Phospho Sandwich

NF-κB p65 (Phospho-Ser276)

Colorimetric Sandwich ELISA Kit

Catalog #: PS-1631

Detection of NF-κB p65 (Phospho-Ser276) in Cell Supernatants

Store entire kit at 4°C until use.
Kit expiration is 3 months from date of shipment

Manual Version: 1.8.622

Research Purposes Only. Not Intended for Diagnostic or Clinical Procedures.

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INTRODUCTION

Phospho Sandwich ELISA Principles

The Phospho Sandwich NF-κB p65 (Phospho-Ser276) ELISA Kit contains the components necessary for semi-quantitative determination of NF-κB p65 (Phospho-Ser276) concentrations within experimental cell lysate samples. This particular immunoassay utilizes the traditional "Sandwich" Enzyme-Linked Immunosorbent Assay (ELISA) where the target protein (antigen) is bound in a "sandwich" format by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies added subsequently by the investigator.

The capture antibodies coated to the bottom of each well are specific for a particular epitope NF-κB p65 (Phospho-Ser276) while the user-added detection antibodies bind to epitopes on the captured target protein. Amid each step of the procedure, a series of wash steps must be performed to ensure the elimination of non-specific binding between proteins to other proteins or to the solid phase.

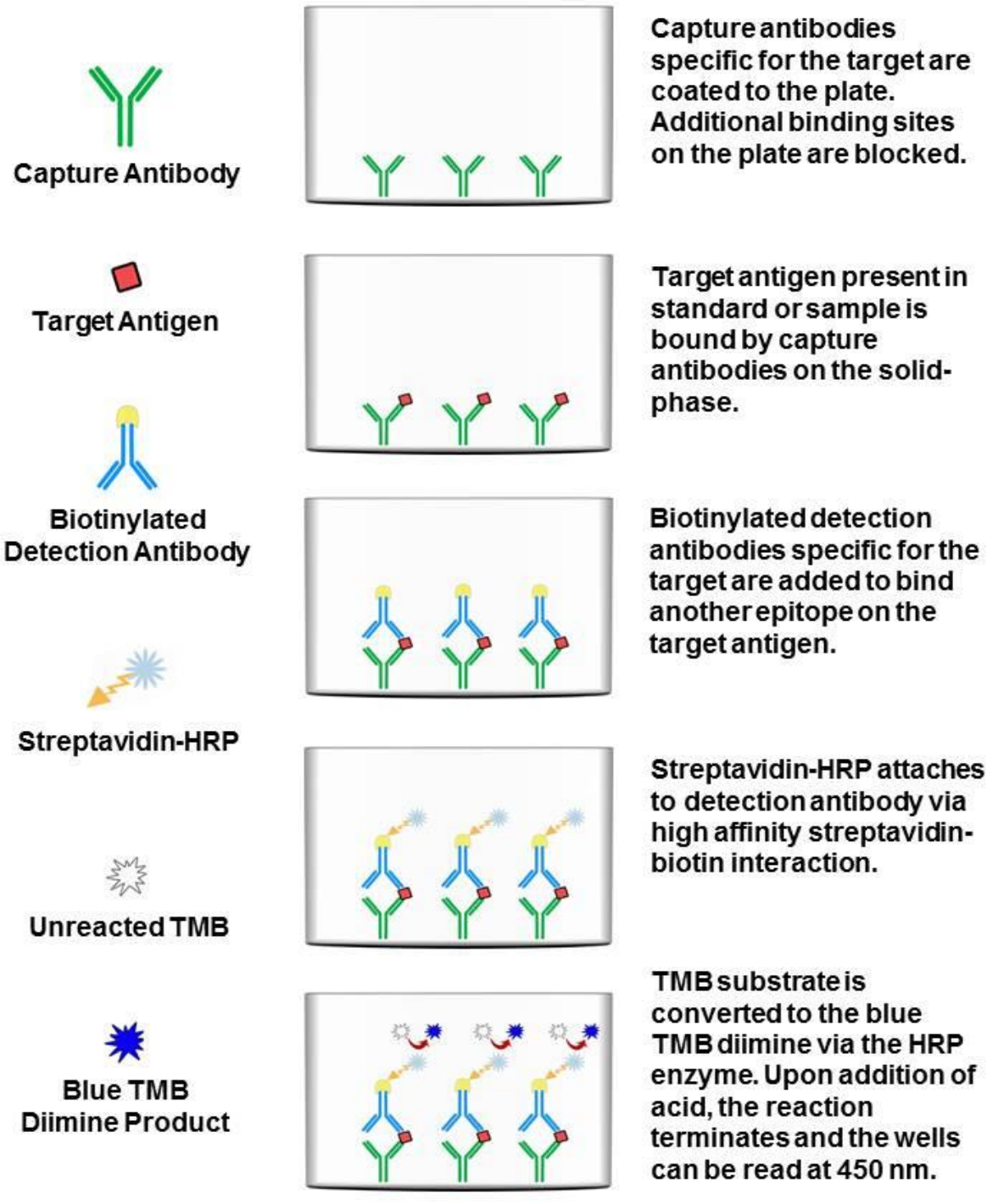
After incubation and "sandwiching" of the target antigen, a peroxidase enzyme is conjugated to the constant heavy chain of the secondary antibody (either covalently or via Avidin/Streptavidin-Biotin interactions), allowing for a colorimetric reaction to ensue upon substrate addition. When the substrate TMB (3, 3', 5, 5'-Tetramethylbenzidine) is added, the reaction catalyzed by peroxidase yields a blue color that is representative of the antigen concentration.

Upon sufficient color development, the reaction can be terminated through addition of Stop Solution (2 N Sulfuric Acid) where the color of the solution will turn yellow. The absorbance of each well can then be read by a spectrophotometer where data analysis may begin.

NF-κB p65 (Phospho-Ser276) ELISA Specificity

The NF-κB p65 (Phospho-Ser276) Phospho Sandwich ELISA Kit is a convenient, lysate- free, high throughput and sensitive assay kit that can detect NF-κB p65 protein phosphorylation and expression profile in cell lysates. The kit can be used for measuring the relative amounts of phosphorylated NF-κB p65 in cultured cells as well as screening for the effects that various treatments, inhibitors (ie. siRNA or chemicals), or activators have on NF-κB p65 phosphorylation.

ASSAY FORMAT



ANTIBODY SPECIFICITY

Figure 1. Jurkat cells were grown to 90% confluency and were stimulated with U.V. 150 J/s and incubated for 1 hr. Cells were immediately lysed thereafter and measured for Total Protein Concentration and O.D. 450 nm of NF-kB p65 P-Ser276 versus untreated Jurkat lysa Lysates were compared side by side for NF-κB p65 (Phospho-Ser276) with Phospho Sandwich ELISA kit (Cat. # PS-1631) and the results are as follows.

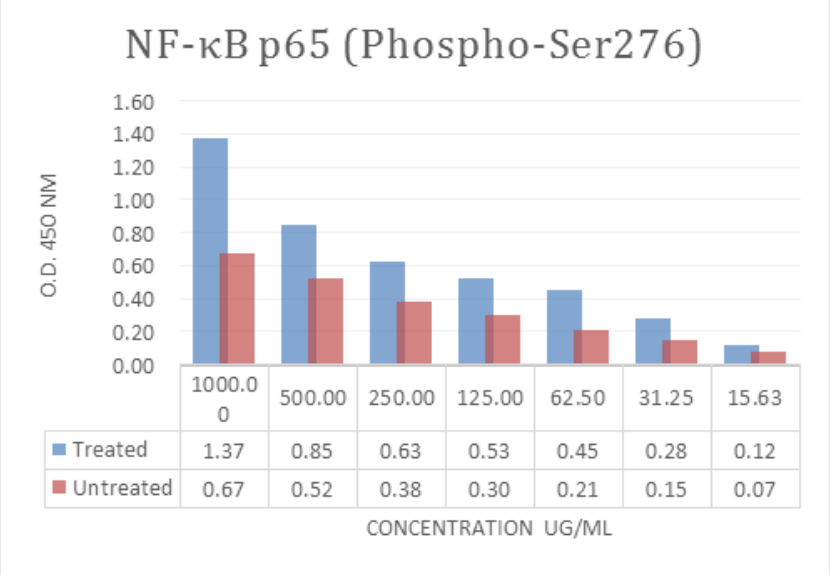
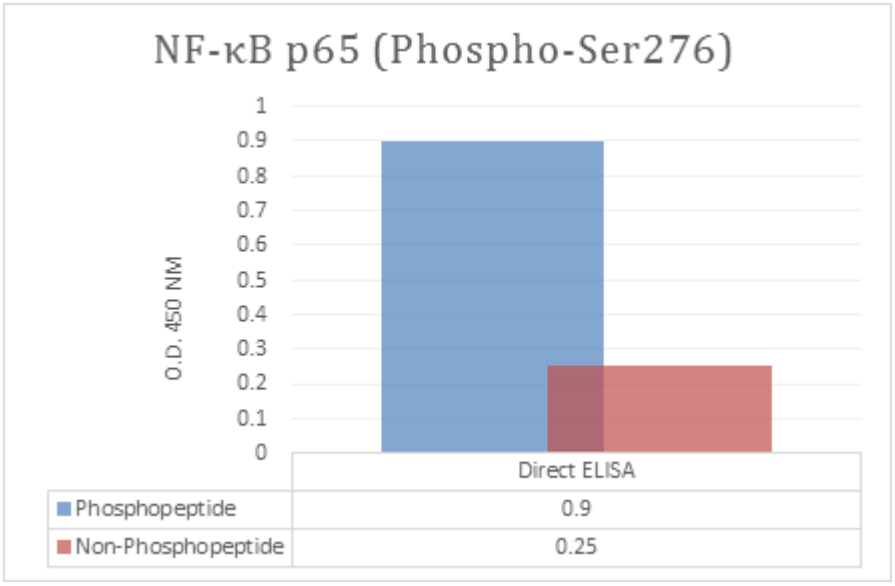


Figure 2. Enzyme-Linked Immunosorbent Assay (ELISA) for immunogen phosphor-peptide (left) and non-phospho peptide (right), using Anti-NF-κB p65 (Phospho-Ser276) Antibody.(Cat # A7169).



MATERIALS INCLUDED

Packaged Component	Quantity Per Kit	Storage/Stability after first use
Microstrips Coated w/ Capture Antibody	12 x 8-Well Microstrips	1 month at 4°C
100X Biotinylated Detection Antibody	110 µl	
400X Streptavidin-HRP	30 µl	
1X Lysis Buffer	12 mL	
Wash Buffer (15X)	50 ml	
Assay Diluent 1TD	50 ml	
Ready-to-Use Substrate	12 ml	
Stop Solution	12 ml	
Adhesive Plate Sealers	2 Sheets	-
Technical Manual	1 Manual	-

*****Bring all materials to room temperature (RT) prior to use and if component is not fully used store them in 4°C *****

Microstrips Coated w/ Capture Antibody – Use strips as necessary and wrap remaining strips tightly to ensure stability during storage.

100x Biotinylated Detection Antibody – Mix thoroughly, incubate for 5 mins, spin down, and then dilute with Assay Diluent 1TD to 1X. Enough Detection Antibody is provided to produce 11 mL of working concentration.

400X Streptavidin-HRP – Mix thoroughly, spin down, and then dilute with Assay Diluent 1TD to 1X. Enough Streptavidin-HRP is provided to produce 12mL of working concentration.

15X Wash Buffer – Briefly mix and dilute with ddH_2O to 1X. Enough Wash Buffer is provided to produce 750 mL of working concentration.

1X Lysis Buffer – Add PMSF to a final concentration of 1mM prior to use. **1X Lysis Buffer should be stored in -20°C until use.**

Assay Diluent 1TD – Dilute samples, Standard, Detection Antibody, and Streptavidin-HRP. Mix prior to use.

Ready-to-use Substrate – Light and temperature sensitive. Allow 30 minutes for solution to come to RT prior to use.

Stop Solution – Corrosive acid. Handle with care.

ADDITIONAL MATERIALS

The following materials and/or equipment are NOT provided in this kit but are necessary to successfully conduct the experiment:

- Microplate reader able to measure absorbance at 450 nm
- Micropipettes with capability of measuring volumes ranging from 1 µl to 1 ml
- Distilled, deionized, and or purified water recommended TOC 1-50 ppb, MΩ-cm 18.0
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent paper or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 ml
- Bench-top centrifuge (optional)
- Bench-top vortex (optional)
- Orbital shaker (optional)

LYSATE PRODUCTION

Adherent Cells

1. Grow cells to 80-90% confluency, aspirate media, and stimulate cells as desired.
2. Rinse plate(s) with cold PBS and aspirate.
3. Add 3 mL of 2 mM EDTA to detach cells or add 3 mL of PBS and gently scrape off the plate.
4. Collect cells and spin down at 1000 rpm for 5 minutes (4°C)
5. Wash the cell pellet with cold PBS and spin down again.
6. Decant the PBS and add 1 mL of 1X Lysis Buffer + 1 mM PMSF per 15 cm/ culture plate.
7. Briefly mix and incubate on ice for 5 minutes.
8. Sonicate cells on ice.
9. Spin mixture at 14,000 rpm for 10 mins. at 4°C and transfer supernatant to a fresh tube.
10. Mix thoroughly and create single use aliquots. Store at -80°C until use.

Suspension Cells

1. Grow cells to 80-90% confluency, remove media via centrifugation (1000 rpm, 5 mins) and stimulate cells as desired.
2. Collect cells and spin down at 1000 rpm, 5 mins, 4°C.
3. Wash the cell pellet with cold PBS and spin down again.
4. Decant the PBS and add 1 mL of 1X Lysis Buffer + 1 mM PMSF per $1X10^7$ cells.
5. Briefly mix and incubate on ice for 5 minutes.
6. Sonicate cells on ice.
7. Spin mixture at 14,000 rpm for 10 mins. at 4°C and transfer supernatant to a fresh tube.
8. Mix thoroughly and create single use aliquots. Store at -80°C until use.

ASSAY PROCEDURE

1. We recommend measuring total protein for sample lysates due to variations in cell growth and types of stimulations.
2. Assemble Assay Diluent 1TD, sample lysates, and desired microstrips for experimental design.
3. Data Images provided in manual were generated from serial dilution of lysates with Assay Diluent 1TD in duplicate. End users may look to the concentration range depicted in Figure 1 for reference.
4. Dilute lysates as desired with Assay Diluent 1TD and dispense 100 µl/well, wrap with parafilm, and incubate for 2 hrs. RT shaking or overnight 4°C shaking.
5. Wash plate 3 times with 1X Wash Buffer 300 µl/well with a vacuum based plate washer. If no vacuum based plate washer is available the following method is described.
   a. Aspirate contents of plate
   b. Dispense 300 µl/well of 1X Wash Buffer, shake briefly, and aspirate the contents
   c. Repeat this process 3 times.
   d. After the last wash, strike the plate on absorbent paper to remove as much residual liquid as possible.
6. Dilute the Detection Antibody to 1X and dispense 100 µl/well, wrap with parafilm, and incubate on a shaker for 2 hrs. at RT.
7. Repeat Washing Step
8. Dilute Streptavidin-HRP to 1X and dispense 100 µl/well, wrap with parafilm, and incubate on a shaker for 30 mins. at RT.
9. Repeat Washing Step
10. Bring Ready-to-use Substrate to RT and dispense 100 µl/well and allow the reaction to incubate on an orbital shaker for 20-30 mins.
11. Stop the reaction with Stop Solution 100 µl/well. The wells will turn the wells from blue to yellow, signaling the end of the reaction.
12. The plate is now ready to read at 450 nm.

SHORT PROTOCOL

Grow cells to 80-90% confluency and stimulate as desired. Lyse cells and create single use aliquots

Take protein concentration of lysates and perform dilution of samples as desired

Add 100ul of sample or control to each well and incubate for 2 hours at room temperature or overnight at 4°C

Aspirate samples or controls out and wash plate 4 times.

Dilute Biotinylated Detection Antibody as specified. Add 100ul to each well and incubate for 2 hours at room temperature.

Aspirate Biotinylated Detection Antibody out and wash plate 4 times.

Dilute 400X Streptavidin-HRP as specified. Add 100ul of 1X Streptavidin-HRP to each well and incubate at room temperature for 30 minutes.

Aspirate 1X Streptavidin-HRP out and wash plate 4 times.

Add 100ul of Ready-to-Use Substrate to each well and incubate at room temperature for color development.

Add 100ul of Stop Solution and read plate at 450nm.

HEALTH AND SAFETY PRECAUTIONS

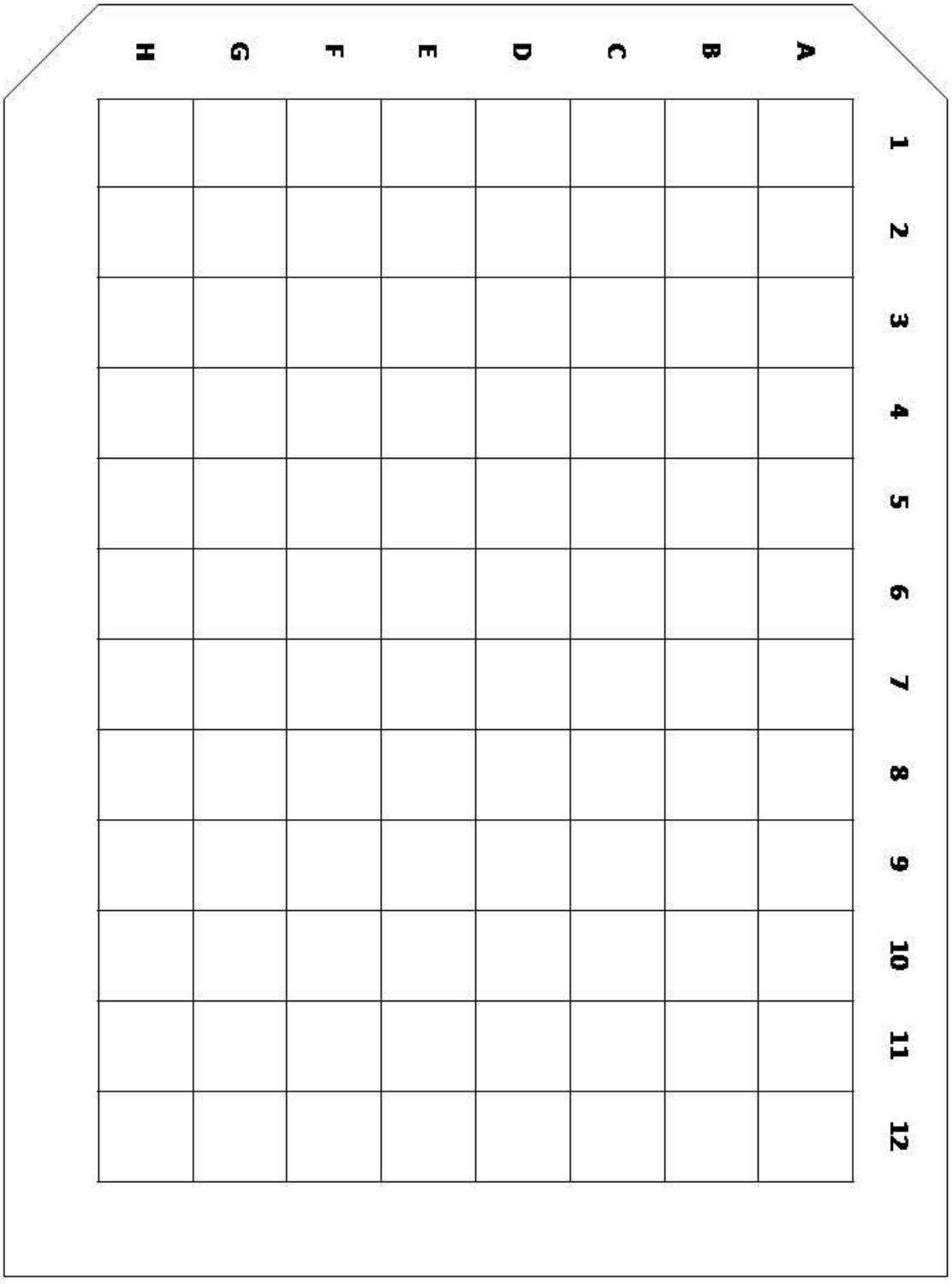
- Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.

- Stop Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent. Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.

ASSAY RESTRICTIONS

- This ELISA kit is intended for research purposes only, NOT diagnostic or clinical procedures of any kind.
- Materials included in this kit should NOT be used past the expiration date on the kit label.
- Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.
- Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.
- The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any biological samples. However, the possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.
- Individual results may vary due to differences in technique, plasticware and water sources.

ELISA PLATE TEMPLATE



TECHNICAL SUPPORT

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