NR Sandwich

PR ELISA

(version A)

Catalog Nos. 49396 & 49896

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Overview

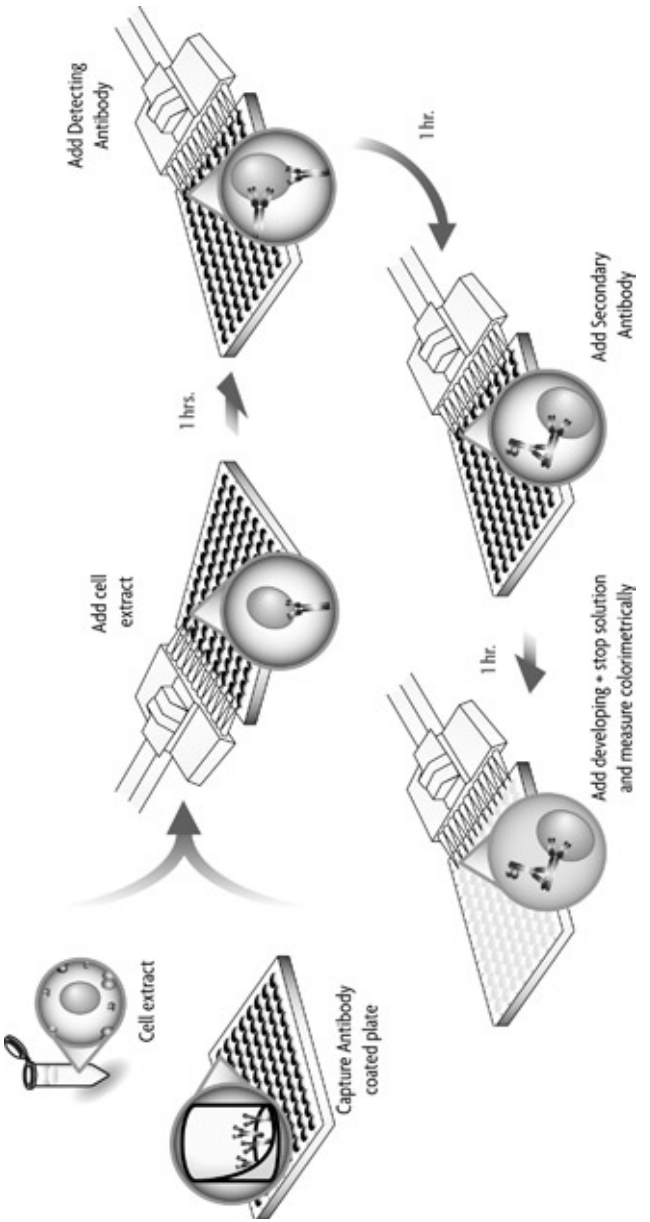
Progesterone Receptor (PR) belongs to the nuclear receptor (NR) superfamily of structurally related ligand-inducible transcription factors[1]. NRs act in combination with other transcription factors to regulate the expression of gene networks involved in cell growth and development, apoptosis, homeostasis, inflammation, lipid metabolism, the reproductive cycle and other fundamental biological processes. Quantitative detection of PR levels is very important for predicting prognosis and evaluating the outcome of endocrine therapy in breast and uterine cancer patients. Because of PR's critical role in cell biology, it is important to measure the total amounts of PR contained in different cell types and tissues. Traditional methods for monitoring PR protein levels, such as Western blotting, EMSA, immunohistochemistry (IHC) and reporter gene assays, are time consuming and not suitable to high-throughput applications.

With its NR Sandwich ELISAs, Active Motif is introducing the first ELISA-based kits to detect total amounts of nuclear receptor proteins. The NR Sandwich PR Kit simplifies the measurement of PR contained in cell and tissue samples by using the "Sandwich ELISA" method for detecting a protein. This method uses two antibodies that each recognize a distinct epitope on the protein of interest. The kit provides an ELISA plate that is coated with the first antibody, called the Capture Antibody, which is used to capture the protein from the sample. The second antibody, called the Detecting Antibody, is used to detect the protein bound by the Capture Antibody. An HRP-conjugated Secondary Antibody is then used to quantitate the amount of bound Detecting Antibody. Subsequent incubation with developing solution provides an easily quantified colorimetric readout.

product	format	catalog no.
NR Sandwich PR	1 x 96-well plate	49396
	5 x 96 well plates	49896

See Active Motif products related to PR in Appendix, Section B.

Flow Chart of Process



Progesterone Receptor

Progesterone is an essential regulator of the reproductive events associated with the establishment and maintenance of pregnancy, including ovulation and uterine and mammary gland development[2]. Progesterone exerts its effects via the intracellular progesterone receptor (PR), which belongs to the nuclear receptor (NR) superfamily of ligand-regulated transcription factors[3]. Like other members of the family, PR consists of an N-terminal modulating domain, a central DNA binding domain, a hinge region and a C-terminal ligand-binding domain[4].

PR mainly acts through two isoforms, A and B, each with unique cellular effects[5]. The difference between the two isoforms is that 164 amino acids of the N-terminal of PR B are absent in PR A. Both PR isoforms are derived from a single gene but are generated from either alternative transcriptional or translational start sites, and are regulated by different estrogen-induced promoters[6, 7]. The B form of PR acts as a transcriptional activator in different cellular contexts whereas the A form functions as a strong inhibitor of transcriptional activity.

Absence of PR expression in primary breast cancer is associated with disease progression and may be a marker of an aggressive tumor phenotype[8]. PR protein level expression in breast cancer has been found to be inversely correlated to tumor size and histological grade[9]. For example, cells that possess PRs usually respond to hormonal therapy and are thought to depend on progesterone for growth. In contrast, breast cancer cells that do not possess PRs usually do not respond to hormonal therapy and do not require progesterone to grow. Estimation of PR levels in patient samples is routine in the management of patients with breast cancer, where it is used as a predictive factor for response to therapeutic hormonal therapy. A significant proportion of breast cancer tumors have been shown to express very low levels of the B form of PR[10], thus indicating that the PR isoform expressed directly influences the tumor phenotype[11].

PR is one of the most widely documented prognostic and predictive factors in endometrial cancer, the most common malignancy of the female genital tract. PR expression in endometrial and ovarian carcinomas has been associated with increased survival[12, 13]. Specifically, high PR B levels correlated significantly with survival of endometrial cancer patients. Progesterone acts principally through PR B to inhibit cancer cell invasiveness modulated by adhesion molecules, and loss of PR B expression has been linked to the development of a lethal form of cancer[5]. Therefore, the measurement of PR isoforms may have prognostic value[14].

Traditional Nuclear Receptor Assays

To date, several methods are widely used to measure PR expression, either directly or indirectly:

1. Cellular levels of PR protein can be determined by Western blot by using antibodies specific for PR 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 PR 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 PR binding. If PR 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. Immunohistochemistry is also commonly performed to analyze the PR content of tissue samples. Although this method is highly sensitive and can reveal the subcellular distribution of PR protein, it is technically demanding, requires specialized equipment, and is not suitable for the analysis of a large number of samples

4. Another method used to assay PR activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing an PR consensus binding site. The promoter can be artificial, made of a GC box and a TATA box, 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; therefore, assays have to be carefully standardized.

NR Sandwich PR ELISAs

PR plays a key role in cancer development and progression. Quantitative detection of PR levels is very important for predicting prognosis and evaluating the outcome of endocrine therapy of breast and uterine cancer patients. However, there is a lack of standardized assays that measure cellular levels of PR.

To overcome this, Active Motif has introduced the NR Sandwich PR to monitor the expression levels of PR in cell and tissue samples. The NR Sandwich PR ELISA Kit uses the "Sandwich ELISA" method for detecting a protein. This method uses two antibodies that each recognize a distinct epitope on the protein of interest. The kit provides an ELISA plate that is coated with the first antibody, called the Capture Antibody, which is used to capture the protein from the sample. The second antibody, called the Detecting Antibody, is used to detect the protein bound by the Capture Antibody. An HRP-conjugated Secondary Antibody is then used to quantitate the amount of bound Detecting Antibody. Subsequent incubation with developing solution provides an easily quantified colorimetric readout. Once the samples are prepared, this assay is completed in less than 4 hours. As this assay is performed in 96-well plates, a large number of samples can be handled simultaneously, enabling high-throughput automation.

The NR Sandwich PR has many applications including the study of PR transcriptional activity regulation and protein structure/function studies of PR in cancer development and progression.

Kit Performance and Benefits

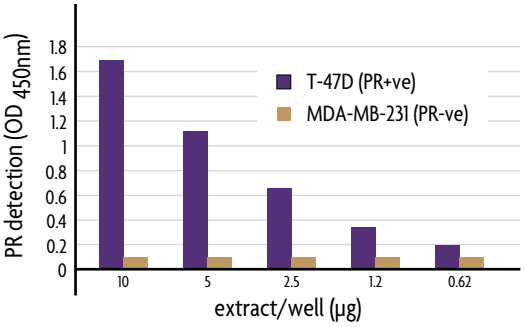
The NR Sandwich PR Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: > 0.6 µg nuclear extract/well.

Range of detection: NR Sandwich PR provides quantitative results from 0.625 to 10 µg of nuclear extract/well (see graph below).

Cross-reactivity: NR Sandwich PR detects both the A and B forms of PR in samples from human origin.

Assay time: < 4 hours.



Monitoring protein expression levels of PR using NR Sandwich. Different amounts of nuclear extracts from two different breast cancer cell lines: T-47D and MDA-MB-231 were analyzed for levels of total PR protein using the NR Sandwich PR Kit. This data is provided for demonstration purposes only.

Kit Components and Storage

Except for the nuclear extract that must be kept at -80°C, 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.

Reagents	Quantity (1 plate / 5 plates)	Storage / Stability
PR detecting antibody	52 µl / 260 µl	4°C for 6 months
HRP-conjugated antibody	6 µl / 30 µl (0.2 µg/µl)	4°C for 6 months
T-47D nuclear extract	40 µl / 200 µl (5 µg/µl)	-80°C for 6 months
Diluent Buffer	22 ml / 110 ml	-20°C for 6 months
10X Wash Buffer AM1	22 ml / 110 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 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)
- Lysis Buffer

Buffer Preparation and Recommendations

Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 10 ml of 1X Wash Buffer required, dilute 1 ml 10X Wash Buffer AM1 with 9 ml distilled water (see the Quick Chart for Preparing Buffers and Controls 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 AM1 may form clumps, therefore homogenize the buffer by vortexing for 2 minutes prior to use.

Preparation of the Antibody Binding Buffers

Dilute the PR detecting antibody to 1:100 and HRP-conjugated secondary antibody to 1:1000 with the Diluent Buffer (see the Quick Chart in this section). Use 50 µl of diluted antibody per well. 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.

Developing Solution

The Developing Solution must be warmed to room temperature before use. This 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 solution indicates that it has been contaminated and must be discarded. Prior to use, transfer the amount of Developing Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section), avoid direct exposure to intense light and leave at room temperature for at least 1 hour. After use, discard any remaining solution that was transferred into the secondary container.

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 and Controls 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 T-47D nuclear extract is provided as a positive control to ensure that the kit reagents are functional. Sufficient extract is provided for 40 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 -80°C. Avoid multiple freeze/thaw cycles of the extract. Various cell extracts are available from Active Motif (see Appendix, Section B. Related Products).

Quick Chart for Preparing Buffers and Controls

Reagents to Prepare	Components	For 1 well	For 1 strip (8 wells)	For 6 strips (48 wells)	For 12 strips (96 wells)
1X Wash Buffer	Distilled water	2.025 ml	16.2 ml	93.6 ml	187.2 ml
	10X Wash Buffer AM1	225.0 µl	1.8 ml	10.4 ml	20.8 ml
	TOTAL REQUIRED	**2.25 ml**	**18.0 ml**	**104.0 ml**	**208.0 ml**
PR Detecting Antibody	PR antibody	0.55 µl	4.5 µl	26 µl	52 µl
	Diluent Buffer	55.0 µl	450.0 µl	2.574 ml	5.15 ml
	TOTAL REQUIRED	**55.55 µl**	**454.5 µl**	**2.60 ml**	**5.2 ml**
Secondary Antibody	HRP-conjugated antibody	0.05 µl	0.45 µl	2.6 µl	5.2 µl
	Diluent Buffer	55.0 µl	450 µl	2.6 ml	5.2 ml
	TOTAL REQUIRED	**55.05 µl**	**450.45 µl**	**2.6 ml**	**5.2 ml**
Developing Solution	**TOTAL REQUIRED**	**110 µl**	**900 µl**	**5.2 ml**	**10.4 ml**
Stop Solution	**TOTAL REQUIRED**	**110 µl**	**900 µl**	**5.2 ml**	**10.4 ml**

* The above volumes include an excess of components

NR Sandwich PR 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 are to be used, cover the unused wells with a portion of the plate sealer while the assay is performed. The content of these wells is stable at room temperature if kept dry. Store the unused strips in the aluminum pouch at 4°C. Use the strip holder for the assay.

Multi-channel pipettor reservoirs may be used for dispensing the 1X Wash Buffer, Antibody Binding Buffers and Developing and Stop Solution into the wells being used.

Step 1: Binding of PR to the capture antibody

1. Please refer to the Buffer Preparation and Recommendation section before starting the protocol.

   Sample wells: Add 50 µl of sample diluted in Diluent Buffer to each well to be used. We recommend using 5 to 50 µg of nuclear extract diluted in Diluent Buffer per well. A protocol for preparing nuclear extracts can be found on page 10.

   Control wells: Add 5 µg of the provided T-47D nuclear extract diluted in 50 µl of Diluent Buffer to each well to be used (1 µl of extract in 49 µl of Diluent Buffer per well) .

   Blank wells: Add 50 µl Diluent Buffer only per well.

2. 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).

3. Wash each well 3 times with 200 µl 1X Wash Buffer. For each wash, flip 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 50 µl diluted PR antibody (1:100 dilution in Diluent Buffer) to all wells being used.

2. Cover the plate and incubate for 1 hour at room temperature with gentle rocking.

3. Wash the wells 3 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 3).

Step 3: Binding of secondary antibody

1. Add 50 µl of diluted HRP-conjugated antibody (1:1000 dilution in Diluent Buffer) to all wells being used.

2. Cover the plate and incubate for 1 hour at room temperature with gentle rocking.

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. 3).

Step 4: Colorimetric detection

1. Transfer the amount of Developing Solution required for the assay into a secondary container. Add 100 µl Developing Solution to all wells being used.

2. Incubate 2-10 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 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) which can be used for preparing nuclear, cytoplasmic and whole-cell extract. 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. Discard the supernatant (which contains the cytoplasm and RNA) carefully without disturbing the pellet. 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 beta-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.

Lysis Buffer	**For 50 ml, mix:**
20 mM Hepes, pH 7.5	0.24 g
400 mM NaCl	1.17 g
0.1 mM EDTA	1.5 mg
10 mM NaF	21 mg
10 µM Na_2MoO_4	0.12 mg
1 mM $NaVO_3$	6.1 mg
20% glycerol	10 ml
10 mM PNPP	0.23 g
10 mM beta-glycerophosphate	0.11 g

Adjust pH to 7.5 with 1 N NaOH. Adjust volume to 50 ml with distilled water. Store at 4°C. Just before use, make up Complete Lysis Buffer by adding 1 µl of 1 M DTT and 10 µl of Protease Inhibitor Cocktail (Sigma, Cat. No. P8340) per ml of Lysis Buffer.

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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	Make sure that correct volume is delivered by pipette
	Developing time too short	Increase the development time up to 30 minutes
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 sample per well	Decrease amount of sample
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1:200 for primary antibody. The sensitivity of the assay will be decreased
No signal or weak signal in sample wells	Not enough PR in the sample added per well	Increase amount of sample
	PR is poorly expressed or inactivated in samples	Check PR expression in the studied sample
	Samples are not from correct origin	Refer to cross-reactivity information on page 5

Section B. Related Products

Nuclear Receptor ELISAs	Unit	Catalog No.
NR Peptide ERα	1 x 96 rxns	49096
	5 x 96 rxns	49596
NR Peptide ERα Chemi	1 x 96 rxns	49097
	5 x 96 rxns	49597
NR Sandwich ERα	1 x 96 rxns	49296
	5 x 96 rxns	49796
NR Sandwich AR	1 x 96 rxns	49196
	5 x 96 rxns	49696
TransAM Kits		
TransAM™ AP-1 Family	2 x 96 rxns	44296
TransAM™ AP-1 c-Fos	1 x 96 rxns	44096
	5 x 96 rxns	44596
TransAM™ AP-1 c-Jun	1 x 96 rxns	46096
	5 x 96 rxns	46596
TransAM™ AP-1 FosB	1 x 96 rxns	45096
	5 x 96 rxns	45596
TransAM™ AP-1 JunD	1 x 96 rxns	43496
	5 x 96 rxns	43996
TransAM™ ATF-2	1 x 96 rxns	42396
	5 x 96 rxns	42896
TransAM™ c-Myc	1 x 96 rxns	43396
	5 x 96 rxns	43896
TransAM™ C/EBP α/β	1 x 96 rxns	44196
	5 x 96 rxns	44696
TransAM™ CREB	1 x 96 rxns	42096
	5 x 96 rxns	42596
TransAM™ pCREB	1 x 96 rxns	43096
	5 x 96 rxns	43596
TransAM™ Elk-1	1 x 96 rxns	44396
	5 x 96 rxns	44896
TransAM™ ER	1 x 96 rxns	41396
	5 x 96 rxns	41996
TransAM™ GR	1 x 96 rxns	45496
	5 x 96 rxns	45996
TransAM™ PPARβ(δ)	1 x 96 rxns	47496
	5 x 96 rxns	47996
TransAM™ PPARγ	1 x 96 rxns	40196
	5 x 96 rxns	40696
TransAM™ Sp1	1 x 96 rxns	41296
	5 x 96 rxns	41796
TransAM™ Sp1/Sp3	1 x 96 rxns	40496
	5 x 96 rxns	40996
Extracts & Proteins		
Nuclear Extract Kit	100 rxns	40010
	400 rxns	40410
Mitochondrial Fractionation Kit	100 rxns	40015
T-47D nuclear extract	200 µg	36027
MDA-MB-231 nuclear extract	200 µg	36080

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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