RayBio® Human NF-κB RelB Transcription Factor Activity Assay Kit

Catalog #: TFEH-RELB

User Manual
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RayBiotech
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RayBio® Human NF-κB RelB TF Activity Assay Kit Protocol

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Please read the entire manual carefully before starting your experiment.

I. INTRODUCTION

The non-canonical NF-κB pathway is an important arm of NF-κB signaling that predominantly functions through activation of the p52/RelB NF-κB complex. This pathway regulates important biological functions, such as lymphoid organogenesis, B-cell survival and maturation, dendritic cell activation, and bone metabolism. Moreover, deregulated non-canonical NF-κB signaling is associated with lymphoid malignancies. In contrast to the canonical NF-κB pathway, in which NF-κB is inactive and retained in the cytoplasm by its inhibitor, IκB, and only transported to the nucleus through modification and degradation of IκB upon stimulation of various factors, the non-canonical pathway depends on the inducible processing of p100, that functions as both the precursor of p52 and a RelB-specific inhibitor. When cells are stimulated by of a subset of TNF receptor family members, the downstream kinase IκB kinase-α (IKKα) is activated and triggers p100 phosphorylation and processing, which generates RelB and results in RelB/RelB NF-κB complex translocation to the nucleus where it regulates the corresponding downstream genes. It is increasingly clear that this pathway of NF-κB activation differs significantly from the canonical NF-κB pathway in its signaling mechanisms. Therefore, better understanding of the mechanism regulating non-canonical NF-κB activation has important therapeutic value.

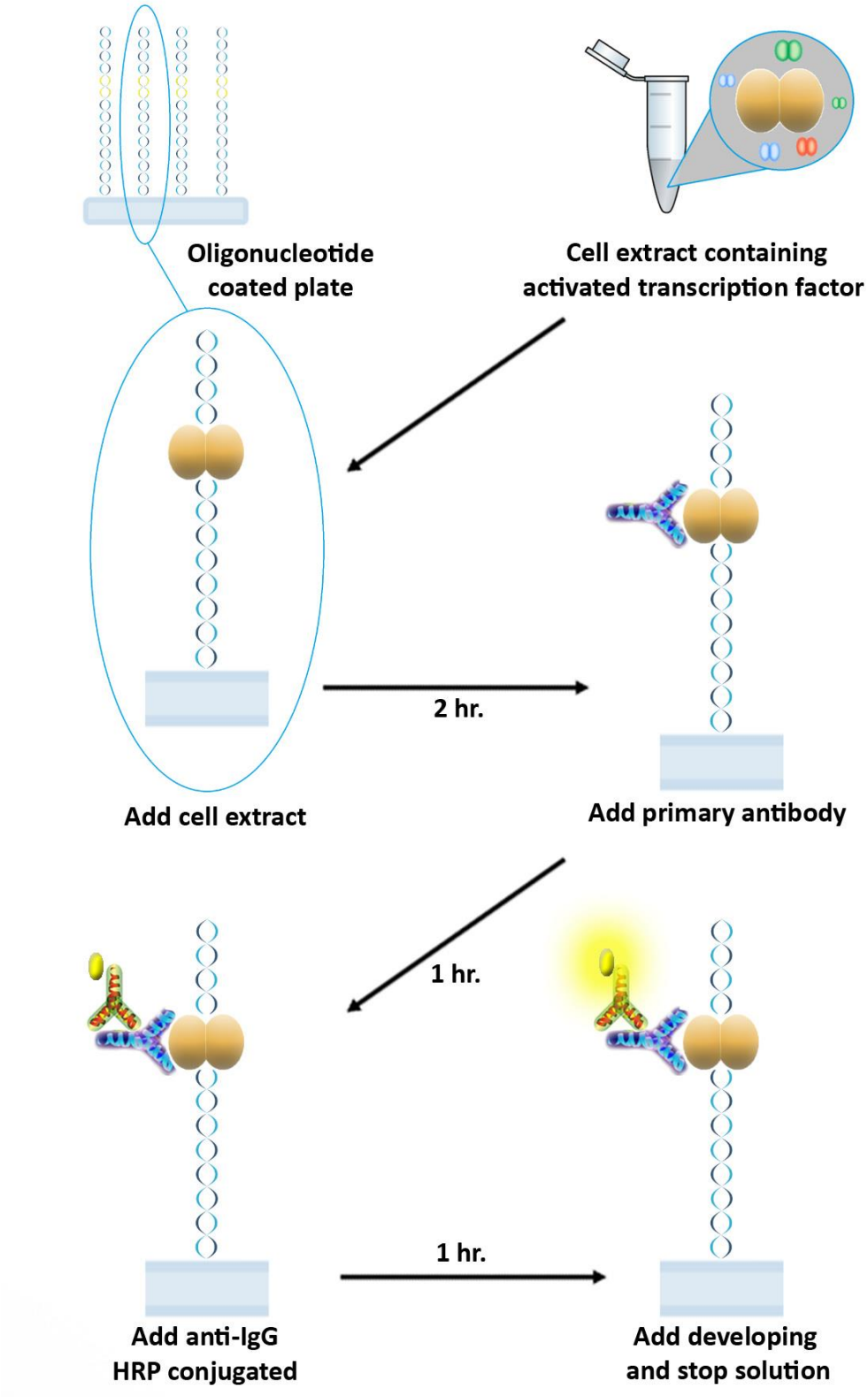
Accurate monitoring the level of activated p52 or RelB in cells, tissues or animal models is required for both basic science research investigating signal transduction pathways and applications such as drug development, and simple, quick and high-throughput methods are needed for this purpose.

Traditionally, western blot has been used to detect the phosphorylation of NF-κB or degradation of p100, electrophoretic mobility shift assay (EMSA) has been used to detect the DNA binding capacity of NF-κB and transfection of reporter genes such as luciferase and β-galactosidase with NF-κB binding sites in culture cells have been used in the evaluation of NF-κB reactivity. However,

these methods are time consuming, laborious, and sometimes require the use of radioactivity.

The RayBio® NF-κB RelB TF Activity Assay kit is a non-radioactive transcription factor assay with an ELISA format. It offers an easy, quick, sensitive and high-throughput method to detect the activation of transcription factors. Double-stranded oligonucleotides containing the NF-κB binding sequence have been coated on 96-well plates. These oligonucleotides specifically capture the active NF-κB RelB contained in whole cell lysate or nuclear extracts after a short incubation. Subsequently, the primary antibody against RelB recognizes the RelB-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 NF-κB RelB 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 removed and stored at -20° or -80°C. The remainder of the kit can be stored for up to 6 months at 2-8°C from the date of shipment. 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.
Note: The kit can be used within one year if the whole kit is stored at -20°C upon receipt. Avoid repeated freeze-thaw cycles.

IV. REAGENTS

Component	Description	Size
NF-κB RelB DNA Probe Microplate	96 wells (12 strips X 8 wells) coated with NF-κB 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 directly with the coated NF-κB probes. Can bind activated NF-κB.	1 vial
Non-specific Competitor DNA Probe	Free DNA probes with mutations of the coated DNA probe. Cannot bind activated NF-κB.	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
NF-κB RelB Primary Antibody	Anti-NF-κB RelB 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 NF-κB RelB 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 binding reaction system:
   Bring all reagents to room temperature (18 - 25°C) before use. Thaw the positive control and samples and keep them on ice before adding into wells. Prepare 100 μl transcription factor binding reaction system for each well with 5x DNA Binding Buffer, Assay Reagent, DTT, Specific Competitor DNA Probe, Non-specific Competitor DNA Probe, and Positive Control or samples containing targeted proteins. Typical examples are shown in the table below.

   Note:
   Each reaction may be prepared in a labeled microfuge tube or directly in the coated plate well. If the reaction system is prepared directly in the coated plate wells, please add the reagents sequentially as shown in the table to get the best results.

	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 DNA Probe	-	-	10 μl	-	-
Non-specific Competitor DNA Probe	-	-	-	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

* Please note that the amount of total protein containing the target protein to be used in this test can be optimized and must be determined by the investigator.

3. Preparation of primary antibody:
   Briefly spin down the NF-κB RelB 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:
   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.

   Note:
   All reagents containing protein (positive control, samples) should be kept on ice to maintain protein stability.

   If the reaction system is prepared directly in the coated plate wells, please add the reagents sequentially as shown in the table to get the best results.

   To observe the specificity of the DNA binding activity, the amount of protein used in wells of sample, specific competitor and non-specific competitor must be the same.

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

VII. ASSAY PROCEDURE:

1. Bring the 96-well plate 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 to 8°C or -20°C. It is recommended that all positive control and samples be run at least in duplicate.

2. Add 100 μl of each prepared transcription factor binding reaction system (see Reagent Preparation step 2) including 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 prepared NF-κB RelB Primary Antibody (Reagent Preparation step 3) to each well. Incubate for 1 hour at room temperature with gentle shaking.

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

6. Add 100 μl of prepared 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 preparation 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

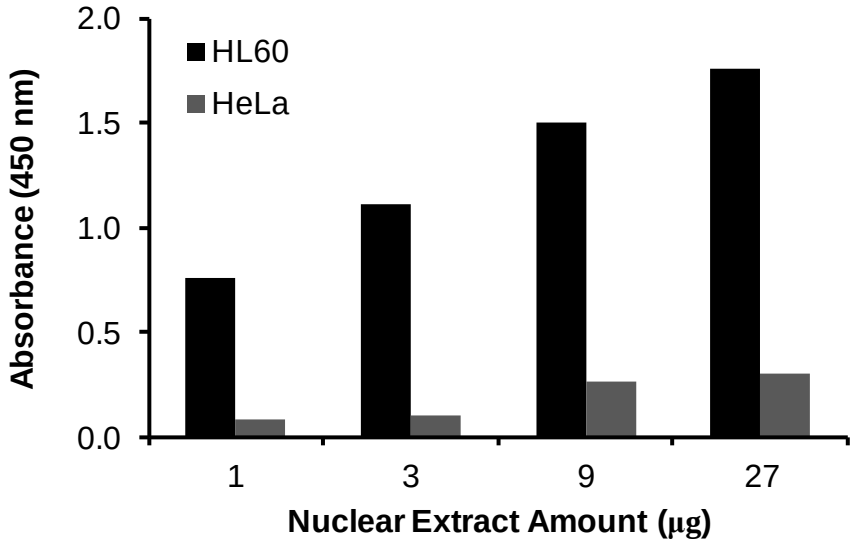

Figure 1: Transcription factor assay of NF-κB RelB from nuclear extracts of HL60 cells or HeLa cells with the RayBio® NF-κB RelB TF Activity Assay Kit (cat # TFEH-RELB).

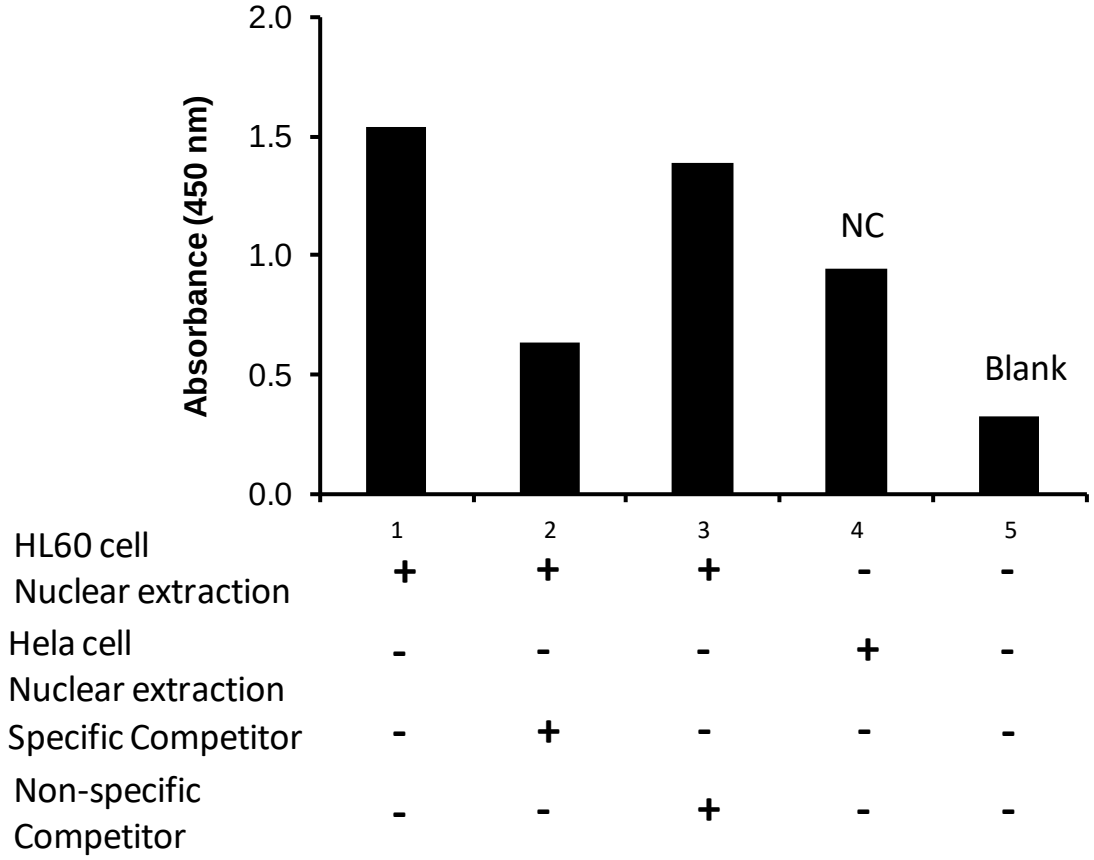

Fig. 2: Transcription factor assay of NF-κB RelB from nuclear extracts of HL60 cells or HeLa cells with the specific competitor or non-specific competitor. The result shows specific binding of NF-κB RelB to the NF-κB non-canonical DNA binding site.

TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Low signal	1.Too brief incubation times	1. Ensure sufficient incubation time; change incubation time in assay procedure step 2 to overnight
	2. Missed key reagent, inadequate reagent volumes or improper dilution	2. Check to ensure 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.

This product is for research use only