropriate Transcription HIF-1 alpha Primary Antibody 1:100 in 1X ABB as outlined in Table 2 below. Add 100 μ l of diluted HIF-1 alpha Primary 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
HIF-1 alpha Primary Antibody	1 μ l	8 μ l	96 μ l
Total Required	100 μ l	800 μ l	9600 μ l

Table 2. Dilution of Primary 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 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 wells.

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 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.

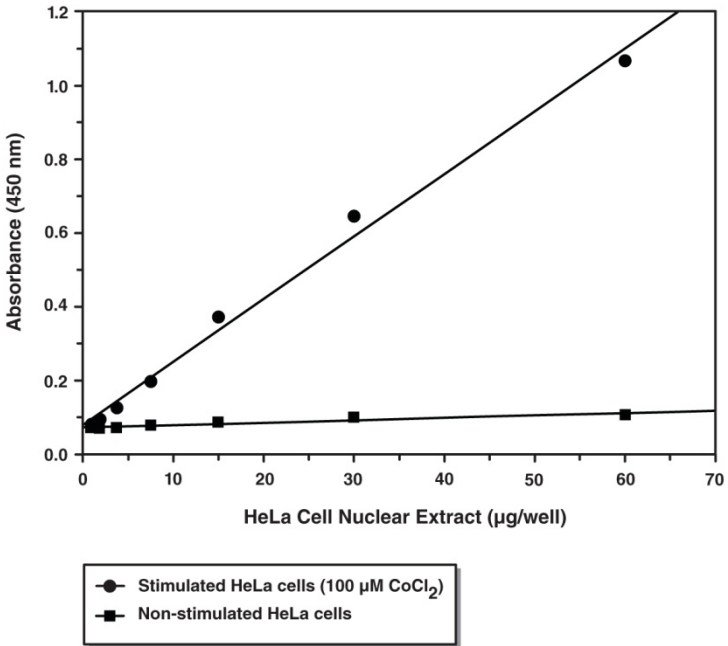
Develop and Read the Plate

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 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 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



Cross Reactivity: (+) Murine and rat HIF-1 α

Figure 3. Assay of nuclear extract from stimulated HeLa cells (100 µM CoCl₂) and non-stimulated HeLa cells.

B. 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 ($\leq 1.5\%$)	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)

0.05 M β-glycerophosphate and 1 M NaF, 0.05 M Na₃VO₄. Store at -80°C.

PBS/Phosphatase Inhibitor Solution

Add 250 μl of 50X Phosphatase Inhibitor Solution to 10 ml of 1X PBS, mix well, and keep on ice. Make fresh daily.

Nuclear Extraction Hypotonic Buffer (pH 7.5)

20 mM HEPES, pH 7.5, containing 5 mM NaF, 100 μM Na₂MoO₄, and 1 mM EDTA. Store at 4°C.

Extraction Buffer

10 mM HEPES, pH 7.9, containing 0.1 mM EDTA, 1.5 mM MgCl_2 , 420 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 1 $\mu\text{g}/\text{ml}$ Pepstatin A, 1 $\mu\text{g}/\text{ml}$ Leupeptin, 10 $\mu\text{g}/\text{ml}$ Aprotinin, 20 mM NaF, 1 mM β -glycerophosphate, 10 mM Na_3VO_4 and 20% glycerol (v/v).

This buffer cannot be stored for extended periods of time and must be made fresh on the day of use.

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 $\sim 1.5 \times 10^7$ 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 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 μ l
Total Volume	150 μ l

Table 5. Preparation of Complete Nuclear Extraction Buffer

Purification of Cellular Nuclear Extracts

The procedure 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 μ g 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 μ l 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 μ l 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 (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 used cold. F. Developing reagent not added at 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 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	<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>
High background (NSB)	Incorrect dilution of antibody (too high).	Check antibody dilutions and use amounts outlined in the instructions.

<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 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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