

TransAM™ HIF-1

(version F2)

Catalog Nos. 47096 & 47596

Copyright 2011 Active Motif, Inc.

Information in this manual is subject to change without notice and does not constitute a commitment on the part of Active Motif, Inc. It is supplied on an “as is” basis without any warranty of any kind, either explicit or implied. Information may be changed or updated in this manual at any time.

This documentation may not be copied, transferred, reproduced, disclosed, or duplicated, in whole or in part, without the prior written consent of Active Motif, Inc. This documentation is proprietary information and protected by the copyright laws of the United States and international treaties.

The manufacturer of this documentation is Active Motif, Inc.

© 2011 Active Motif, Inc., 1914 Palomar Oaks Way, Suite 150; Carlsbad, CA 92008. All rights reserved.

All trademarks, trade names, service marks or logos referenced herein belong to their respective companies.

TABLE OF CONTENTS	Page
Overview	1
Flow Chart of Process	2
Introduction	
HIF-1 Transcription Factor	3
Transcription Factor Assays	3
TransAM HIF-1	4
Kit Performance and Benefits	5
Kit Components and Storage	6
Additional Materials Required	6
Protocols	
Buffer Preparation and Recommendations	7
Quick Chart for Preparing Buffers	9
HIF-1 Transcription Factor Assay	9
Preparation of Nuclear Extract	11
References	11
Appendix	
Section A. Preparation of Nuclear Extract	11
Section B. Troubleshooting Guide	13
Technical Services	16

Overview

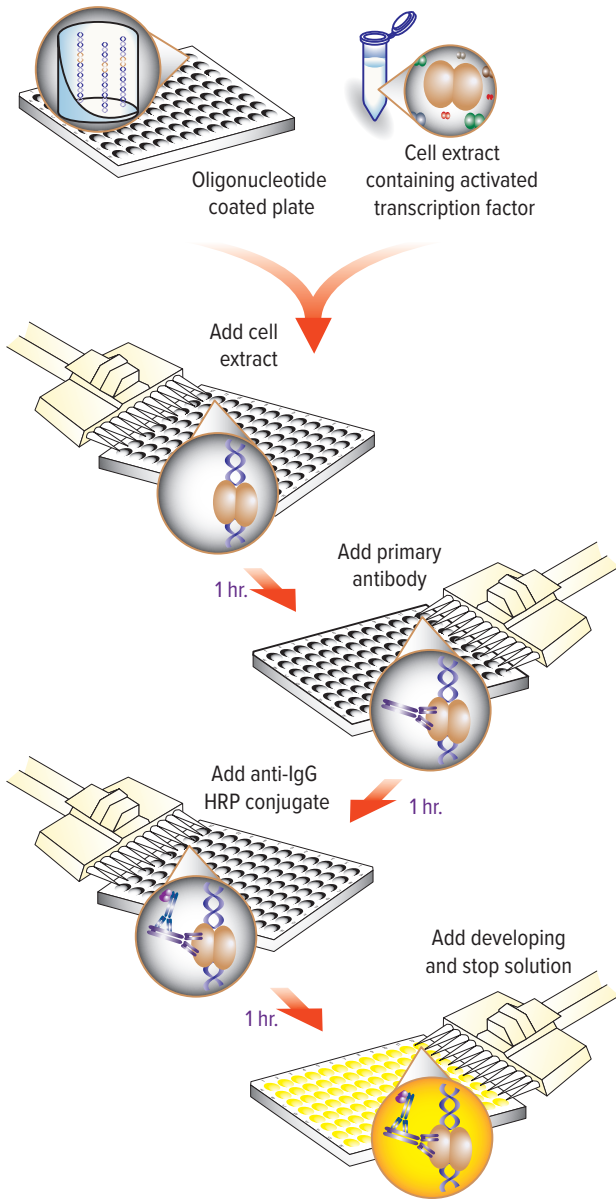
Hypoxia-Inducible Factor 1 (HIF-1) is a transcription factor that is activated in response to low oxygen levels, which occur during hypoxic pulmonary hypertension, or cerebral and myocardial ischemia. Angiogenesis is the most common physiological response observed upon up-regulation of HIF-1 α . Therefore, accurate monitoring of HIF activation in cells, tissues or animals is crucial for drug development and biomedical research. To date, such research projects are tedious and time consuming, and lack high-throughput screening methods.

With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive and specific assay. TransAM HIF-1 Kits are designed specifically for the study of HIF-1. They contain a 96-well plate to which oligonucleotide containing the hypoxia response element (HRE) has been immobilized. HIF dimers contained in nuclear extracts bind specifically to this oligonucleotide and are detected through the use of an antibody directed against HIF-1 α . Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout that is easily quantified by spectrophotometry. The 96-well plate with individual strips of 8 wells is suitable for manual use or for high-throughput screening applications. TransAM HIF-1 Kits are available in two sizes:

product	format	catalog no.
TransAM™ HIF-1	1 x 96 rxns	47096
TransAM™ HIF-1	5 x 96 rxns	47596

* Technology covered by AAT-filed patents and licensed to Active Motif.

Flow Chart of Process



Introduction

HIF-1 Transcription Factor

All organisms have mechanisms for sensing oxygen concentrations and for responding to low oxygen levels (hypoxia) with changes in gene expression. The transcription factor Hypoxia-Inducible Factor 1 (HIF-1) is one of the key regulators of oxygen homeostasis¹. It is required for the development of key physiological systems, such as vasculogenesis and pneumogenesis, during fetal and postnatal life. HIF-1 also regulates the physiological responses to hypoxia and the pathophysiology of heart attack, cancer, stroke and chronic lung disease (see 2 for review).

HIF-1 is a heterodimeric protein that consists of two subunits, HIF-1 α and HIF-1 β (also known as ARNT1, the aryl hydrocarbon nuclear translocator)³. They belong to a larger family of transcription factors that contain basic helix-loop-helix (bHLH) and PER-ARNT-SIM homology domains². Class I members of this family such as HIF-1 α , HIF-2 α and HIF-3 α heterodimerize with one of the class II sub-family such as ARNT1, ARNT2 and ARNT3, resulting in different DNA-binding and transcriptional properties depending on the dimer formation. HIF-1 α and HIF-1 β mRNA are expressed in most human and rodent tissues⁴. HIF-2 α , HIF-3 α , ARNT2 and ARNT3 are restricted to certain tissues, and therefore have a more specialized role in oxygen homeostasis.

Whereas HIF-1 β is constitutively expressed, the expression of HIF-1 α is induced by hypoxia (oxygen concentration below 6%)⁵. Under nonhypoxic conditions, HIF-1 α is hydroxylated by a prolyl hydroxylase enzyme⁶. This modification is required for the binding of the von Hippel-Lindau (VHL) protein to HIF-1 α and its subsequent ubiquitination and degradation in the proteasome⁷. Iron chelators and cobalt chloride prevent HIF-1 α ubiquitination besides inducing its expression⁸. Therefore these chemicals have been used in mammalian cells to generate HIF study models. HIF heterodimers bind to the hypoxia response element (HRE), a 5'-RCGTG-3' consensus sequence⁹. Several dozen HIF-1-regulated genes have been identified so far, including genes coding for proteins involved in angiogenesis, energy metabolism, erythropoiesis, cell proliferation and viability, vascular remodeling and vasomotor responses (see 2 for review).

Transcription Factor Assays

To date, three methods are widely used to measure HIF-1 activation, either directly or indirectly:

1. **Western blot:** HIF-1 activation can be determined by Western Blot by using antibodies specific for HIF-1 α protein. This method is time consuming (up to 2 days once the nuclear extracts are prepared), and is not suitable for processing large numbers of samples.
2. **Gel retardation:** The DNA-binding capacity of HIF-1 can be assayed by gel retardation, also called electrophoretic mobility shift assay (EMSA). In this case, nuclear extracts are incubated with a radioactive double-stranded oligonucleotide probe containing the consensus sequence for HIF binding. If HIF-1 is active in the nuclear extract, it will bind to the probe. Samples are then resolved by electrophoresis on a native polyacrylamide gel, followed by autoradiography. This method is sensitive, but like the previous procedure, it is time consuming (multiple days of gel exposure may be required to achieve sufficient sensitivity) and it cannot be applied to high-throughput screening. Gel-shift assays also require special precautions and equipment for handling radioactivity.

3. Reporter assays: Another largely used method to assay HIF-1 activation is based on reporter genes, typically luciferase or β -galactosidase, placed under the control of a promoter containing a HRE. The promoter can be artificial, made of several HRE cis-elements and a TATA box, or natural, like the erythropoietin promoter sequence. However, the procedure is limited by the following issues: (i) reporter gene assays have to be repeated several times to obtain statistically reliable data; and (ii) reporter gene assays are sensitive to confounding factors that may influence the expression level of the reporter gene, and therefore assays have to be carefully standardized. Reporter gene assays are sensitive and easy to perform with a large number of samples, but require efficient cell transfection with the reporter plasmid..

TransAM HIF-1

HIF-1 is involved in every major cause of mortality in the industrialized world such as heart attack, cancer, stroke and chronic lung disease, and therefore represents an excellent pharmacological target. However, pharmaceutical research in this field has been hampered by the lack of convenient assays suitable for high sample number experiments.

To overcome this, Active Motif is introducing a high-throughput assay to quantify HIF-1 activation¹⁰. TransAM Kits combine a fast, user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM HIF-1 Kits contain a 96-well plate on which has been immobilized an oligonucleotide containing a HRE (5'-TACGTGCT-3') from the EPO gene. HIF contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used in TransAM HIF-1 Kits recognizes an epitope on HIF-1 α protein that is accessible upon DNA binding. Addition of a secondary HRP-conjugated antibody provides a sensitive colorimetric readout easily quantified by spectrophotometry. Once the nuclear extracts are prepared, this assay is completed in less than 3.5 hours. As the assay is performed in 96-well plates, a large number of samples can be handled simultaneously, enabling high-throughput automation. The assay is specific for HIF-1 α activation and has been shown to be more sensitive and faster than the gel-retardation technique. With the 3.5-hour TransAM procedure, we could detect HIF-1 activation with as little as 2.5 μ g of nuclear extract from CoCl₂-treated cells. A comparative assay using EMSA required 5 μ g of nuclear extract and a 3-day autoradiography.

TransAM has many applications including the study of drug potency toward HIF-1-related enzymes, HIF-1 transcriptional activity regulation and protein structure/function of HIF-1 α and its cofactors in areas such as angiogenesis, erythropoiesis, tumorigenesis and embryogenesis.

Kit Performance and Benefits

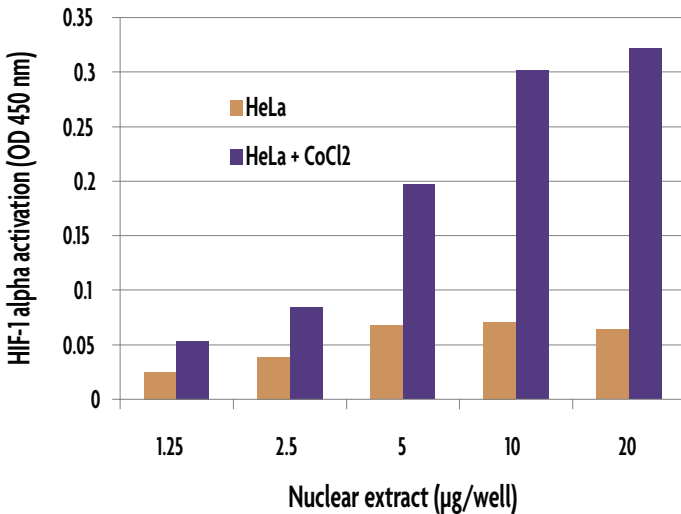
The TransAM HIF-1 Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: > 2.5 µg nuclear extract/well.

Range of detection: TransAM provides quantitative results within a 2.5 - 20 µg nuclear extract/well range (see graph below).

Cross-reactivity: TransAM HIF-1 detects HIF-1α in human and monkey extracts. It does not cross-react with other species or HIF subunits.

Assay time: 3.5 hours. TransAM is 20-fold faster than EMSA.



Monitoring HIF-1α activation with TransAM HIF-1 Kit.

Different amounts of nuclear extract from untreated and CoCl₂-treated HeLa cells are tested for HIF activation using the TransAM HIF-1 Kit. This data is provided for demonstration only.

Kit Components and Storage

TransAM HIF-1 Kits are for research use only. Not for use in diagnostic procedures. The nuclear extract must be kept at -80°C . Other Kit components can be stored at -20°C prior to first use. Then, we recommend storing each component at the temperature indicated in the table below.

All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity 1 plate / 5 plates	Storage
HIF-1 antibody (0.25 mg/ml)	22 μl / 110 μl	-20°C for 6 months
Anti-mouse HRP-conj. IgG (0.4 mg/ml)	11 μl / 55 μl	4°C for 6 months
Wild-type oligonucleotide AM13 (10 pmol/ μl)	100 μl / 500 μl	-20°C for 6 months
Mutated oligonucleotide AM13 (10 pmol/ μl)	100 μl / 500 μl	-20°C for 6 months
HeLa (CoCl_2) nuclear extract (2.5 mg/ml)	50 μl / 250 μl	-80°C for 6 months
Dithiothreitol (DTT) (1 M)	100 μl / 500 μl	-20°C for 6 months
Protease Inhibitor Cocktail	100 μl / 500 μl	-20°C for 6 months
Lysis Buffer AM1	10 ml / 50 ml	4°C
Binding Buffer AM4	10 ml / 50 ml	4°C
10X Wash Buffer AM2	22 ml / 110 ml	4°C
10X Antibody Binding Buffer AM3	2.2 ml / 11 ml	4°C
Developing Solution	11 ml / 55 ml	4°C
Stop Solution	11 ml / 55 ml	4°C
96-well IRF-3 assay plate	1 / 5	
Plate sealer	1 / 5	

Additional materials required

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform
- Microplate spectrophotometer capable of reading at 450 nm (655 nm as optional reference wavelength)

Protocols

Buffer Preparation and Recommendations

Preparation of Complete Lysis Buffer

We provide an excess of Lysis Buffer AM1 in order to perform the assay AND to prepare customized nuclear extracts. Our Nuclear Extract Kit can also be purchased separately (Cat. Nos. 40010 & 40410). The presence of phosphatase inhibitors gives a yellow coloration to Lysis Buffer AM1. Prepare the amount of Complete Lysis Buffer required for the assay (see the Quick Chart for Preparing Buffers in this section) by adding 1 μ l 1 M DTT and 10 μ l Protease Inhibitor Cocktail per ml of Lysis Buffer AM1. Some of the protease inhibitors lose their activity after 24 hours once diluted. Therefore, we recommend using the Complete Lysis Buffer immediately for cell lysis. The remaining amount should be discarded if not used in the same day.

Preparation of Complete Binding Buffer

Prepare the amount of Complete Binding Buffer required for the assay (see the Quick Chart for Preparing Buffers in this section) by adding 1 μ l 1 M DTT per ml of Binding Buffer AM4. After use, discard remaining Complete Binding Buffer.

Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay (see the Quick Chart for Preparing Buffers in this section) as follows: For every 100 ml of 1X Wash Buffer required, dilute 10 ml 10X Wash Buffer AM2 with 90 ml of distilled water. Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in the 10X Wash Buffer AM2 may form clumps, therefore homogenize the buffer by incubating at 50°C for 2 minutes and mixing prior to use.

Preparation of 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay (see the Quick Chart for Preparing Buffers in this section*) as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer AM3 with 9 ml of distilled water. Mix gently to avoid foaming. Discard remaining 1X Antibody Binding Buffer after use. The BSA contained in the 10X Antibody Binding Buffer AM3 may form clumps, therefore homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use. Dilute the primary antibody to 1:500 and the HRP-conjugated secondary antibody to 1:1000 with the 1X Antibody Binding Buffer. Depending on the particular assay, the signal:noise ratio may be optimized by using higher dilutions of the antibodies. This may decrease the sensitivity of the assay.

* Volumes listed refer to the preparation of buffer for diluting both the primary & secondary antibodies.

Developing Solution

The Developing Solution should be warmed to room temperature before use. The Developing Solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. A blue color present in the Developing Solution indicates that it has been contaminated and must be discarded. Prior to use, place the Developing Solution at room temperature for at least 1 hour. Transfer the amount of Developing Solution required for the assay into a secondary container before aliquoting into the wells (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Developing Solution.

Stop Solution

Prior to use, transfer the amount of Stop Solution required for the assay (see the Quick Chart for Preparing Buffers in this section) into a secondary container. After use, discard remaining Stop Solution.

WARNING: The Stop Solution is corrosive. Wear personal protective equipment when handling, *i.e.* safety glasses, gloves and labcoat.

HeLa nuclear extract

The HeLa (CoCl₂) nuclear extract is provided as a positive control for HIF-1 activation. Sufficient extract is supplied for 10 reactions per plate. This extract is optimized to give a strong signal when used at 10 µg/well. We recommend aliquoting the extract in 10 µl fractions and storing at -80°C. Avoid multiple freeze/thaw cycles of the extract. Various cell extracts are available from Active Motif (see Appendix, Section B. Related Products).

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for HIF binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent HIF binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on HIF binding. Prepare the required amount of wild-type and/or mutated consensus oligonucleotide by adding 2 µl of the appropriate oligonucleotide to 43 µl Complete Binding Buffer per well being used (see the Quick Chart for Preparing Buffers in this section). To allow for optimum competition, add the oligonucleotide to the well prior to addition of the nuclear extract.

Quick Chart for Preparing Buffers

Reagents to prepare	Components	1 well	1 strip (8 wells)	6 strips (48 wells)	12 strips (96 wells)
Complete Lysis Buffer	DTT	0.01 µl	0.1 µl	0.6 µl	1.2 µl
	Protease Inhibitor Cocktail	0.12 µl	0.9 µl	5.4 µl	10.8 µl
	Lysis Buffer	11.12 µl	89.0 µl	534.0 µl	1.07 ml
	Total Required	11.25 µl	90.0 µl	540.0 µl	1.08 ml
Complete Binding Buffer	DTT	0.04 µl	0.3 µl	2.16 µl	4.3 µl
	Binding Buffer	44.5 µl	356.1 µl	2.14 ml	4.27 ml
	Total Required	45 µl	360 µl	2.16 ml	4.32 ml
Binding Buffer with IRF wt or mut oligont	wt or mut oligont	2.0 µl	16.0 µl	96.0 µl	N/A
	Complete Binding Buffer	43.0 µl	344.0 µl	2.064 µl	N/A
	Total Required	45.0 µl	360.0 µl	2.16 µl	N/A
1X Washing Buffer	Distilled Water	2.025 ml	16.2 ml	97.2 ml	194.4 ml
	10X Washing Buffer	225.0 µl	1.8 ml	10.8 ml	21.6 ml
	Total Required	2.25 ml	18.0 ml	108.0 ml	216.0 ml
1X Antibody Binding Buffer*	Distilled Water	202.5 µl	1.62 ml	9.72 ml	19.44 ml
	10X Antibody Binding Buffer	22.5 µl	180.0 µl	1.08 ml	2.16 ml
	Total Required	225.0 µl	1.8 ml	10.8 ml	21.6 ml
Developing Solution	Total Required	112.5 µl	900.0 µl	5.4 ml	10.8 ml
Stop Solution	Total Required	112.5 µl	900.0 µl	5.4 ml	10.8 ml

* Volumes listed refer to the preparation of buffer for diluting both the primary & secondary antibodies.

HIF-1 Transcription Factor Assay

Determine the appropriate number of microwell strips required for testing samples, controls and blanks in duplicate. If less than 8 wells in a strip need to be used, cover the unused wells with a portion of the plate sealer while you perform the assay. The content of these wells is stable at room temperature if kept dry and, therefore, can be used later for a separate assay. Store the unused strips in the aluminum pouch at 4°C. Use the strip holder for the assay.

Prepare the Complete Lysis Buffer, Complete Binding Buffer, 1X Wash Buffer and 1X Antibody Binding Buffer as described above in the section Buffer Preparation and Recommendations. Multi-channel pipettor reservoirs may be used for dispensing the Complete Binding Buffer, Wash Buffer, Antibody Binding Buffer, Developing Solution and Stop Solution into the wells being used.

Step 1: Binding of HIF-1 to its consensus sequence

1. Add 40 μ l Complete Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 40 μ l Complete Binding Buffer that contains 20 pmol (2 μ l) of the wild-type or mutated oligonucleotide (see the Buffer Preparation section above for a description of competitive binding).
2. Sample wells: Add 10 μ l of sample diluted in Complete Lysis Buffer per well. We recommend using 2-20 μ g of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts is provided on page 11.
Positive control wells: Add 10 μ g of the provided nuclear extract diluted in 10 μ l of Complete Lysis Buffer per well (4 μ l of nuclear extract in 6 μ l of Complete Lysis Buffer per well).
Blank wells: Add 10 μ l Complete Lysis Buffer only per well.
3. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform).
4. Wash each well 3 times with 200 μ l 1X Wash Buffer. For each wash, flick the plate over a sink to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.

Step 2: Binding of primary antibody

1. Add 100 μ l diluted HIF-1 antibody (1:500 dilution in 1X Antibody Binding Buffer) to each well being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. Wash the wells 3 times with 200 μ l 1X Wash Buffer (as described in Step 1, No. 4).

Step 3: Binding of secondary antibody

1. Add 100 μ l of diluted HRP-conjugated antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. During this incubation, place the Developing Solution at room temperature.
4. Wash the wells 4 times with 200 μ l 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric reaction

1. Add 100 μ l Developing Solution to all wells being used.
2. Incubate 8-12 minutes at room temperature protected from direct light. Monitor the blue color development in the sample and positive control wells until it turns medium to dark blue. Do not overdevelop.
3. Add 100 μ l Stop Solution. In presence of the acid, the blue color turns yellow.
4. Read absorbance on a spectrophotometer within 5 minutes at 450 nm with a reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

References

1. Richard D.E., Berra E. and Pouysségur J. (1999) *Biochem. Biophys. Res. Commun.* 266: 718-22.
2. Semenza G.L. (2000) *J. Appl. Physiol.* 88: 1474-80.
3. Wang G.L., Jiang B.H., Rue E.A. and Semenza G.L. (1995) *Proc. Natl. Acad. Sci. USA* 92: 5510-4.
4. Wiener C.M., Booth G. and Semenza G.L. (1996) *Biochem. Biophys. Res. Commun.* 225: 485-8.
5. Jiang B.H., Semenza G.L., Bauer C. and Marti H.H. (1996) *Am. J. Physiol.* 271: C1172-80.
6. Zhu H. and Bunn H.F. (2001) *Science* 292: 449-51.
7. Tanimoto K., Makino Y., Pereira T. and Poellinger L. (2000) *EMBO J.* 19: 4298-4309.
8. Wang G.L. and Semenza G.L. (1993) *Blood* 82: 3610-5.
9. Semenza G.L., Jiang B.H., Leung S.W., Passantino R., Concordet J-P., Maire P. and Giallongo A. (1996) *J. Biol. Chem.* 271: 32529-37.
10. Renard P., Ernest I., Houbion A., Art M., Le Calvez H., Raes M. and Remacle J. (2001) *Nucleic Acids Res.* 29: (4) e21.

Appendix

Section A. Preparation of Nuclear Extract

For your convenience, Active Motif offers a Nuclear Extract Kit (Cat. Nos. 40010 & 40410). This kit contains buffers optimized for use in TransAM Kits, which serves to reduce inconsistencies in the assay that may arise from using homemade or other buffers. If you prefer to make your own buffers, please refer to the following protocol.

This procedure can be used for a confluent cell layer of 75 cm² (100-mm dish). The yield is approximately 0.15 mg of nuclear proteins for 9 x 10⁶ cells.

1. Wash cells with 10 ml ice-cold PBS/PIB. Discard PBS/PIB.
2. Add 10 ml ice-cold PBS/PIB and scrape the cells off the dish with a cell lifter. Transfer cells into a pre-chilled 15 ml tube and spin at 300 x g for 5 minutes at 4°C.
3. Resuspend the pellet in 1 ml ice-cold HB buffer by gentle pipetting and transfer the cells into a pre-chilled 1.5 ml tube.
4. Allow the cells to swell on ice for 15 minutes.
5. Add 50 µl 10% Nonidet P-40 (0.5 % final) and vortex the tube vigorously for 10 seconds.
6. Centrifuge the homogenate for 30 seconds at 4°C in a microcentrifuge. Remove the supernatant (cytoplasmic fraction) and, if you wish to save this for other uses, transfer it into a pre-chilled microcentrifuge tube. (Store the cytoplasmic fraction at -80°C.)
7. Resuspend the nuclear pellet in 50 µl Complete Lysis Buffer and rock the tube gently on ice for 30 minutes on a shaking platform.
8. Centrifuge for 10 minutes at 14,000 x g at 4°C and save the supernatant (nuclear extract). Aliquot and store at -80°C. Avoid freeze/thaw cycles.

9. Determine the protein concentration of the extract by using a Bradford-based assay.

Preparation of Buffers for Nuclear Extract

10X PBS

0.1 M phosphate buffer, pH 7.5

1.5 M NaCl

27 mM KCl

For 250 ml, mix:

3.55 g Na_2HPO_4 + 0.61 g KH_2PO_4

21.9 g

0.5 g

Adjust to 250 ml with distilled water. Prepare a 1X PBS solution by adding 10 ml 10X PBS to 90 ml distilled water. Sterilize the 1X PBS by filtering through a 0.2 μm filter. The 1X PBS is at pH 7.5. Store the filter-sterilized 1X PBS solution at 4°C.

PIB (Phosphatase Inhibitor Buffer)

125 mM NaF

250 mM β -glycerophosphate

250 mM p-nitrophenyl phosphate (PNPP)

25 mM NaVO_3

For 10 ml, mix:

52 mg

0.55 g

1.15 g

31 mg

Adjust to 10 ml with distilled water. Mix the chemicals by vortexing. Incubate the solution at 50°C for 5 minutes. Mix again. Store at -20°C.

PBS/PIB

Prior to use, add 0.5 ml PIB to 10 ml 1X PBS.

HB (Hypotonic Buffer)

20 mM Hepes, pH 7.5

5 mM NaF

10 μM Na_2MoO_4

0.1 mM EDTA

For 50 ml, mix:

0.24 g

12 mg

5 μl of a 0.1 M solution

10 μl of a 0.5 M solution

Adjust pH to 7.5 with 1 N NaOH. Adjust volume to 50 ml with distilled water. Sterilize by filtering through a 0.2 μm filter. Store the filter-sterilized solution at 4°C.

Section B: Troubleshooting Guide

Problem/question	Possible cause	Recommendation
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in all wells in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction, follow our recommendations to prepare buffers
	Plate reader settings not optimal	Verify the wavelength and filter settings in the plate reader
	Incorrect assay temperature	Bring substrate to room temperature
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette
High background in all wells	Developing time too long	Stop enzymatic reaction as soon as the positive wells turn medium-dark blue
	Concentration of antibodies is too high	Increase antibody dilutions
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
Uneven color development	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Well cross-contamination	Follow washing recommendations
High background in sample wells	Too much nuclear extract per well	Decrease amount of nuclear extract down to 1-2 µg/well
	Concentration of antibodies is too high	Perform antibody titration to determine optimal working concentration. Start using 1:1000 for primary antibody and 1:5000 for the secondary antibody. The sensitivity of the assay will be decreased
No signal or weak signal in sample wells	Not enough nuclear extract per well	Increase amount of nuclear extract to 50 µg/well
	HIF is poorly activated or inactivated in nuclear fractions	Perform a time course for HIF activation in the studied cell line
	Nuclear extracts are not from correct species	

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

Active Motif North America

Toll free: 877.222.9543
Direct: 760.431.1263
Fax: 760.431.1351
E-mail: tech_service@activemotif.com

Active Motif Europe

UK Free Phone: 0800/169 31 47
France Free Phone: 0800/90 99 79
Germany Free Phone: 0800/181 99 10
Direct: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050
E-mail: eurotech@activemotif.com

Active Motif Japan

Direct: +81 (0)3 5225 3638
Fax: +81 (0)3 5261 8733
E-mail: japantech@activemotif.com

Active Motif China

Direct: (86)-21-20926090
Cell Phone: 18521362870
E-mail: techchina@activemotif.com

Visit Active Motif online at activemotif.com