RayBio® Human NRF2 Transcription Factor Activity Assay Kit

Catalog #: TFEH-NRF2

User Manual
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RayBio® Human NRF2 TF-Activity Assay Kit Protocol

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Please read the entire manual carefully before starting your experiment.

I. INTRODUCTION

Cellular oxidative and electrophilic stress caused by drugs and other xenobiotics, inflammation, and ionizing radiation are associated with an accumulation of reactive oxygen species and electrophilic insults, which contribute to the pathogenesis of various diseases such as cancer, neurodegenerative disease, and atherosclerosis. In order to protect cells from reactive oxygen species and electrophilic insults, the endogenous cellular antioxidant defense system initiates a response to cellular oxidative and electrophilic stress. NRF2 (nuclear factor (erythroid-derived 2)-like 2; NFE2L2) is a key transcriptional factor regulating hundreds of antioxidant and Phase II detoxification genes. Under normal conditions, NRF2 is sequestered in the cytoplasm through binding with Keap1, an actin-binding protein and finally is degraded through the Keap1-dependent ubiquitination. In response to a stimulus, degradation of Keap1 is markedly increased. This leads to the disruption of the Keap1-NRF2 complex and nuclear translocation of NRF2. NRF2 then dimerizes with small Maf proteins and binds to the ARE (antioxidative response element) in promoters of downstream genes to initiate expression.

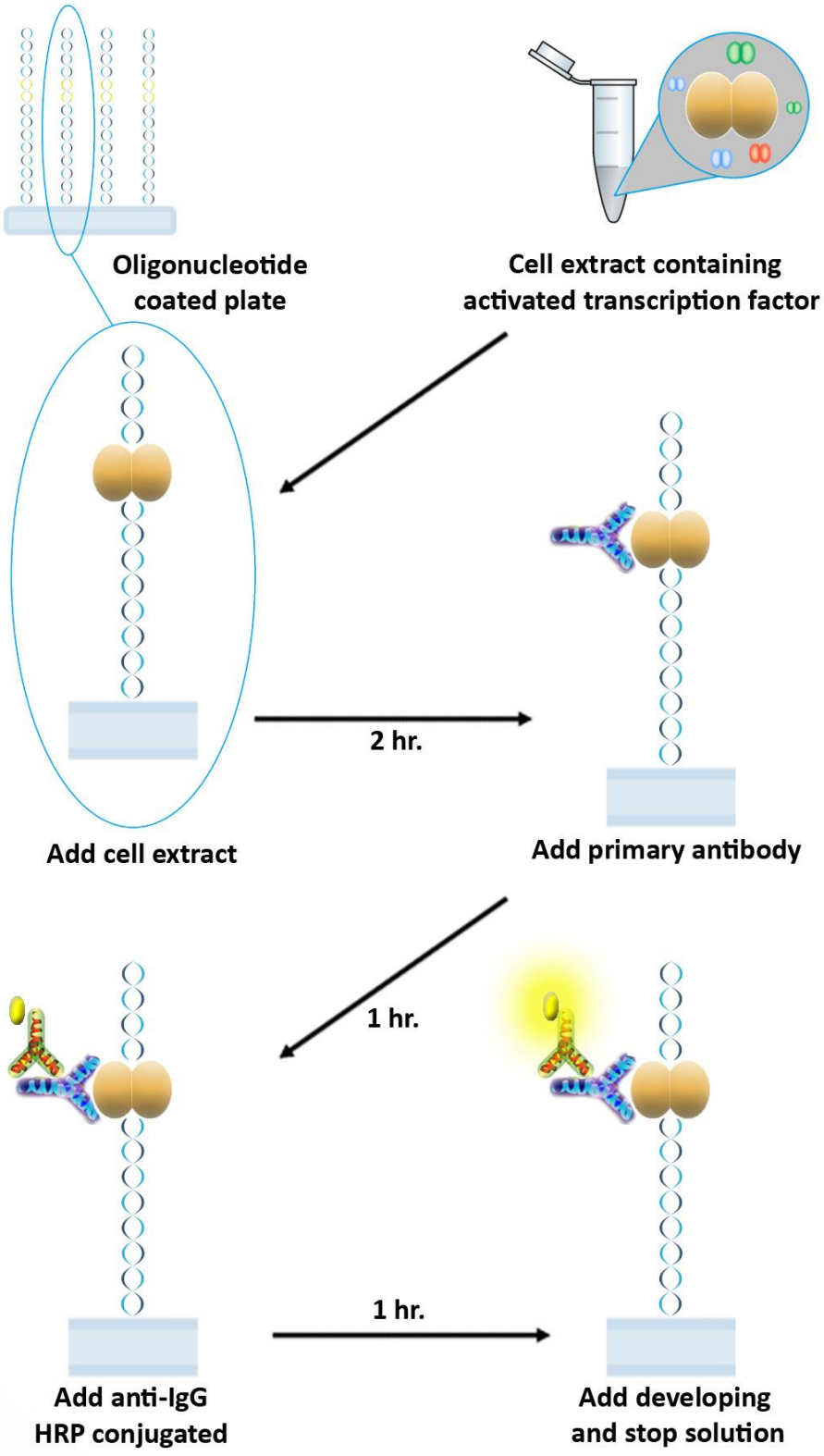
Accurate monitoring of the level of activated NRF2 in cells, tissues or animal models is required for both science research investigating signal transduction pathways and applications such as drug development. Simple, speedy and high-throughput methods are needed for these purposes.

Traditionally, western blots to detect the degradation of NRF2, electrophoretic mobility shift assays (EMSA) to detect the DNA binding capacity of NRF2, and transfection of reporter genes such as luciferase and β-galactosidase with NRF2 binding sites in culture cells have been used in evaluation of NRF2 reactivity. However, these methods are time consuming, laborious, and sometimes require the use of radioactivity.

The RayBio® NRF2 TF-Activity Assay (Transcription Factor-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 NRF2 binding sequence (5'-TGACTCAG-3') have been coated. These oligonucleotides specifically capture the active NRF2 contained in whole cell lysate or nuclear extracts after a short incubation. Subsequently, the primary antibody against NRF2 recognizes the NRF2-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 NRF2 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
NRF2 DNA Probe Microplate	96 wells (12 strips X 8 wells) coated with NRF2 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 NRF2.	1 vial
Non-specific Competitor DNA Probe	Free DNA probes with mutations of the coated DNA probe. Cannot bind activated NRF2.	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
NRF2 Primary Antibody	Anti-NRF2 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 NRF2 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 TF-Activity Assay DNA Binding Buffer	20 µl	20 µl	20 µl	20 µl	20 µl
TF-Activity Assay 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	-
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 TF-Activity Assay NRF2 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 TF-Activity Assay 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 TF-Activity Assay NRF2 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 TF-Activity Assay 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 controls as instructed.

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

⇩

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

⇩

4. Add 100 μl diluted 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.

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

VIII. TYPICAL DATA

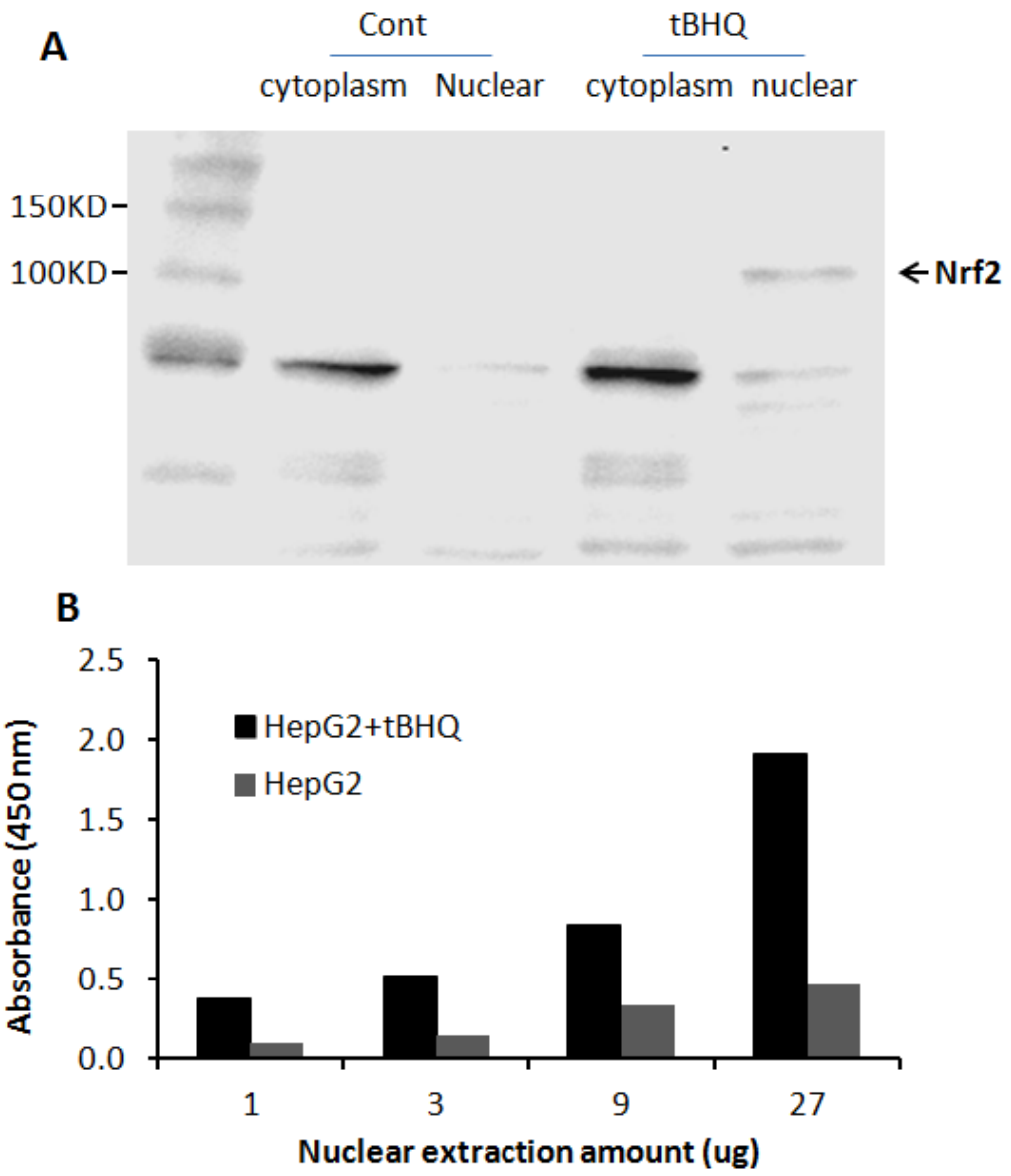

Figure 1: Transcription factor activity assay of NRF2 from nuclear extracts of HepG2 cells or HepG2 cells treated with tBHQ (90uM) for 24 hr. After stimulation activated NRF2 is translocated into the nucleus where it binds with its corresponding DNA. A. Western-blot result of NRF2 from cytoplasmic and nuclear fractions. B. Transcription factor activity assay of NRF2 from nuclear fractions with the RayBio® NRF2 TF-Activity Assay Kit (cat # TFEH- NRF2).

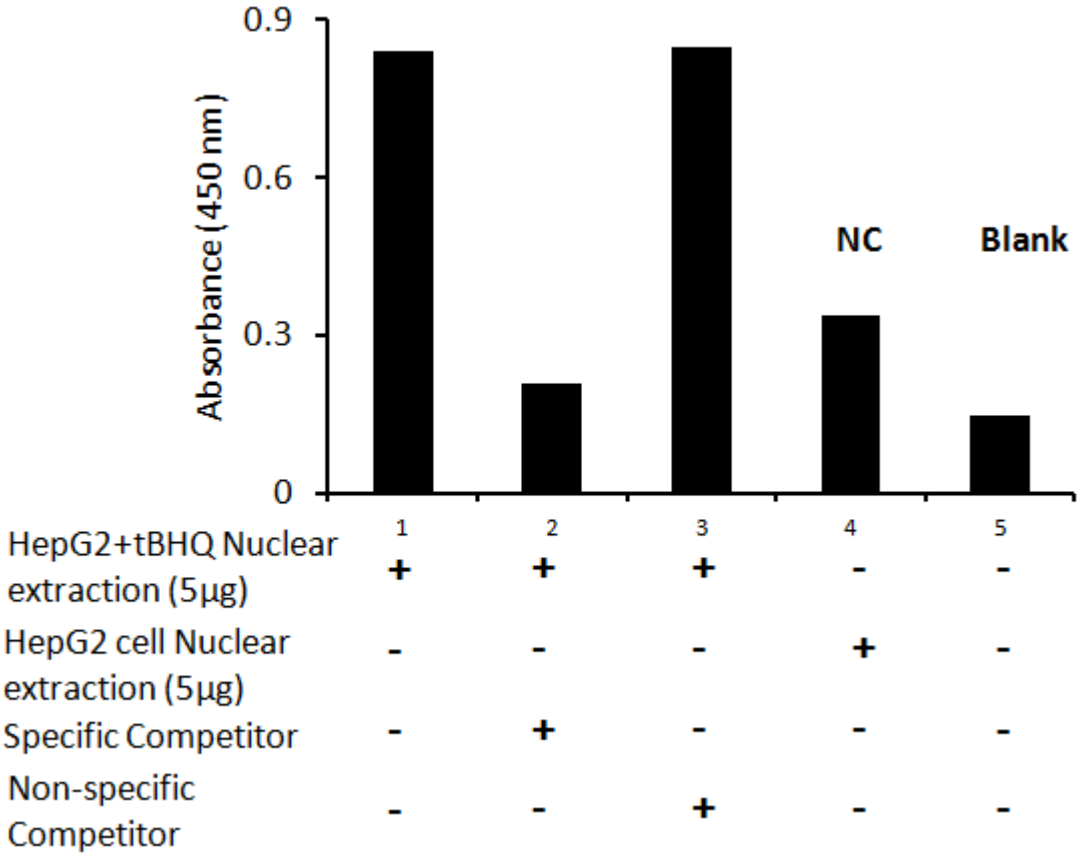

Fig. 2: Transcription factor activity assay of NRF2 from nuclear extracts of HepG2 cells or HepG2 cells treated with tBHQ (90uM) for 24 hr with the specific competitor or non-specific competitor. The result shows specific binding of NRF2 to the ARE binding site detected by using the RayBio® NRF2 TF-Activity Assay Kit (cat # TFEH- NRF2).

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

RayBio® TF-Activity Assay kits:

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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