TransAM™
ER Transcription Factor Assay Kits

(version C3)

Catalog Nos. 41396 & 41996

Revision History

Revision	Date	Description of Change
C2	June 2019	Corrected recipe for Complete Lysis Buffer

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Overview

The Estrogen Receptor (ER) is expressed in two forms, ERα and ERβ. These are encoded by two distinct genes and are members of the steroid/thyroid hormone superfamily of nuclear receptors[1]. The activity of these proteins (dimerization, DNA binding and interaction with other transcription factors) is modulated by their corresponding ligands. Estrogens and estrogen-like molecules exert their effects through binding to and activating ER[2]. They affect growth, differentiation and the development of reproductive tissues. Binding of 17β-estradiol (E_2) to ER induces a conformational change leading to dissociation of the receptor from the heat shock protein complex (hsp56-59, hsp70 and hsp90). The activated ERα can interact with basal transcription factors (TFIIB, hTAF 30) which permit RNA polymerase to initiate transcription of several genes such as: progesterone receptor, ovalbumin, uteroglobin, lactoferrin, glucose-6-phosphate dehydrogenase and Xenopus vitellogenin A2. Also, ERs are detected in 50-80% of all breast tumors and are clinically important as markers for patients with a higher probability of responding to hormonal or endocrine treatments[3]. Therefore, accurate monitoring of ER activity in cells, tissues or animals is crucial for biomedical research and drug development.

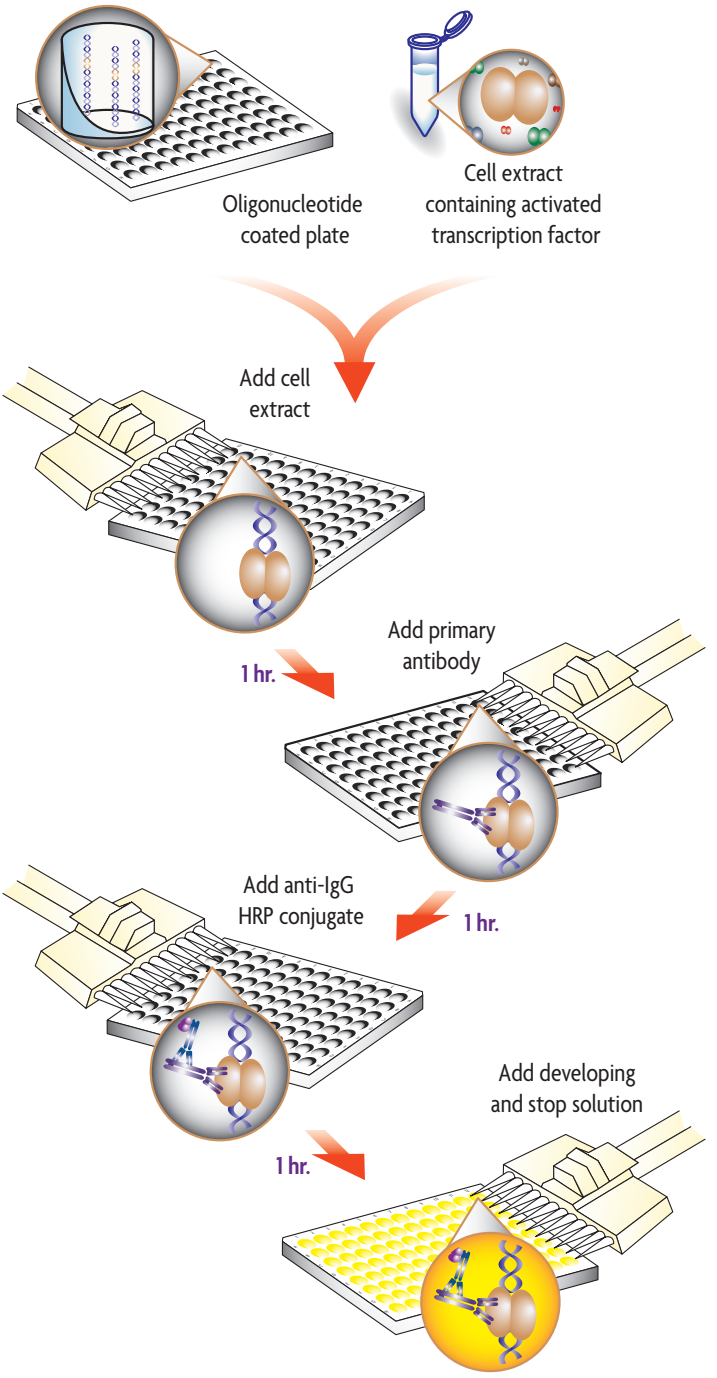
With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM ER Kits are designed specifically for the study of ER expression and activity. They contain a 96-well plate to which an oligonucleotide containing the ER consensus binding site has been immobilized. ER contained in nuclear extracts binds specifically to this oligonucleotide and is detected through use of an antibody directed against ERα. 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 ER Kits are available in two sizes:

product	format	catalog no.
TransAM ER	1 x 96-well plate	41396
	5 x 96 well plates	41996

See Active Motif products related to the ER transcription factor in Appendix, Section C.

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

Flow Chart of Process



Introduction

ER Transcription Factor

ER belongs to the superfamily of ligand-inducible transcription factors. Human ERα is comprised of 595 amino acids and displays an approximate molecular weight of 66-70 kDa. Six functional regions have been identified[4]. A hypervariable domain (aa 1-184) contains activation function 1 (AF1). The DNA binding domain (DBD, aa 185-263) contains two zinc finger motifs and is highly conserved across the nuclear receptor superfamily[5]. It is responsible for the binding of the receptor to estrogen response elements (EREs) and contributes to dimerization and activation[5]. Typically, EREs consist of two inverted half-sites separated by 3 bp (5´-GGTCAnnnTGACC-3´)[6]. The region which separates the ligand binding domain (LBD) and the DBD is called the hinge region (aa 264-302). The LBD (aa 303-553) consists of 12 α-helices, which form a hydrophobic pocket responsible for ligand binding. The function of the final domain (aa 554-595) is not clear but is thought to play a role in distinguishing between agonist and antagonist binding[7]. Human ERβ is expressed as multiple isoforms[8]. Structure and function studies have shown that the DBD of ERα and ERβ are highly homologous, approaching 96%, whereas the LBD showed only 59% homology. The general mechanism of action of ERβ is thought to be similar to that of ERα. ERα and ERβ have the ability to interact with target promoters in three different complexes: ERα homodimers, ERβ homodimers and ERα/ERβ heterodimers.

The transcriptional effects of ER can be mediated through several mechanisms other than E_2-ER complexes binding to EREs. E_2-ER complexes can also transactivate genes through protein-protein interactions with transcription factors such as AP-1 or Sp1 that bind DNA, with coaccessory proteins (Src, ACTR), some of which have histone acetylase activity, and with RNA Polymerase II complex proteins[9]. In addition, ERs serve to repress genes, which also plays an important role in E_2 action[10, 11].

Transcription Factor Assays

To date, three methods are widely used to measure ER expression, either directly or indirectly:

1. ER expression can be determined by Western blot by using antibodies specific for ER 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. The DNA-binding capacity of ER 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 ER binding. If ER 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 ER activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing an ER consensus binding site. The promoter can be artificial, made of a GC box and a TATA box, or natural, like promoter sequences from viral regulator elements, such as the HIV-1 LTR promoter. 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; 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 ER

ER-regulated genes are involved in a variety of cellular pathways that are currently being deciphered by academic and pharmaceutical laboratories for new target discovery. However, this field has been hampered by the lack of convenient assays suitable for discriminating the ER family members and performing high numbers of experiments.

To overcome this, Active Motif is introducing a high-throughput assay to quantify ER activ-ation[12]. TransAM Kits combine a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM ER Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains an ER consensus binding site (5´-GGTCACAGTGACC-3´). ER contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used in the TransAM ER Kit recognizes an accessible epitope on ERα 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 ERα 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 ER activation using as little as 0.6 µg of nuclear extract from MCF-7 cells. A comparable assay using EMSA required 2.5 µg of nuclear extract and a 3-day autoradiography.

TransAM ER has many applications including the study of ER transcriptional activity regulation and protein structure/function studies of ER and its mutated variants in areas such as osteoporosis, arteriosclerosis, breast cancer propagation and many more.

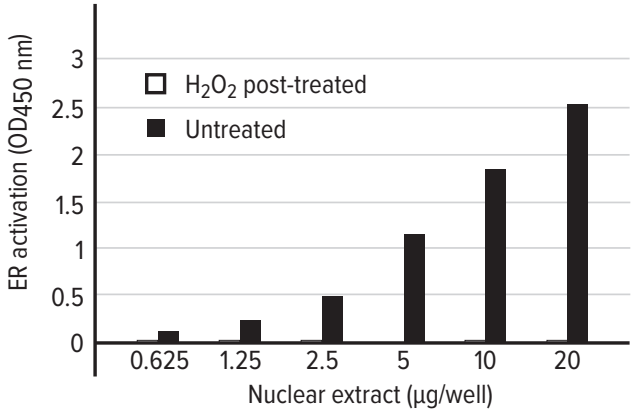
Kit Performance and Benefits

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

Range of detection: TransAM provides quantitative results from 0.6 to 20 µg of nuclear extract/well (see graph below).

Cross-reactivity: TransAM ER detects ERα from human, mouse and rat origin.

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



Monitoring ERα activation with the TransAM ER Kit: Different amounts of untreated and H_2O_2 post-treated nuclear extracts from MCF-7 cells are tested for ER activity by using the TransAM ER Kit. This data is provided for demonstration only.

Kit Components and Storage

TransAM NFκB Kits are for research use only. Not for use in diagnostic procedures. 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. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity 1 plate / 5 plates	Storage
ERα antibody	11 µl / 55 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 AM11	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM11	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
MCF-7 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
Poly [d(I-C)]	100 µl / 500 µl (17 µg/ml)	-20°C for 6 months
Lysis Buffer AM1	10 ml / 50 ml	4°C for 6 months
Binding Buffer AM1	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 ER 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
- Nuclear or whole-cell extracts
- Microplate spectrophotometer capable of reading at 450 nm (655 nm as an optional reference wavelength)

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 nuclear extracts. Please refer to the Appendix Section A for a protocol to prepare a nuclear extract. Our Nuclear Extract Kit can also be purchased separately (Cat. Nos. 40010 & 40410). When preparing extracts, it is suggested to perform the final lysis step using the Lysis Buffer AM2 provided in the TransAM Kit. All subsequent dilutions should also be performed using Lysis Buffer AM1 from the TransAM Kit. 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 AM1 (see the Quick Chart for Preparing Buffers in this section). Some of the protease inhibitors lose their activity after a few hours once diluted. Therefore, we recommend adding protease inhibitors immediately prior to use. Any remaining Complete Lysis Buffer 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 2 µl of DTT and 10 µl of 17 µg/ml poly[d(I-C)] per ml of Binding Buffer AM1 (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 it is necessary to completely resuspend any precipitates 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 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 the 10X Antibody Binding Buffer AM3 may form clumps, therefore it is necessary to completely resuspend the buffer by warming to room temperature and vortexing for 1 minute prior to use.

* 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.

Nuclear extract

The MCF-7 nuclear extract is provided as a positive control for ER activation. 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 10 µl fractions and storing at -80ºC. Avoid multiple freeze/thaw cycles of the extract. Various cell extracts are available from Active Motif (see Appendix, Section C. Related Products).

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for ER binding in order to monitor the specificity of the assay. This competition assay will confirm that the protein subunits binding to the plate are specific for the ER consensus binding sequence. Used at 20 pmol/well, the oligonucleotide will prevent ER binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on ER binding. Prepare the required amount of wild-type and/or mutated consensus oligonucleotide by adding 2 µl of appropriate oligonucleotide to 43 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 first, then add the cell extract. It is not necessary to perform an incubation step of the oligonucleotide in the well prior to addition of the cell extract. The oligonucleotide competition only needs to be performed as a control. It is suggested to test the oligonucleotide competition each new cell type used.

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 µl	534 µl	1.068 ml
	TOTAL REQUIRED	**11.25 µl**	**90 µl**	**540 µl**	**1.08 ml**
Complete Binding Buffer	DTT	0.09 µl	0.7 µl	4.3 µl	8.6 µl
	Poly [d(I-C)]	0.45 µl	3.6 µl	21.6 µl	43.2 µl
	Binding Buffer AM1	44.5 µl	356 µl	2.134 ml	4.268 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	2 µl	16 µl	96 µl	N/A
	Complete Binding Buffer	43 µl	344 µl	2.064 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 AM3	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.

ER 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 ER 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 20 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 10 µl of sample diluted in Complete Lysis Buffer per well. We recommend using 2-10 µg of nuclear extract diluted in Complete Lysis Buffer per well. A protocol for preparing nuclear extracts can be found on page 13.

   Positive control wells: Add 5 µg of the provided MCF-7 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.

Reagents	Blank wells	Positive Control no competition	Sample no competition	(Optional) wild-type	(Optional) mutated
Complete Binding Buffer	40 µl	40 µl	40 µl	38 µl	38 µl
Wild-type oligonucleotide	–	–	–	2 µl	–
Mutated oligonucleotide	–	–	–	–	2 µl
Complete Lysis Buffer	10 µl	–	–	–	–
Sample in Complete Lysis Buffer	**–**	**10 µl**	**10 µl**	**10 µl**	**10 µl**

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). Keep any unused wells covered during the remaining steps in order to preserve those wells for future assays. Any unused strips from the stripwell plate can be placed in the foil bag, sealed with tape and stored at 4°C.
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 ERα antibody (1:2000 dilution in 1X Antibody Binding Buffer) to each well being used, including blank wells.
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 anti-rabbit HRP-conjugated antibody (1:2000 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 5-10 minutes at room temperature protected from direct light. Please read the Certificate of Analysis supplied with this kit for optimal development time for this specific kit lot. Monitor the blue color development in the sample 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 an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

References

1. Cowley S.M., Hoare S., Mosselman S. and Parker M.G. (1997) Estrogen Receptors α and β Form Heterodimers on DNA. *J. Biol. Chem.* 272: 19858-19862.
2. Boris J., Cheskis J., Karathanasis S. and Lyttle C.R. (1997) Estrogen Receptor Ligands Modulate Its Interaction with DNA. *J. Biol. Chem.* 272: 11384-11391.
3. Traish A.M., Newton A.W., Styperek K., Beazley R. and Kavanah, M. (1995) Estrogen receptor functional status in human breast cancer. *Diag. Mol. Pathol.* 4(3): 220-228.
4. Kumar V., Green S., Stack G., Berry M, Jin J.R., Chambon P., (1987) Functional domains of the human estrogen receptor. *Cell* 51: 941-951.
5. Greene G.L., Gilna P., Waterfield M., Baker A., Hort Y. and Shine J. (1986) Sequence and expression of human estrogen receptor complementary DNA. *Science* 231:1150-1154.
6. Fawell S. E., Lees J. A., White R. and Parker M.G. (1990). Characterization and Colocalization of Steroid Binding and Dimerization Activities In the Mouse Estrogen Receptor. *Cell* 60: 953-962,
7. Brzozowski A.M., Pike A.C., Dauter Z. *et al.* (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 389: 753-758.
8. Mosselman S., Polman J., Dijkema R. (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* 392: 49-53.
9. McKenna N.J. and O'Malley B.W. (2000) From ligand to response: generating diversity in nuclear receptor coregulator function. J Steroid Biochem. *Mol. Biol.* 74: 351-356.
10. Valentine J.E., Kalkoven E., White R., Hoare S. and Parker M.G. (2000) Mutations in the estrogen receptor ligand binding domain discriminate between hormone-dependent transactivation and transrepression. *J. Biol. Chem.* 275: 25322-25329.
11. Wang Y, Miksicek R.J. 1991 Identification of a dominant negative form of the human estrogen receptor. *Mol Endocrinol.* 5:1707-1715.
12. 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 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. Do not use Trypsin to remove cells as it may alter transcription factor activation states.
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. Check the cells under a microscope to monitor the cell lysis. The cell membrane should be completely lysed, while the nuclear membrane remains intact.
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. Discard the debris pellet.
9. Determine the protein concentration of the extract by using a Bradford-based assay. It is recommended to perform a 1:50 dilution of your samples for protein determination. A blank sample consisting of Complete Lysis Buffer diluted 1:50 should be run as control.

Preparation of Buffers for Nuclear Extract

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 p-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.

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 by mixing HRP and Developing Solution together
	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
	Incorrect assay temperature	Bring Developing Solution and Stop Solution to room temperature before using
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette
High background in all wells	Measurement time too long	Stop enzymatic reactions 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 1:2,000 for primary antibody and 1:5,000 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
	Too many freeze/thaw cycles of extract	Aliquot extract into 5 µl aliquots and store at -80°C to avoid multiple freeze/thaws
	ER is poorly activated or inactivated in nuclear fractions	Perform a time course for ER activation in the studied cell line
	Nuclear extracts are not from correct species	Perform study with a human, mouse or rat model..
	Salt concentration too high in binding reaction	Reduce amount of extract per well or dialyze extract before use

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

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