HIF1A Transcription Factor Assay Kit

Catalog Number KA1321

96 assays

Version: 06

Intended for research use only

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Introduction

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.[1,2] 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.[3-5] 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.[5] This hypoxic transcriptional response is mediated primarily by the HIF transcription complex, comprised of HIF-1α, and HIF-1β subunits. HIF-1β, also called the aryl hydrocarbon receptor and nuclear translocator (ARNT), is constitutively expressed, whereas HIF-1α is tightly regulated. HIF-1α is stabilized under low oxygen (<5% O_2) leading to the formation of a functional heterodimer with ARNT and upregulation of hypoxic genes. When oxygen levels are normal, HIF-1α 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 proteosome.[6] HIF-1α has emerged as an important drug target in breast and prostate cancer, cardiovascular disease, and ischemia.[7-9]

Principle of the Assay

HIF1A Transcription Factor Assay Kit 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-1α response element (5'-ACGTG-3') is immobilized to the wells of a 96-well plate (see Figure 1). HIF-1α contained in a nuclear extract, binds specifically to the HIF-1α response element. The HIF transcription factor complex is detected by addition of a specific primary antibody directed against HIF-1α. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. HIF1A Transcription Factor Assay Kit detects human, murine, and rat HIF-1α.

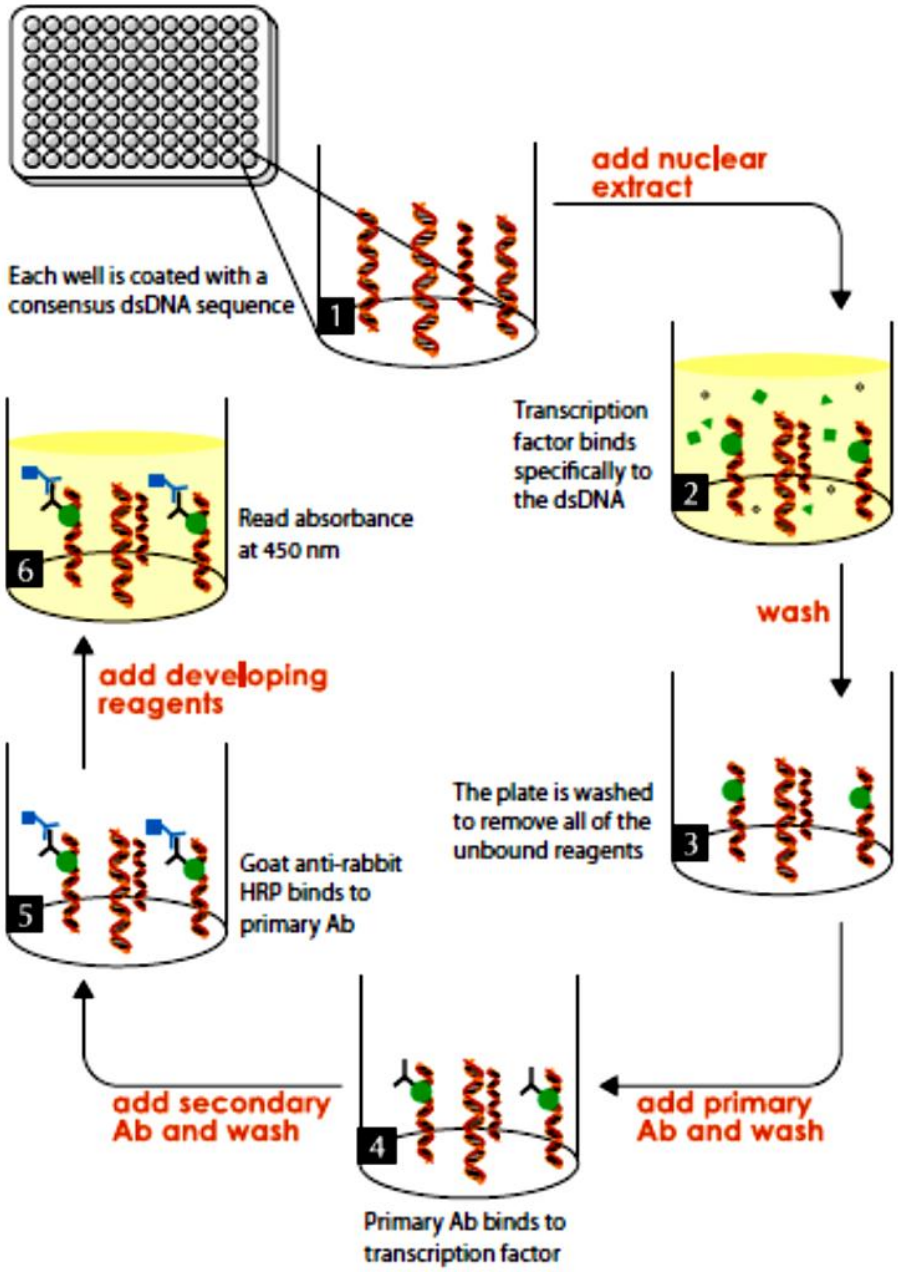


Figure 1. Schematic of the Transcription Factor Assay

General Information

Materials Supplied

List of component

Item	Quantity
Transcription Factor Binding Assay Buffer (4X)	1 vial/3 mL
Transcription Factor Reagent A	1 vial/120 µL
Transcription Factor HIF-1α Positive Control	1 vial/150 µL
Transcription Factor Antibody Binding Buffer (10X)	1 vial/3 mL
Transcription Factor HIF-1α Primary Antibody	1 vial/ 120 µL
Wash Buffer Concentrate (400X)	1 vial/5 mL
Polysorbate 20	1 vial/3 mL
Transcription Factor HIF-1α Competitor dsDNA	1 vial/120 µL
Transcription Factor Goat Anti-Rabbit HRP Conjugate	1 vial/120 µL
Transcription Factor HIF-1α 96-Well Strip Plate	1 plate
96-Well Cover Sheet	1 cover
Transcription Factor Developing Solution	1 vial/12 mL
Transcription Factor Stop Solution	1 vial/12 mL

Storage Instruction

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	Storage
Transcription Factor Binding Assay Buffer (4X)	4°C
Transcription Factor Reagent A	-20°C
Transcription Factor HIF-1α Positive Control	-80°C
Transcription Factor Antibody Binding Buffer (10X)	4°C
Transcription Factor HIF-1α Primary Antibody	-20°C
Wash Buffer Concentrate (400X)	RT
Polysorbate 20	RT
Transcription Factor HIF-1α Competitor dsDNA	-20°C
Transcription Factor Goat Anti-Rabbit HRP Conjugate	-20°C
Transcription Factor HIF-1α 96-Well Strip Plate	4°C
96-Well Cover Sheet	RT
Transcription Factor Developing Solution	4°C

Transcription Factor Stop Solution	RT

Materials Required but Not Supplied

- ✓ A plate reader capable of measuring absorbance at 450 nm
- ✓ Adjustable pipettes and a repeat pipettor
- ✓ A source of UltraPure water; glass Milli-Q or HPLC-grade water is acceptable
- ✓ 300 mM dithiothreitol (DTT)
- ✓ Buffers for preparation of nuclear extracts

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

Precautions for Use

- ✓ WARNING: This product is for laboratory research use only: not for administration to humans. Not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

- ✓ Precautinos

- 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. After use we recommend each kit component be stored according to the temperatures listed in the booklet.
- For research use only. Not for human or diagnostic use.

- ✓ 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.

Assay Protocol

Reagent Preparation

- ✓ Transcription Factor Antibody Binding Buffer (10X)

  One vial contains 3 mL of a 10X stock of 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 liters with UltraPure water and add 1 mL of Polysorbate 20. *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.

- ✓ 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.

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

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	1 µL	8 µL	96 µL
300 mM DTT	1 µL	8 µL	96 µL
Total Required	100 µL	800 µL	9,600 µL

- ✓ Transcription Factor HIF-1α Positive Control

  One vial contains 150 µL of DMOG-stimulated HeLa cell nuclear extract. This extract is provided as a positive control for HIF-1α 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 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.

- ✓ Plate Set Up

  There is no specific pattern for using the wells on the plate. A typical layout of HIF-1α Positive Control (PC), Competitor dsDNA (C1), and samples of nuclear extracts (S1-S44) to be measured in duplicate is

given in Plate Layout. We suggest you record the contents of each well on the template sheet provided on page 16.

Sample Preparation

✓ Sample Buffer Preparation

All buffers and reagents below are required for preparation of Nuclear Extracts:

- 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.
- Nuclear Extraction PBS (1X)
  Dilute 100 mL of 10X stock with 900 mL distilled H_2O.
- Nuclear Extraction Phosphatase Inhibitor Solution (50X)
  0.05 M β-glycerophosphate
  0.5 M NaF.
  0.05 M Na_3VO_4
  Store at -80°C
- 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.
- 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
- 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 4°C
- Complete Hypotonic Buffer (1X)
  Prepare as outlined in Table 2. The phosphatase and protease inhibitors lose activity shortly after dilution; therefore any unused 1X Complete Hypotonic Buffer should be discarded.

Reagent	150 nm 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

- 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.

  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 4°C.

- Complete Nuclear Extraction Buffer (1X)

  Prepare as outlined in Table 3. 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 3. Prepareation 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 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 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.

Assay Procedure

✓ Binding of active HIF-1α 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:
   Blks - add 100 µL of CTFB to designated wells.
   NSB - add 100 µL of CTFB to designated wells. Do not add HIF-1α samples or Positive Control to these wells.
   C1 - Add 80 µL of CTFB prior to adding 10 µL of Transcription Factor HIF-1αCompetitor dsDNA to designated wells. Add 10 µL of control nuclear extract, 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.
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 HIF-1α Primary Antibody

1. Dilute the Transcription Factor HIF-1α Primary Antibody 1:100 in 1X ABB as outlined in Table 4 below. Add 100 µl of diluted HIF-1α Primary Antibody to each well except the Blk wells.

Table 4. Dilution of Primary Antibody

Component	Volume/Well	Volume/Strip	Volume/96-well plate
1X ABB	99 µL	792 µL	9,504 µL
HIF-1α Primary Antibody	1 µL	8 µL	96 µL
Total required	100 µL	800 µL	9,600 µL

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 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 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.

Table 5. Dilution of Secondary Antibody

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

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.

✓ Develop and Read the Plate

1. To each well being used add 100 µL 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 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. Prepare CTFB as described in the Reagent Preparation section, Table 1.
2. Add 90 µL CTFB per sample well (80 µL if adding Competitor dsDNA), 100 µL to Blk and NSB wells).
3. Add 10 µL of Competitor dsDNA (optional) to appropriate wells.
4. Add 10 µL of Positive Control to appropriate wells.
5. Add 10 µL of Sample containing HIF-1α to appropriate wells
6. Incubate overnight at 4°C or 1 hour at room temperature without agitation.
7. Wash each well five times with 200 µL of 1X Wash Buffer.
8. Add 100 µL of diluted HIF-1α Antibody per well (except Blk wells).

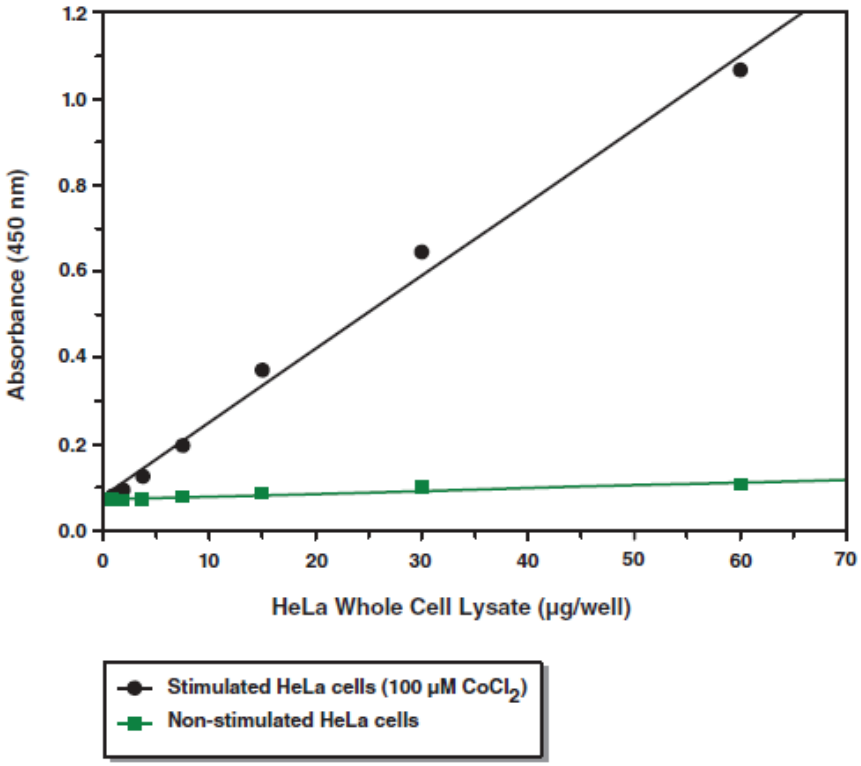
9. Incubate one hour at room temperature without agitation.
10. Wash each well five times with 200 µL of 1X Wash Buffer.
11. Add 100 µL of diluted Secondary Antibody (except Blk wells).
12. Incubate one hour at room temperature without agitation.
13. Wash each well five times with 200 µL of 1X Wash Buffer.
14. Add 100 µL of Developing Solution per well.
15. Incubate 15 to 45 minutes with gentle agitation.
16. Add 100 µL of Stop Solution per well.
17. Measure the absorbance at 450 nm.

Table 6. Quick Protocol Guide

Steps	Reagent	Blk	NSB	PC	Cl	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 agitation					
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 room temperature without agitation					
6. Wash	Wash all wells five times					
7. Add reagents	Goat anti-Rabbit HRP conjugate		100 µL	100 µL	100 µL	100 µL
8. Incubate	Cover plate and incubate one hour at room temperature without agitation					
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					
12. Add reagents	Stop solution	100 µL	100 µL	100 µL	100 µL	100 µL
13. Read	Read plate at wavelength of 450 nm					

Data Analysis

Performance Characteristics



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

✓ 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

Resources

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No signal or weak signal in all wells	• Omission of key reagent • Plate reader settings not correct • Reagent/reagents expired • Salt concentrations affected binding between DNA and protein • Developing reagent used cold • Developing reagent not added to correct volume	• Check that all reagents have been added and in the correct order. Perform the assay using the positive control • Check wavelength setting on plate reader and change to 450 nm • Check expiration date on reagents • 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) • Warm the Developing Solution to room temperature prior to use • Check pipettes to ensure correct amount of developing solution was added to wells
High signal in all wells	• Incorrect dilution of antibody (too high) • Improper/inadequate washing of wells • Overdeveloping	• Check antibody dilutions and use amounts outlined in instructions • Follow the protocol for washing wells using the correct number of times and volumes • 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.	• Sample concentration is too low • Incorrect dilution of antibody	• 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. • Check antibody dilutions and use amounts outlined in the instructions

	• Salt concentrations affecting binding between DNA and protein	• 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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Plate Layout

	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

S1-44=Sample Wells

NSB=Non-specific Binding Wells

PC=Positive Control Wells

Blk=Blank Wells

C1=Competitor dsDNA Wells