TransAM™ FKHR

Transcription Factor Assay Kits

(version C2)

Catalog Nos. 46396 & 46896

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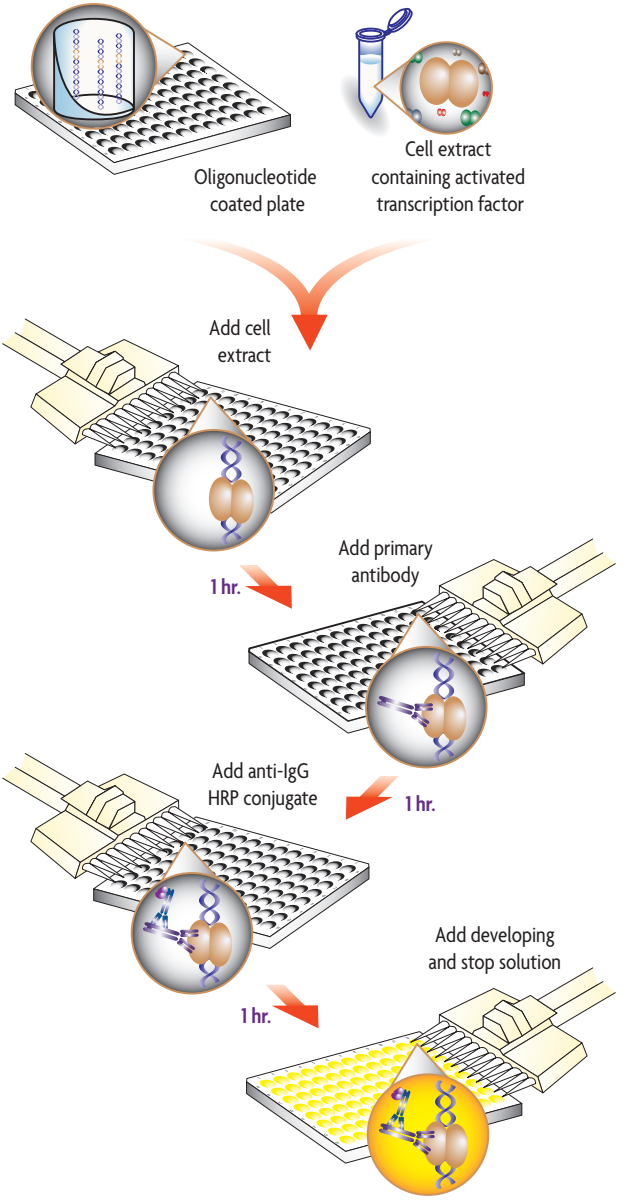
Overview

The Forkhead transcription factor FKHR regulates expression of gluconeogenic factors, and plays a key role in insulin regulation of glucose levels. Besides its role in glucose homeostasis, FKHR has been shown to be involved in pathological processes such as type 2 diabetes, cancer and organismal aging. The transcriptional output of FKHR is controlled by a two-tiered mechanism of phosphorylation and acetylation, and alterations in this balance can result in profound effects.[1] Therefore, accurate monitoring of FKHR activity in cells, tissues or animals is crucial for biomedical research and drug development. 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, specific assay. TransAM FKHR Kits are designed specifically for the study of FKHR. They contain a 96-well plate to which a Forkhead Box (FOX) element has been immobilized. FKHR contained in nuclear extracts binds specifically to this DNA molecule and is detected through use of an antibody directed against FKHR. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provides a 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 FKHR Kits are available in two sizes:

product	format	catalog no.
TransAM FKHR	1 x 96-well plate	46396
	5 x 96-well plates	46896

Flow Chart of Process



FKHR Transcription Factor

The Forkhead family of transcription factors are involved in regulation of the cell cycle, cell death, cell metabolism and oxidative stress (see 2 for review). The FOXO (Forkhead Box, class O) proteins form a subfamily of Forkhead transcription factors that are direct targets of phosphoinositide 3-kinase (PI3K) mediated signal transduction.[3] In the presence of growth factor/survival signals, PI3K activation leads to PDK mediated downstream activation of PKB/c-AKT. Phosphorylation and activation of PKB causes nuclear translocation of PKB, which phosphorylates and inactivates nuclear FOXO. FOXO then binds 14-3-3 proteins, and the FOXO/14-3-3 complex is exported to the cytoplasm. Phosphorylated, inactive FOXO proteins remain bound to 14-3-3 proteins in the cytoplasm, thereby preventing nuclear import of FOXO. In the absence of survival signals, cytoplasmic FOXO is dephosphorylated, causing dissociation from 14-3-3 proteins and allowing nuclear import of FOXO to activate gene expression.[3]

There are three AKT phosphorylation sites in the FKHR protein: Thr24, Ser256 and Ser319.[4] Specifically, phosphorylation at Ser256 is thought to play a role in masking a FKHR nuclear localization signal.[5] In addition, phosphorylation at Ser256 may also mediate the effects of insulin on gene expression.[6] Phosphorylation at Thr24 is critical for FKHR interaction with 14-3-3 proteins.[5]

The most well studied FOXO members include acute-lymphocytic-leukaemia-1 fused gene from chromosome X (AFX/FOXO4), Forkhead in rhabdomyosarcoma (FKHR/FOXO1) and FKHR-Like 1 (FKHRL1/FOXO3a). AFX mRNA is expressed at high levels in heart and skeletal muscle, and moderately in brown and white adipose tissue. FKHR mRNA is detected at its highest levels in brown adipose tissue, white adipose tissue and spleen, with lower levels in liver and skeletal muscle. FKHRL1 mRNA is expressed at its highest levels in brain, heart, kidney and spleen, with moderate expression in white adipose tissue and testis.[7]

In different cell types, FOXO proteins modulate various cellular activities. In hepatocytes, FOXO proteins regulate the expression of factors involved in gluconeogenesis, such as peroxisome proliferators-activated receptor-γ coactivator-1, glucose-6-phosphate and phosphoenolpyruvate carboxykinase .[8-11] Using genetic gain and loss of function analysis in mice, FKHR has also been shown to control β cell compensation for insulin resistance and glucose production in type 2 diabetes.[12] In addition, FOXO proteins such as FKHRL1 have been shown to regulate catalase and superoxide dismutase gene expression that protect cells from oxidative stress, suggesting that FOXO factors act to control the mammalian lifespan.[13, 14]

While Forkhead transcription factors do not bind to a clear consensus sequence, the subclasses have been shown to bind to specific sequence elements. For example, FOXO and HNF subclasses of Forkhead proteins have been shown to bind to the insulin response elements (IREs) of insulin-like growth factor-binding protein-1 (IGFBP-1).[11] FOXO transcription factors bind to a consensus core sequence of 5′-TTGTTTAC-3′[15], which includes a sequence TRTTTAY (with R a purine base and Y a pyrimidine base) conserved among various Forkhead members.[7]

Transcription Factor Assays

To date, three methods are widely used to measure FKHR activity, either directly or indirectly:

1. Expression can be measured by Western blot, using antibodies raised against FKHR. 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. The DNA-binding capacity of FKHR can be assayed by gel retardation, also called electrophoretic mobility shift assay (EMSA). In this method, nuclear extracts are incubated with a radioactive double-stranded oligonucleotide probe containing a specific sequence element for FKHR binding. If FKHR 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. Gelshift assays also require special precautions and equipment for handling radioactivity.
3. Another method used to assay FKHR activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing a FKHR consensus binding element. This promoter can be artificial, made of several FKHR cis-elements and a TATA box, or natural. Limitations of this procedure are: (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. Therefore, assays have to be carefully standardized. This method is sensitive and easy to perform with a large number of samples but requires efficient cell transfection with the reporter plasmid.

TransAM FKHR (FOXO1)

FKHR is involved in cell survival and regulation of glucose levels, and therefore represents an excellent pharmacological target for developing drugs to treat cancer and diabetes. However, pharmaceutical research in this field has been limited by the lack of convenient assays suitable for processing large numbers of samples.

To overcome this, Active Motif is introducing a high-throughput assay to quantify FKHR activation. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM FKHR (FOXO1) Kits contain a 96-well plate on which has been immobilized oligonucleotide that contains a FOXO element. The active form of FKHR contained in the nuclear extract specifically binds to this oligonucleotide. The primary antibodies used to detect FKHR will recognize an epitope on FKHR protein that is accessible only when FKHR is activated and bound to the target DNA. 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 this assay is performed in 96-well plates, a large number of samples can be handled simultaneously, enabling high-throughput automation. With the TransAM procedure, activated FKHR was detected in 0.5 µg of nuclear extract.

Kit Performance and Benefits

The TransAM FKHR Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: <5 µg nuclear extract/well.

Range of detection: TransAM provides quantitative results from 5 to 20 µg of nuclear extract/well.

Cross-reactivity: TransAM FKHR detects FKHR (FoxO1) from human, mouse and rat origin.

Assay time: 3.5 hours.

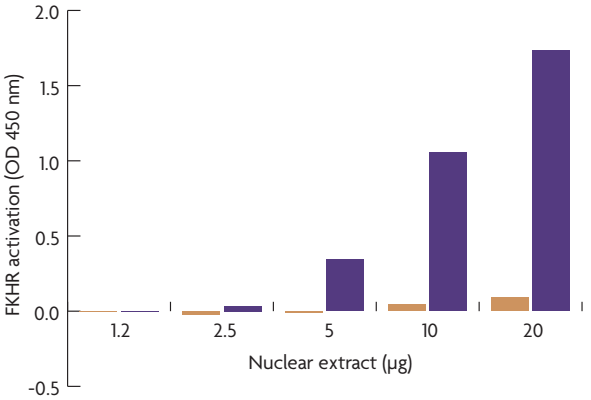


Figure 1: Monitoring FKHR activity with the TransAM FKHR Kit.
Different amounts of nuclear extracts from untreated Raji (dark bars) and HeLa (light bars) cells are tested for FKHR activity by using the TransAM FKHR Kit. This data is provided for demonstration only.

Kit Components and Storage

Except for the nuclear extract that must be kept at -80ºC, kit components can be stored at -20ºC prior to first use. Then, we recommend storing each component at the temperature indicated in the table below.

Reagents	Quantity 1 plate / 5 plates	Storage / Stability
FKHR (FOXO1) antibody	12 µl / 60 µl	-20°C for 6 months
Anti-rabbit HRP-conjugated IgG	11 µl / 55 µl (0.2 µg/µl)	4°C for 6 months
Wild-type oligonucleotide AM19	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM19	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Raji nuclear extract	40 µl / 200 µl (2.5 µg/µl)	-80°C for 6 months
Dithiothreitol (DTT)	100 µl / 500 µl (1 M)	-20°C for 6 months
Protease Inhibitor Cocktail	100 µl / 500 µl	-20°C for 6 months
Herring sperm DNA	100 µl / 500 µl (1 µg/µl)	-20°C for 6 months
Lysis Buffer AM1	10 ml / 50 ml	4°C for 6 months
Binding Buffer AM6	10 ml / 50 ml	4°C for 6 months
10X Wash Buffer AM2	22 ml / 110 ml	4°C for 6 months
10X Antibody Binding Buffer AM2	2.2 ml / 11 ml	4°C for 6 months
Developing Solution	11 ml / 55 ml	4°C for 6 months
Stop Solution	11 ml / 55 ml	4°C for 6 months
96-well FKHR assay plate	1 / 5	4°C for 6 months
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)

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). Prepare the amount of Complete Lysis Buffer required for the assay by adding 1 µl of 1 M DTT and 10 µl of Protease Inhibitor Cocktail per ml of Lysis Buffer AM1 (see the Quick Chart for Preparing Buffers in this section). 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 by adding 10 µl of 1 µg/µl Herring sperm DNA per ml of Binding Buffer AM6 (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Complete Binding Buffer.

Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 100 ml of 1X Wash Buffer required, dilute 10 ml 10X Wash Buffer AM2 with 90 ml distilled water (see the Quick Chart for Preparing Buffers in this section). 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 vortexing for 2 minutes prior to use.

Preparation of 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer AM2 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section)*. Mix gently to avoid foaming. Discard remaining 1X Antibody Binding Buffer after use. The BSA contained in the 10X Antibody Binding Buffer AM2 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 1:1000 and the 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 both 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 into a secondary container (see the Quick Chart for Preparing Buffers in this section). 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.

Raji nuclear extract

The Raji nuclear extract is provided as a positive control for FKHR activation. Sufficient extract is supplied for 20 reactions. This extract is optimized to give a strong signal when used at 5 µg/well. We recommend aliquoting the extract in 5 µl fractions and storing at -80ºC to help avoid multiple freeze/thaw cycles of the extracts. Various nuclear 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 FKHR binding in order to monitor the specificity of the assay. Used at 10 pmol/well, the oligonucleotide will prevent FKHR binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on FKHR binding. Prepare the required amount of wild-type and/or mutated consensus oligonucleotide by adding 1.13 µl of appropriate oligonucleotide to 43.88 µl of 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	For 1 well	For 1 strip (8 wells)	For 6 strips (48 wells)	For 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 AM1	11.12 µl	89.0 µl	534.0 µl	1.068 ml
	TOTAL REQUIRED	**11.25 µl**	**90.0 µl**	**540.0 µl**	**1.08 ml**
Complete Binding Buffer	Herring Sperm DNA	0.45 µl	3.6 µl	21.6 µl	43.2 µl
	Binding Buffer AM6	44.55 µl	356.4 µl	2.138 ml	4.277 ml
	TOTAL REQUIRED	**45 µl**	**360 µl**	**2.16 ml**	**4.32 ml**
Complete Binding Buffer with wild-type or mutated oligonucleotide	Wild-type or mutated oligo	1.13 µl	9 µl	54 µl	N/A
	Complete Binding Buffer	43.88 µl	351 µl	2.106 ml	N/A
	TOTAL REQUIRED	**45 µl**	**360 µl**	**2.16 ml**	**N/A**
1X Wash Buffer	Distilled water	2.025 ml	16.2 ml	97.2 ml	194.4 ml
	10X Wash Buffer AM2	225 µl	1.8 ml	10.8 ml	21.6 ml
	TOTAL REQUIRED	**2.25 ml**	**18 ml**	**108 ml**	**216 ml**
1X Antibody Binding Buffer*	Distilled water	202.5 µl	1.62 ml	9.72 ml	19.44 ml
	10X Ab Binding Buffer AM2	22.5 µl	180 µl	1.08 ml	2.16 ml
	TOTAL REQUIRED	**225 µl**	**1.8 ml**	**10.8 ml**	**21.6 ml**
Developing Solution	**TOTAL REQUIRED**	**112.5 µl**	**900 µl**	**5.4 ml**	**10.8 ml**
Stop Solution	**TOTAL REQUIRED**	**112.5 µl**	**900 µl**	**5.4 ml**	**10.8 ml**

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

FKHR 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 Binding Buffer, Wash Buffer, Antibody Binding Buffer, Developing Solution and Stop Solution into the wells being used.

Step 1: Binding of FKHR to its consensus sequence

1. Add 40 µl Complete Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 40 µl Complete Binding Buffer that contains 10 pmol (1 µl) of the wild-type or mutated consensus oligonucleotide (see the Buffer Preparation section above for a description of competitive binding).
2. **Sample wells:** Add 10 µl of sample diluted in Complete Lysis Buffer per well. We recommend using 2-20 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts can be found on page 11.

   Positive control wells: Add 5 µg of the provided nuclear extract diluted in 10 µl of Complete Lysis Buffer per well (2 µl of nuclear extract in 8 µl of Complete Lysis Buffer per well).

   Blank wells: Add 10 µl 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 µl 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 µl diluted FKHR antibody (1:1000 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 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 3: Binding of secondary antibody

1. Add 100 µl 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 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric reaction

1. Add 100 µl room-temperature Developing Solution to all wells being used.
2. Incubate 2-10 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 µl 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.

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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 cm^2 (100 mm dish). The yield is approximately 0.5 mg of nuclear proteins for 10^7 cells.

1. Wash cells with 10 ml of ice-cold PBS/PIB.
2. Add 10 ml of ice-cold PBS/PIB and scrape the cells off the dish with a cell lifter. Transfer the 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 of 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 mix by gentle pipetting.
6. Centrifuge the homogenate for 30 seconds at 4°C in a microcentrifuge.
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.

10X PBS	**For 250 ml, mix:**
0.1 M phosphate buffer, pH 7.5	3.55 g Na_2HPO_4 + 0.61 g KH_2PO_4
1.5 M NaCl	21.9 g
27 mM KCl	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)	**For 10 ml, mix**
125 mM NaF	52 mg
250 mM β-glycerophosphate	0.55 g
250 mM para-nitrophenyl phosphate (PNPP)	1.15 g
25 mM $NaVO_3$	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 of PIB to 10 ml of 1X PBS.

HB (Hypotonic Buffer)	**For 50 ml, mix**
20 mM Hepes, pH 7.5	0.24 g
5 mM NaF	12 mg
10 µM Na_2MoO_4	5 µl of a 0.1 M solution
0.1 mM EDTA	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	POSSIBLE CAUSE	RECOMMENDATION
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added 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 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 and follow washing recommendations	Ensure all wells are filled with Wash Buffer
	Well cross-contamination	Follow washing recommendations
High background in sample wells	Too much nuclear extract per well	Decrease amount of nuclear extract down to 2.5 µg/well
	Concentration of antibodies 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, not to exceed 40 µg/well
	FKHR is poorly activated or inactivated	Perform a time course for FKHR activation in the studied cell line
	Samples are not from correct species	Refer to cross-reactivity information on page 5

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

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