TransAM™ GR

Transcription Factor Assay Kits

(version A2)

Catalog Nos. 45496 & 45996

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Overview

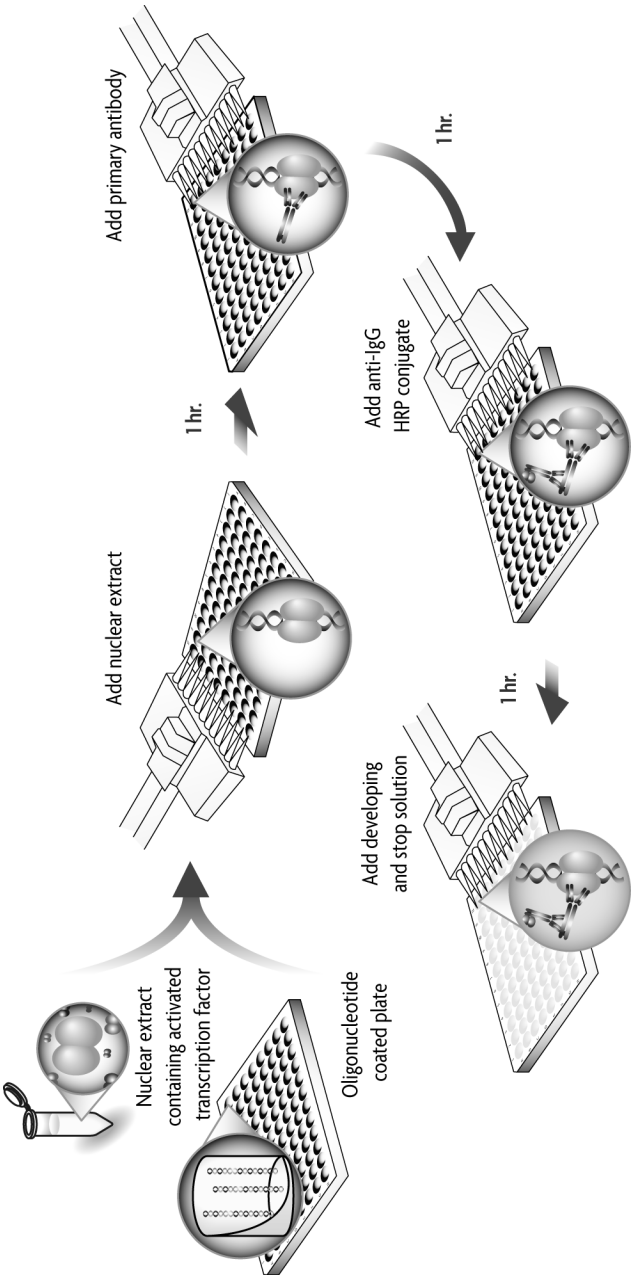
Glucocorticoids play an essential role in maintaining basal and stress-related homeostasis. Glucocorticoid Receptor (GR) is the mediator for most known effects of glucocorticoids. Abnormalities in GR sensitivity can lead to steroid-resistant asthma, rheumatoid arthritis, osteoarthritis, hypertension and obesity[1]. Because of the importance of GR in maintaining homeostasis, accurate monitoring of GR in cells, tissues and animals is crucial for many biomedical research and drug development projects. 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 GR Kits are designed specifically to detect and quantify GR activation. They contain a 96-well plate to which oligonucleotide containing a GRE consensus sequence has been immobilized. GR contained in nuclear extract binds specifically to this oligonucleotide and is detected through use of an antibody directed against GR. 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 GR Kits are available in two sizes:

product	format	catalog no.
TransAM GR	1 x 96-well plate	45496
	5 x 96 well plates	45996

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

Flow Chart of Process



Introduction

GR Transcription Factor

Glucocorticoids can affect a large number of metabolic, cardiovascular, immune, inflammatory and behavioral functions. They are produced by the adrenal cortex and are under the control of the hypothalamus and pituitary (hypothalamus-pituitary-adrenal [HPA] axis). At the cellular level, glucocorticoid effects are mediated by the Glucocorticoid Receptor (GR). GR belongs to the superfamily of nuclear hormone receptors that includes receptors for estrogens, progestins, vitamin D and thyroid hormone[1].

The nuclear hormone receptors share a characteristic three-domain structure. The N-terminal activates target genes and interacts with transcription machinery. Two highly conserved zinc fingers constitute the DNA-binding domain and also participate in dimerization, nuclear translocation and transactivation. The C-terminal contains the ligand-binding domain, and also includes sequences important for heat shock protein (hsp) binding, nuclear translocation, dimerization and transactivation[1].

The unliganded GR is part of a multiprotein complex that consists of the receptor, two molecules of hsp90 and one molecule each of hsp70 and hsp56. Glucocorticoids, when present, are able to cross the cell membrane and interact with GR. When bound, there is a conformational change in the GR molecule that results in dissociation from the hsp complex, hyper-phosphorylation of GR and unmasking of nuclear localization signals. When in the nucleus, the activated GR can act in two ways: directly with specific DNA sequences or indirectly with other transcription factors[1]. GR mutations can result in glucocorticoid resistance or hypersensitivity, and can cause severe disturbances in mood, pathologic alterations of metabolism and, correspondingly, hypotension or hypertension and excessive or suppressed inflammatory/immune responses[2].

Transcription Factor Assays

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

1. GR activation can be determined by Western blot by using antibodies specific for GR proteins. This method is time consuming (up to 2 days once the nuclear cell extracts are prepared), and is not suitable for processing large numbers of samples.

2. The DNA-binding capacity of GR 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 the consensus sequence for GR binding. If GR 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 cannot be applied to high-throughput screening. Gelshift assays also require special precautions and equipment for handling radioactivity.

3. Another method used to assay GR activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing the GR recognition site. The promoter can be artificial, made of several GR elements or natural. 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 GR

GR plays a key role in the regulation of metabolism, cardiovascular function, inflammation and immunity[2], and therefore represents an excellent pharmacological target. However, pharmaceutical research in this field has been limited by the lack of convenient assays suitable for large numbers of samples.

To overcome this, Active Motif is introducing a high-throughput assay to quantify GR activation. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM GR Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains the GR consensus binding site (5′-GGTACAnnnTGTTCT-3′)[3]. GR contained in nuclear extract binds specifically to this oligonucleotide. The primary antibody used in TransAM GR Kits recognizes an accessible epitope on GR protein 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 this assay is performed in 96-well plates, a large number of samples can be handled simultaneously, enabling high-throughput automation. This assay is specific for GR activation and has been shown to be 5-fold more sensitive and 20-fold faster than the gel-retardation technique. With the 3.5-hour TransAM procedure, we could detect GR activation using as little as 0.6 µg of nuclear extract. A comparable assay using EMSA requires 3 µg of nuclear extract and a 5-day autoradiography.

TransAM has many applications including the study of drug potency toward GR, GR transcriptional activity regulation and protein structure/function studies of GR.

Kit Performance and Benefits

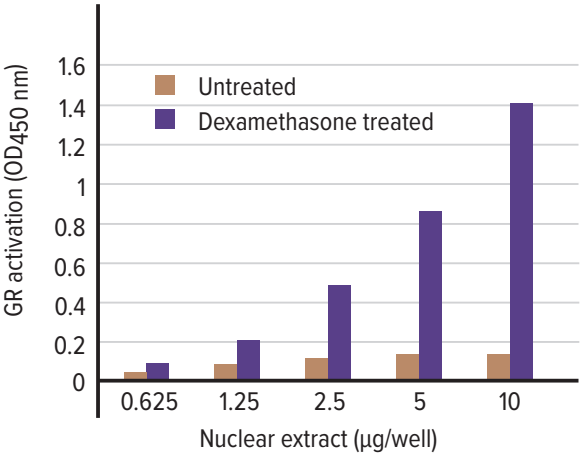
TransAM GR Kits are for research use only. Not for use in diagnostic procedures.

Detection limit: < 0.6 µg nuclear extract/well. TransAM GR is 5-fold more sensitive than EMSA.

Range of detection: TransAM provides quantitative results from 0.6 to 10 µg of nuclear extract/well (see graph below).

Cross-reactivity: The TransAM GR Kit detects GR from human, mouse and rat origin.

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



Monitoring GR activity with the TransAM GR Kit: Different amounts of nuclear extracts from untreated and Dexamethasone treated HeLa cells are tested for GR activity by using the TransAM GR 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
GR antibody	11 µl / 55 µl	4°C for 6 months
Anti-rabbit HRP-conjugated antibody	11 µl / 55 µl (0.25 µg/µl)	4°C for 6 months
Wild-type oligonucleotide AM27	100 µl / 500 µl (20 pmol/µl)	-20ºC for 6 months
Mutated oligonucleotide AM27	100 µl / 500 µl (20 pmol/µl)˙	-20ºC for 6 months
HeLa nuclear extract (Dexamethasone treated)	40 µl / 200 µl (2.5 µg/µl)	-80ºC for 6 months
Dithiothreitol (DTT)	100 µl / 500 µl (1 M)	-20ºC for 6 months
Protease Inhibitor Cocktail	100 µl / 500 µl	-20ºC for 6 months
Herring sperm DNA	100 µl / 500 µl (1 µg/µl)	-20ºC for 6 months
Lysis Buffer AM2	10 ml / 50 ml	4°C for 6 months
Binding Buffer AM2	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 AM3	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 GR 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)

For Nuclear Extract preparation

- Hypotonic Buffer
- Phosphatase Inhibitor Buffer
- 10X PBS
- Detergent (NP-40)

Buffer Preparation and Recommendations

Preparation of Complete Lysis Buffer

We provide an excess of Lysis Buffer AM2 in order to perform the assay AND to prepare customized cell 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 Protease Inhibitor Cocktail per ml of Lysis Buffer AM2 (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 1 µl of 1 M DTT, and 10 µl of 1 µg/µl Herring sperm DNA per ml of Binding Buffer AM2 (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, which makes it necessary to homogenize the buffer by incubating at 50ºC 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 as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer AM3 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 10X Antibody Binding Buffer AM3 may form clumps, therefore you should homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use. Dilute the primary and secondary antibodies with the 1X Antibody Binding Buffer to 1:1000. 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).

HeLa nuclear extract

The HeLa nuclear extract (Dexamethasone treated) is provided as a positive control for GR activation. Nuclear extract was made from HeLa cells treated with 100 nM Dexamethasone for 1 hour at 37°C. Sufficient extract is supplied for 20 reactions per plate. 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 -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 GR binding in order to monitor the specificity of the assay. Used at 40 pmol/well, the oligonucleotide will prevent GR binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on GR binding. Prepare the required amount of wild-type and/or mutated consensus oligonucleotide by adding 2 µl of appropriate oligonucleotide to 31.8 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 cell 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	0.02 µl	0.2 µl	1.2 µl	2.4 µl
	Protease Inhibitor Cocktail	0.23 µl	1.8 µl	10.8 µl	21.6 µl
	Lysis Buffer AM2	22.25 µl	178.0 µl	1.068 ml	2.136 ml
	TOTAL REQUIRED	**22.5 µl**	**180.0 µl**	**1.08 ml**	**2.16 ml**
Complete Binding Buffer	DTT	0.03 µl	0.27 µl	1.6 µl	3.25 µl
	Herring sperm DNA	0.34 µl	2.7 µl	16.2 µl	32.4 µl
	Binding Buffer AM2	33.4 µl	267 µl	1.6 ml	3.2 ml
	TOTAL REQUIRED	**33.8 µl**	**270 µl**	**1.62 ml**	**3.24 ml**
Complete Binding Buffer with wild-type or mutated oligonucleotide	Wild-type or mutated oligo	2.0 µl	16.0 µl	96.0 µl	N/A
	Complete Binding Buffer	31.8 µl	254.0 µl	1.524 ml	N/A
	TOTAL REQUIRED	**33.8 µl**	**270.0 µl**	**1.62 ml**	**N/A**
1X Wash Buffer	Distilled water	2.025 ml	16.2 ml	97.2 ml	194.4 ml
	10X Wash Buffer AM2	225.0 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 Ab Binding Buffer AM3	22.5 µl	180 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.

GR 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, 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 GR to its consensus sequence

1. Add 30 µl Complete Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 30 µl Complete Binding Buffer that contains 40 pmol (2 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 20 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 20 µl of Complete Lysis Buffer per well (2 µl of nuclear extract in 18 µl of Complete Lysis Buffer per well).

   Blank wells: Add only 20 µl Complete Lysis Buffer 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 GR 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. 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 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 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric reaction

1. Add 100 µl Developing Solution to all wells being used.
2. Incubate 2-20 minutes at room temperature protected from direct light. Please read the Certificate of Analysis supplied with this kit for the optimal development time for this specific kit lot, which varies from lot to lot. 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 the 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.

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 0.15 mg of nuclear proteins for 9 x 10^6 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.

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 PIB to 10 ml 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.

References

1. Bamberger C.M. *et al* (1996) *Endocrine Reviews* 17(3): 245-261.
2. Karl M. *et al* (1993) *J. Clinical Endo and Metab.* 76(3): 683-689.
3. Scott D.K. *et al* (1998) *Mol. Endo.* 12: 482-491.

Appendix

Section A. 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	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 5 µg/well
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1:2000 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
	GR is poorly activated or inactivated in nuclear fractions	Perform a time course for GR activation in the studied cell line
	Extracts are not from rat, mouse or human origin	Perform study with a human, mouse or rat model

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.

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