RayBio® Human ER-alpha Transcription Factor Activity Assay Kit

Catalog #: TFEH-ERa

User Manual
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RayBio® Human ER-alpha TF Activity Assay Kit Protocol

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Please read the entire manual carefully before starting your experiment.

I. INTRODUCTION

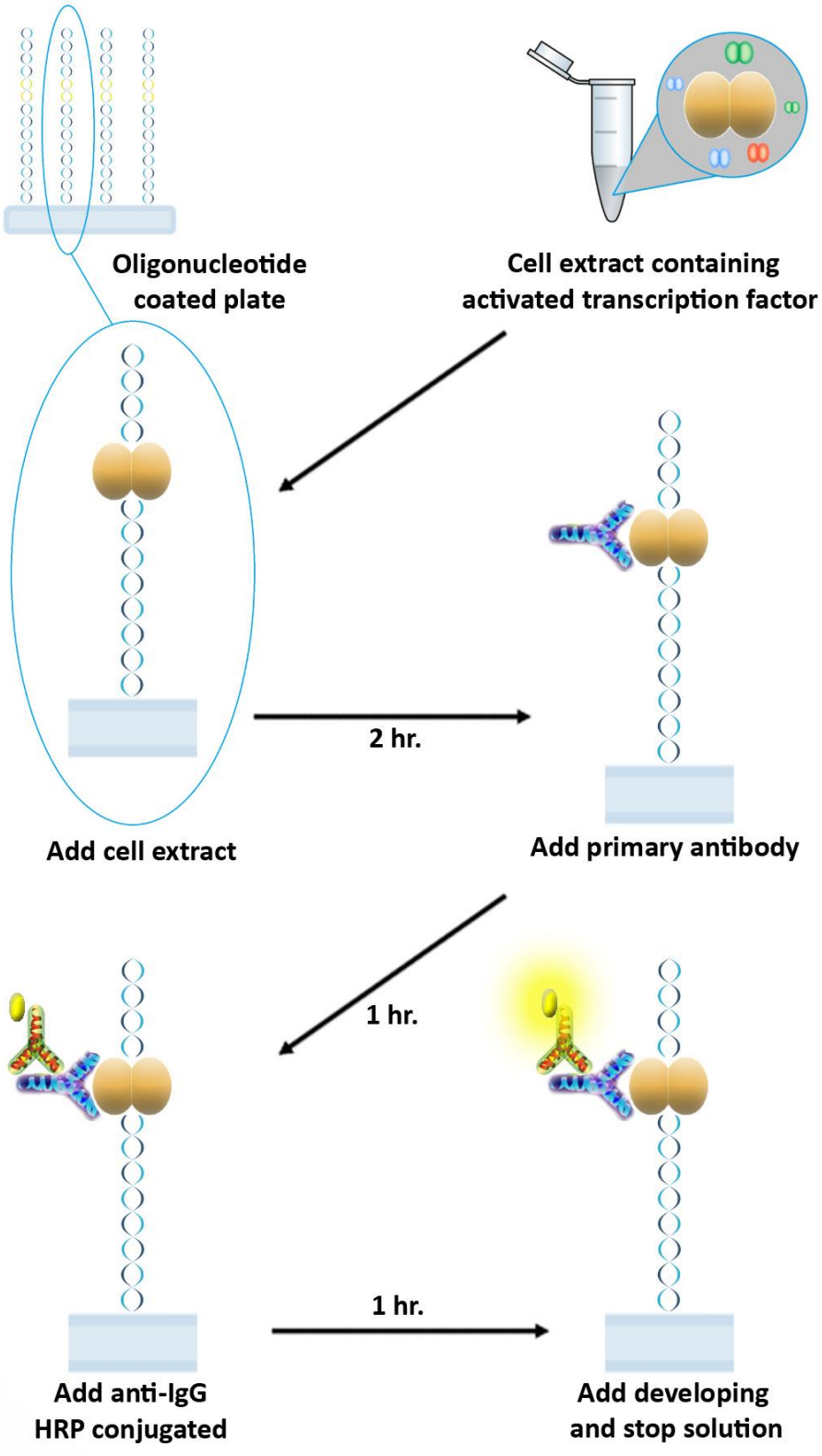
The estrogen receptor (ER) is a ligand-activated enhancer protein that belongs to the steroid/nuclear receptor superfamily and functions as a signal transducer and transcription factor to modulate expression of target genes. Mammalian ER is encoded by two genes: alpha and beta (ER-alpha and ER-beta) which have three major functional domains including an N-terminus that modulates transcription in a gene- and cell-specific manner through Activation Function-1, a highly conserved central DBD through which ER interacts directly with the DNA helix, and the LBD that contains Activation Function-2. In human physiology, ERs not only play a central role in the control of sexual behavior and reproductive functions, but are also widely involved in the differentiation of several tissues and organs, the modulation of inflammation, and brain and cardiovascular functions. In response to ligand binding of 17beta-estradiol (E2) or other agonists, ER changes conformation and dissociates from hsp90, hsp70 and other proteins, so that ligand-occupied ER dimer has been formed to regulate target genes. Activated ER stimulates target gene expression by two mechanisms: genomic and non-genomic (non-genotropic) pathways. In the genomic pathway, ligand-occupied ER directly binds to a specific sequence in the promoters of target genes (specifically, the estrogen response element or ERE), resulting in enhanced transcription. In the non-genomic pathway, ER can indirectly associate with promoters through protein-protein interactions with other DNA-binding transcription factors. For example, ER-alpha interacts with Sp1 in response to estrogen stimulation to regulate the transcription of RAR-alpha, insulin-like growth factor-binding protein-4, transforming growth factor α, LDL receptor genes, and others.

Accurate monitoring of the level of activated ER-alpha in cells, tissues or animal models is required for both science research investigating signal transduction pathways and applications such as drug development, and simple, speedy and high-throughput methods are needed for this purpose.

Traditionally, electrophoretic mobility shift assays (EMSA) to detect the DNA binding capacity of ER-alpha, or transfection of reporter genes such as luciferase and beta-galactosidase with ER-alpha binding sites in culture cells are used in evaluation of ER reactivity. However, these methods are time consuming, laborious, and sometimes require the use of radioactivity.

The RayBio® ER-alpha TF Activity Assay kit is a non-radioactive transcription factor assay with an ELISA format. It offers an easy, speedy, sensitive and high-throughput method to detect the activation of transcription factors. In 96 well plates, double stranded oligonucleotides containing ER-alpha binding sequence have been coated. These oligonucleotides specifically capture the active ER-alpha contained in whole cell lysate or nuclear extracts after a short incubation. Subsequently, the primary antibody against ER-alpha recognizes the ER-alpha-DNA complex in each well, and a HRP-conjugated secondary antibody is then used for detection. After washing away any unbound antibody, signal can be obtained easily through a colorimetric assay with a spectrophotometric plate reader at 450 nm. The specificity of the reaction between active ER-alpha and the DNA probe is additionally stringent because of the establishment of specific competitive DNA and non-specific competitive DNA probes in this reaction system.

II. HOW IT WORKS



III. STORAGE

Upon receipt, the positive control should be stored at -20°C for short-term storage or -80°C for long-term storage. Opened Microplate Wells or reagents may be stored for up to 1 month at 2-8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge. The remainder of the kit can be stored for up to 6 months at 2-8°C from the date of shipment.

Note: The kit can be used within one year if the entire kit is stored at -20°C upon receipt. Avoid repeated freeze-thaw cycles.

IV. REAGENTS

Component	Description	Size
ER-alpha DNA Probe Microplate	96 wells (12 strips X 8 wells) coated with ER-alpha probes	1 plate
DNA Binding Buffer	5X concentrated Buffer	4 ml
Positive Control	Cell nuclear extracts	1 vial (20 µl)
Specific Competitor DNA Probe	Free DNA probes that compete with the coated probes by binding with activated ER-alpha.	1 vial
Non-specific Competitor DNA Probe	Free DNA probes with mutations of the coated DNA probe. Cannot bind activated ER-alpha.	1 vial
Assay Reagent	1X solution	1 vial (200 µl)
DTT	300 mM DTT	1 vial (200 µl)
Wash Buffer Concentrate (20X)	20X concentrated solution	25 ml
ER-alpha Primary Antibody	Anti- ER-alpha antibody	1 vial
HRP-conjugated Secondary Antibody	Anti-IgG HRP conjugated antibody	1 vial
Antibody Diluent Buffer	Buffer solution for diluting primary and secondary antibodies	25 ml
TMB One-Step Substrate Reagent	3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution	12 ml
Stop Solution	0.2 M sulfuric acid	8 ml

V. ADDITIONAL MATERIALS REQUIRED

1) Microplate reader capable of measuring absorbance at 450 nm.
2) Precision pipettes to deliver 1 μl to 1 ml volumes.
3) Adjustable 1-25 ml pipettes for reagent preparation.
4) 100 ml and 1 liter graduated cylinders.
5) Absorbent paper.
6) Distilled or deionized water.
7) Tubes to prepare positive or sample mixtures.

VI. REAGENT PREPARATION

1. Preparation of samples:

   Prepare nuclear extraction or whole lysate containing targeted protein ER-alpha from cell culture or tissue.

   We recommend using the RayBiotech Nuclear Extraction Kit (Cat#: NE-50) to isolate nuclear proteins for subsequent use in this transcription factor assay.

2. Preparation of transcription factor reaction solutions:

   Thaw the positive control and samples and keep them on ice before adding into wells. Bring all other reagents to room temperature (18 – 25°C) before use. Spin down vials to make sure contents are settled before pipetting.

   Prepare 100 μl transcription factor binding reaction solution for each well with the following: 5x DNA Binding Buffer, Assay Reagent, DTT, Specific Competitor DNA Probe, Non-Specific Competitor DNA Probe, and either Positive Control or samples containing targeted proteins. Fill remaining volume to 100uL with deionized water. Typical examples are shown in the table below.

	REACTION				
COMPONENT	Positive control	Sample	Specific competitor	Non-Specific competitor	Blank
5x DNA Binding Buffer	20 μl	20 μl	20 μl	20 μl	20 μl
Assay Reagent	1.5 μl	1.5 μl	1.5 μl	1.5 μl	1.5 μl
DTT	1 μl	1 μl	1 μl	1 μl	1 μl
Specific Competitor	-	-	10 μl	-	-
Non-specific Competitor	-	-	-	10 μl	-
Positive Control/Sample containing proteins	5 μl	* μl	* μl	* μl	-
Total volume	bring final volume to **100μl** with deionized water	bring final volume to **100μl** with deionized water	bring final volume to **100μl** with deionized water	bring final volume to **100μl** with deionized water	bring final volume to **100μl** with deionized water

Note:

Each reaction may be prepared in a labeled microcentrifuge tube or directly in the well of the coated microplate. If the reaction solution is prepared in a tube, add an additional 1-2 μL of deionized water to account for pipetting error when transferring the solution into the well. Once positive control/sample is added, ensure the tube is kept on ice to maintain protein stability. If the reaction solution is prepared directly in the coated plate wells, please add the reagents sequentially as shown in the table to get the best results. Scale volumes accordingly depending on the number of replicates used.

**Please note that the amount of sample containing the target protein used in this test can be optimized and must be determined by the investigator. To observe the specificity of the DNA binding activity, the amount of protein used in wells containing the sample, specific competitor, and non-specific competitor must be the same.*

A positive control should be included every time to confirm correct operation of the experiment, however it is not necessary to run the specific competitor and non-specific competitor for each sample every time.

3. Preparation of primary antibody:

   Once thawed, briefly spin down the ER-alpha Primary Antibody vial. Add 100 μl of Antibody Diluent Buffer into the vial to prepare a primary antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The primary antibody concentrate should then be diluted 100-fold with the Antibody Diluent Buffer and used in step 4 of Part VII Assay Procedure.

4. Preparation of secondary antibody:

   Once thawed, briefly spin down the HRP-conjugated Secondary Antibody vial before use. Add 100 μl of Antibody Diluent Buffer into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should then be diluted 100-fold with the Antibody Diluent Buffer and used in step 6 of Part VII Assay Procedure.

5. Preparation of 1x Wash Buffer:

   Dilute 25 ml of the 20x Wash Buffer Concentrate into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If the Wash Buffer Concentrate (20x) contains visible crystals, warm to room temperature and mix gently until dissolved.

VII. ASSAY PROCEDURE:

1. Thaw positive control and/or samples containing the target protein on ice. Bring the 96-well plate and other kit components to room temperature (18 - 25°C) before use. If the whole plate will not be used in this assay, place remaining wells back to 2-8°C, or -20°C for long-term storage. It is recommended that all positive control and samples be run in at least duplicate.

2. Add 100 μl of each prepared transcription factor binding reaction solution (see Reagent Preparation step 2) that includes positive control, Specific Competitor, Non-specific Competitor and sample into appropriate wells. Cover wells and incubate for 2 hours at room temperature or overnight at 4°C with gentle shaking.

3. Discard the solution and wash 4 times by filling each well with 300 μl of 1x Wash Buffer (Reagent Preparation step 5) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 μl of diluted ER-alpha Primary Antibody (Reagent Preparation step 3) to each well. Incubate for 1 hour at room temperature with gentle shaking.

5. Discard the solution. Repeat the wash as in step 3.

6. Add 100 μl of diluted HRP-conjugated Secondary Antibody (see Reagent Preparation step 4) to each well. Incubate for 1 hour at room temperature with gentle shaking.

7. Discard the solution. Wash as directed in step 3.

8. Add 100 μl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

9. Add 50 μl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

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2. Add 100 μl sample to each well.
   Incubate 2 hours at room temperature or overnight at 4°C.

⇩

3. Add 100 μl prepared primary antibody to each well.
   Incubate 1 hour at room temperature.

⇩

4. Add 100 μl prepared secondary antibody.
   Incubate 1 hour at room temperature.

⇩

5. Add 100 μl TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.

⇩

6. Add 50 μl Stop Solution to each well.
   Read at 450 nm immediately.

VIII. TYPICAL DATA

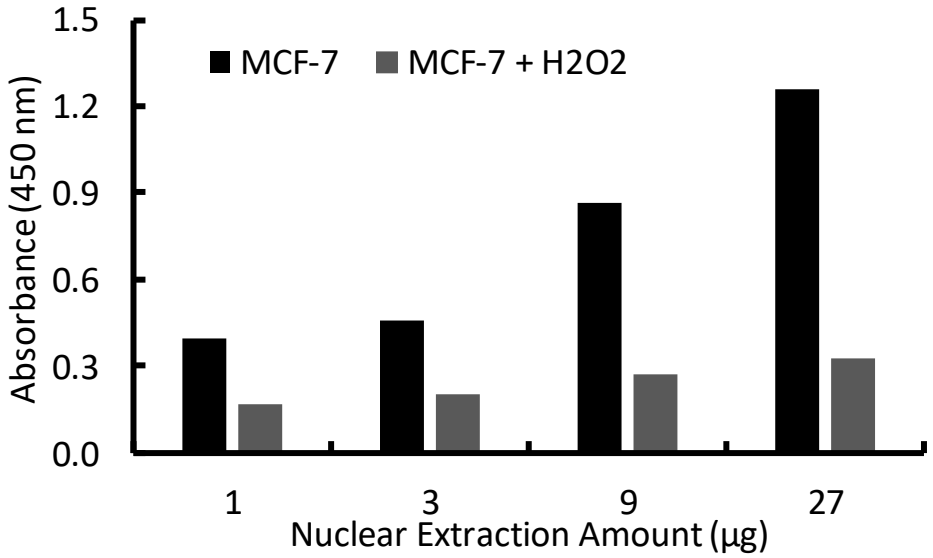

Figure 1: Transcription factor assay of ER-alpha from nuclear extracts of MCF-7 cells or MCF-7 cells treated with H_2O_2 (200µM) for 3 hr with RayBio Activity Assay Kit (cat # TFEH- ERa).

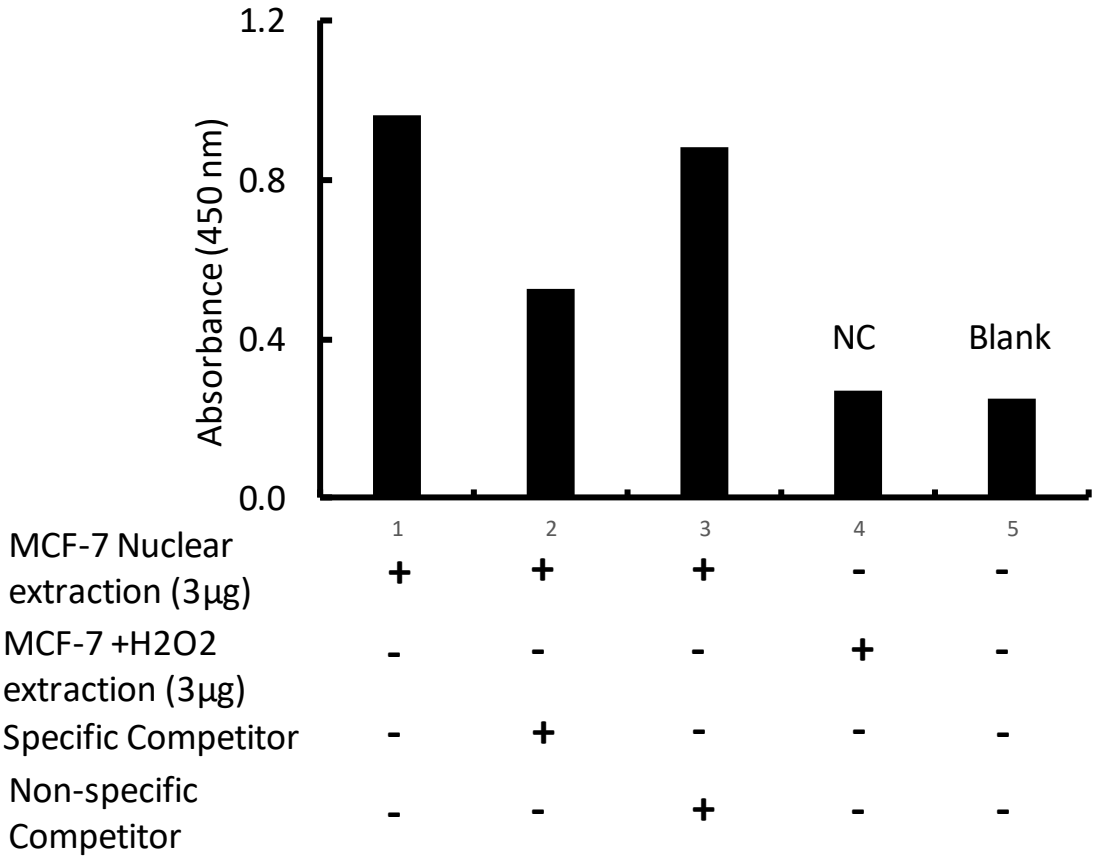

Fig. 2: Transcription factor assay of ER-alpha from nuclear extracts of MCF-7 cells or MCF-7 cells treated with H_2O_2 (200µM) for 3 hr with the specific competitor or non-specific competitor. The result shows specific binding of ER-alpha to the conserve binding site detected by using RayBio ER-alpha TF Activity assay Kit (cat # TFEH- ERa).

TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Low signal	1.Too brief incubation times	1. Ensure sufficient incubation time; assay procedure step 2 change to overnight
	2. Missed key reagent, inadequate reagent volumes or improper dilution	2. Check all reagents have been added and check pipettes to ensure correct preparation
	3. Not enough targeted protein per well	3. Check positive control wells and increase the amount of sample.
	4. Inadequate development in colorimetric assay	4. Ensure correct developing buffer and enough time used
2. Large CV	1. Inaccurate pipetting	1. Check pipettes
	2. Wells cross contamination	2. Be careful when preparing samples between wells
3. High background	1. Plate is insufficiently washed	1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed
	2. Contaminated wash Buffer	2. Make fresh wash buffer
	3. Incorrect antibody dilution	3. Check antibody dilutions

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Choose TF Activity assay kits with more targets for human, mouse, rat and a variety of other species. Visit www.raybiotech.com for the complete list.

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