LSBio™ Mouse/Human/Rat Phospho-NPM1 / NPM / Nucleophosmin Cell-Based Phosphorylation ELISA Kit

Catalog No. LS-F1423

User Manual

Please Read the Manual Carefully
Before Starting your Experiment

LifeSpan BioSciences, Inc.

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INTRODUCTION

Colorimetric Cell-Based ELISAs

The Colorimetric Cell-Based ELISA Kit allows for the detection of various target proteins and the effects that certain stimulation conditions have on target protein expression in different cell lines. Qualitative determination of target protein concentration is achieved by an indirect ELISA format. In essence, the target protein is captured by target-specific primary (1°) antibodies while the HRP-conjugated secondary (2°) antibodies bind the Fc region of the 1° antibody. Through this binding, the HRP enzyme conjugated to the 2° antibody can catalyze a colorimetric reaction upon substrate addition. Due to the qualitative nature of the Cell-Based ELISA, multiple normalization methods are described: 1) a monoclonal antibody specific for human GAPDH is included to serve as an internal positive control in normalizing the target absorbance values. 2) Following the colorimetric measurement of HRP activity via substrate addition, the Crystal Violet whole-cell staining method is used to determine cell density. After staining, the results can be analyzed by normalizing the absorbance values to cell amounts, by which the plating difference can be adjusted. 3) If a phosphorylated target is being detected, an antibody against the non-phosphorylated counterpart will be provided for normalization purposes. The absorbance values obtained for the non-phosphorylated target can be used to normalize the absorbance values for the phosphorylated target.

NPM (Phospho-Thr234) Colorimetric Cell-Based ELISA

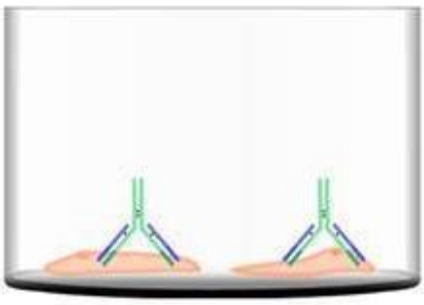
The NPM (Phospho-Thr234) Cell-Based ELISA Kit is a convenient, lysate-free, high throughput and sensitive assay kit that can monitor NPM protein phosphorylation and expression profile in cells. The kit can be used for measuring the relative amounts of phosphorylated NPM in cultured cells as well as screening for the effects that various treatments, inhibitors (ie. siRNA or chemicals), or activators have on NPM phosphorylation.

ASSAY RESTRICTIONS

α-Target
Primary Antibody

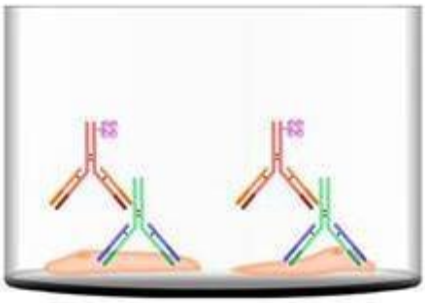
Cells are seeded onto the bottom of each well. The cells are quenched, fixed and the well is blocked.

α-Primary IgG
Secondary Antibody



Primary antibodies specific for the target antigen are added and allowed to bind to their respective epitopes.

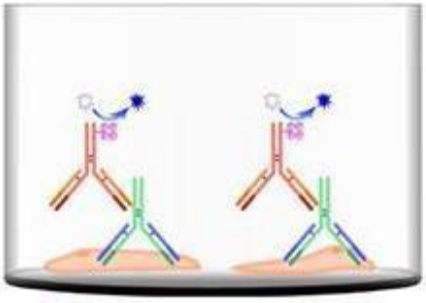
HRP Conjugate



HRP-conjugated secondary antibodies specific for the primary antibody are added and allowed to bind to their respective epitopes.

Unreacted TMB

Blue TMB
Diimine Product



TMB substrate is converted to the blue TMB diimine via the HRP enzyme. Upon addition of acid, the reaction terminates and the wells can be read at 450 nm.

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.

ANTIBODY SPECIFICITY

Anti-NPM (Phospho-Thr234) Antibody

The Anti-NPM (Phospho-Thr234) Antibody is a rabbit polyclonal antibody. It was tested on Western Blots for specificity. The data in Figure 2 shows that a single protein band was detected. This protein band can be blocked by the synthesized immunogen peptide.

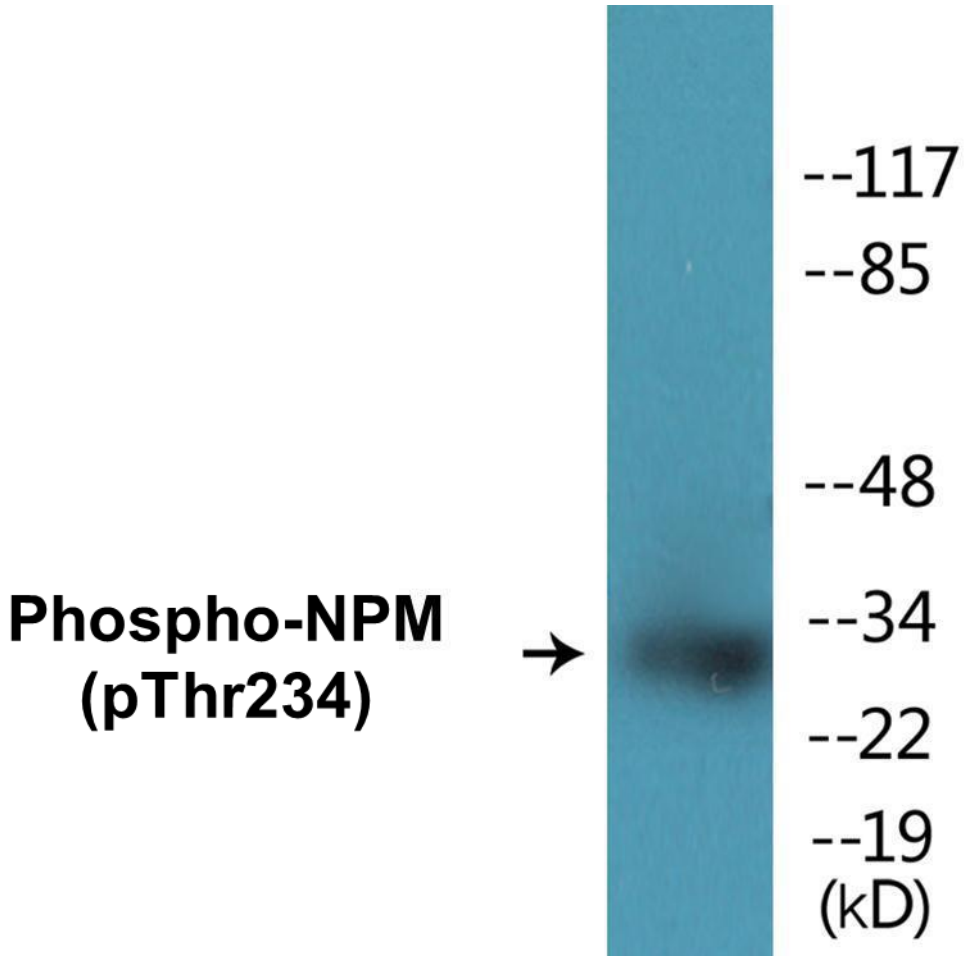


Figure 2. Western blot analysis of extracts from HeLa cells treated with nocodazole 1ug/ml 18h, using the Anti-NPM (Phospho-Thr234) Antibody.

The data in Figure 3 shows that the Anti-NPM (Phospho-Thr234) Antibody is highly specific for the phospho-peptide in comparison to the non-phospho peptide counterpart, through an ELISA.

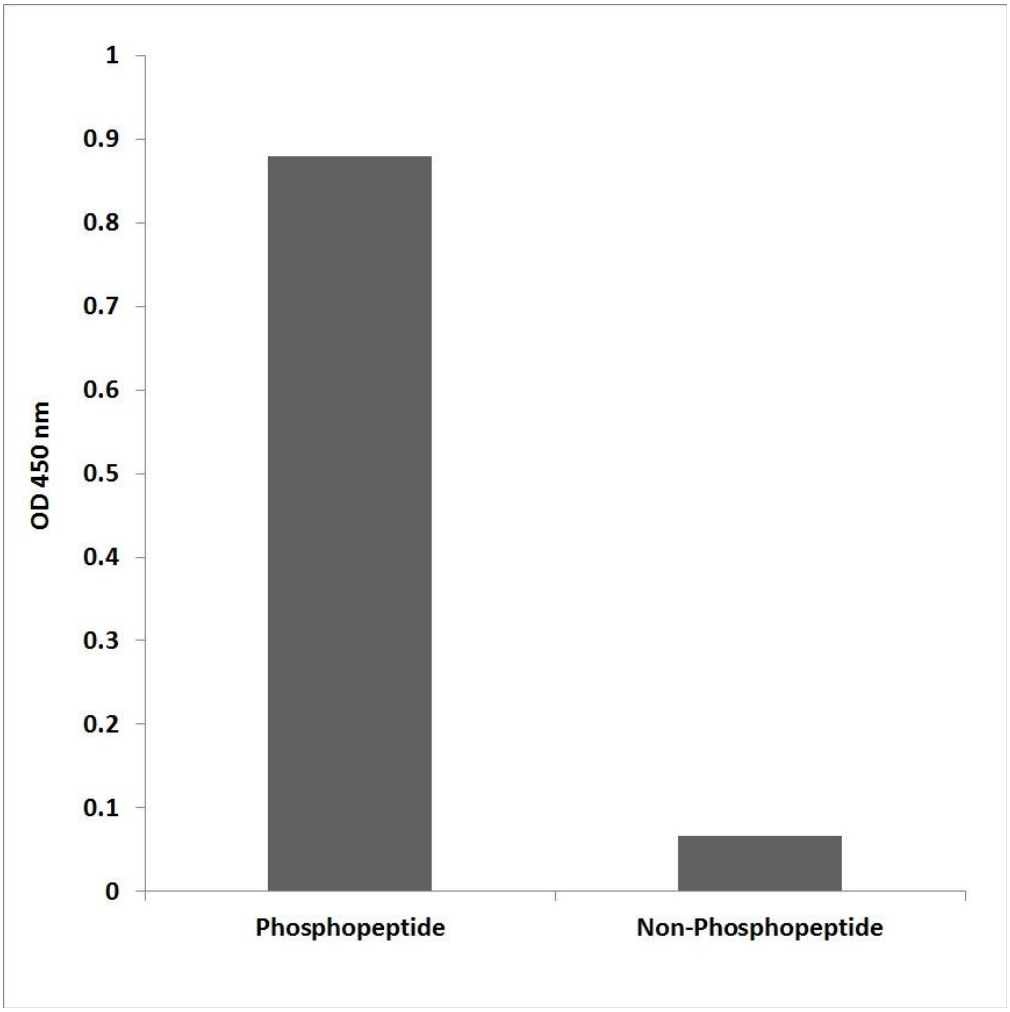


Figure 3. Enzyme-Linked Immunosorbent Assay (ELISA) for immunogen phosphor-peptide (left) and non-phospho peptide (right), using Anti-NPM (Phospho-Thr234) Antibody.

Anti-NPM Antibody

The Anti-NPM Antibody is a rabbit polyclonal antibody. It was tested on Western Blots for specificity. The data in Figure 4 shows that a single protein band was detected. This protein band can be blocked by the synthesized immunogen peptide.

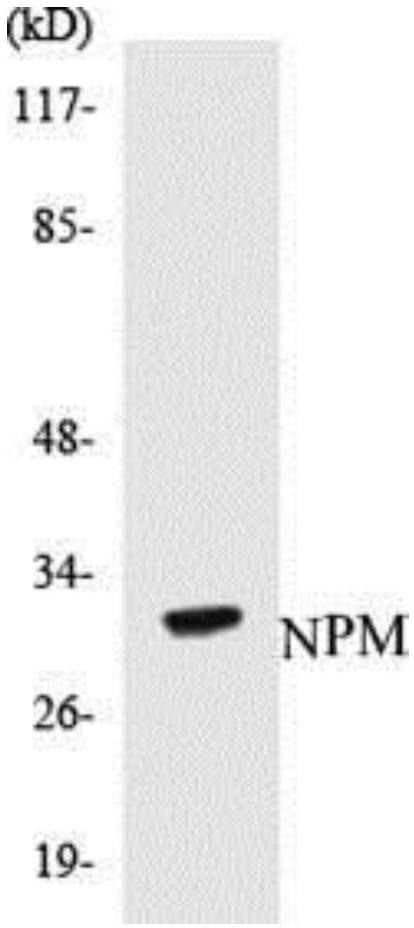


Figure 4. Western blot analysis of extracts from Jurkat cells, using the Anti-NPM Antibody.

Anti-GAPDH Antibody

The Anti-GAPDH Antibody is a mouse monoclonal antibody. It was tested on Western Blots with the tissue lysates from human, mouse, and rat for specificity. The data in Figure 5 shows that a single protein band was detected from all three lysates.

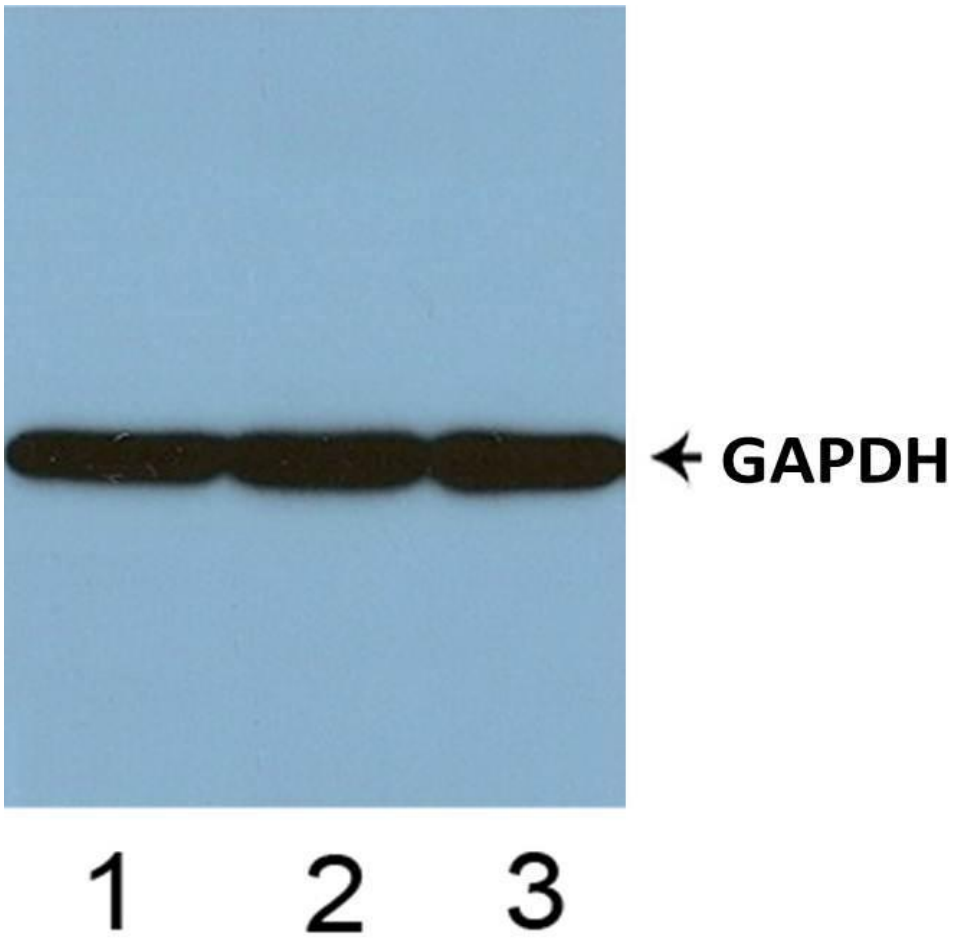


Figure 5. Western blot analysis of tissue lysates from human (1), mouse (2) and rat (3).

MATERIALS INCLUDED

Reagent	Quantity	Container
96-Well Cell Culture Clear-Bottom Microplate	2 Plates	-
10x TBS	24 ml (10x)	Clear
Quenching Buffer	24 ml (1x)	Clear
Blocking Buffer	50 ml (1x)	Clear
10x Wash Buffer	50 ml (10x)	Clear
100x Anti-NPM (Phospho-Thr234) Antibody (Rabbit Polyclonal)	60 µl (100x)	Red
100x Anti-NPM Antibody (Rabbit Polyclonal)	60 µl (100x)	Purple
100x Anti-GAPDH Antibody (Mouse Monoclonal)	60 µl (100x)	Green
HRP-Conjugated Anti-Rabbit IgG Antibody	12 ml (1x)	Glass
HRP-Conjugated Anti-Mouse IgG Antibody	12 ml (1x)	Glass
Primary Antibody Diluent	12 ml (1x)	Clear
Ready-to-Use Substrate	12 ml (1x)	Brown
Stop Solution	12 ml (1x)	Clear
Crystal Violet Solution	12 ml (1x)	Glass
SDS Solution	24 ml (1x)	Clear
Adhesive Plate Seals	4 Seals	-

STORAGE AND STABILITY

Upon receipt, the kit should be stored at 4°C. The un-opened kit will be stable for up to 6 months from the date of shipment if stored at 4°C. Diluted Anti-NPM (Phospho-Thr234) Antibody, Anti-NPM Antibody and diluted Anti-GAPDH Antibody can each be stored at 4°C for up to two weeks. HRP-Conjugated Anti-Rabbit IgG Antibody and HRP-Conjugated Anti-Mouse IgG Antibody will be stable at 4°C for up to six months. The SDS Solution should be stored at room temperature or warmed up to room temperature if stored at 4°C.

BUFFER PREPARATION AND RECOMMENDATIONS

Note: Please remember to allow all solutions to warm up to room temperature prior to use.

1x TBS – 1x TBS is used to wash cells seeded on the plate. 1x TBS can be prepared by adding 1 volume of 10x TBS provided in the kit to 9 volumes of ddH_2O.

Fixing Solution – This solution is **NOT** provided. Fixing Solution is used to fix cells after cell culture. It is prepared by adding formaldehyde to 1x TBS with light mixing. The 4% formaldehyde is used for adherent cells and 8% formaldehyde is used for suspension cells and loosely attached cells. 37% formaldehyde can be purchased from Sigma Cat# F-8775.

Quenching Buffer – This solution is provided as ready-to-use. Quenching Buffer is used to inactivate the endogenous peroxidase activity of the seeded cells.

Blocking Buffer – This solution is provided as ready-to-use. Blocking Buffer is used to block additional binding sites in each well.

Wash Buffer – This buffer is provided as a 10x solution. 1x Wash Buffer can be prepared by adding 1 volume of 10x Wash Buffer provided in the kit to 9 volumes of ddH_2O.

100x Anti-NPM (Phospho-Thr234) Antibody – This antibody is a rabbit polyclonal antibody. This antibody was tested to be specific for the NPM protein phosphorylated at Thr234. The supplied antibody is a 100x solution. Make 1:100 dilutions in Primary Antibody Diluent prior to use. The diluted primary antibody can be stored at 4°C for up to two weeks.

100x Anti-NPM Antibody – This antibody is a rabbit polyclonal antibody. This antibody was tested to be specific for the NPM protein. The supplied antibody is a 100x solution. Make 1:100 dilutions in Primary Antibody Diluent prior to use. The diluted primary antibody can be stored at 4°C for up to two weeks.

100x Anti-GAPDH Antibody – This antibody is a mouse monoclonal antibody. This antibody was tested to be specific for GAPDH. The supplied antibody is a 100x solution. Make 1:100 dilutions in Primary Antibody Diluent prior to use. The diluted primary antibody can be stored at 4°C for up to two weeks.

HRP-Conjugated Anti-Rabbit IgG Antibody – This solution is provided as ready-to-use. HRP-Conjugated Anti-Rabbit IgG Antibody is used as the secondary antibody to detect the target-bound, primary rabbit antibodies.

HRP-Conjugated Anti-Mouse IgG Antibody – This solution is provided as ready-to-use. HRP-Conjugated Anti-Mouse IgG Antibody is used as the secondary antibody to detect the target-bound, primary mouse antibodies.

Primary Antibody Diluent – This solution is provided as ready-to-use. Use this solution to dilute the provided antibodies.

Ready-to-Use Substrate – This solution is provided as ready-to-use. Ready-to-Use Substrate must be warmed to room temperature before use. Keep away from light as this solution is light-sensitive.

Stop Solution – This solution is provided as ready-to-use. Stop Solution must be handled with caution as it contains 2 N Sulfuric Acid (H_2SO_4) and is corrosive. Wear eye protection and gloves when handling.

Crystal Violet Solution – This solution is provided as ready-to-use. Crystal Violet is an intense stain used to stain cell nuclei. Avoid contact with skin and clothing.

SDS Solution – This solution is provided as ready-to-use. SDS is used to solubilize the Crystal Violet in preparation for cell staining. Store this solution at room temperature or warm up to room temperature if stored at 4°C.

ADDITIONAL MATERIALS REQUIRED

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

- Microplate reader able to measure absorbance at 450 nm and/or 595 nm for Crystal Violet Cell Staining (Optional)
- Micropipettes with capability of measuring volumes ranging from 1 µl to 1 ml
- 37% formaldehyde (Sigma Cat# F-8775) or formaldehyde from other sources
- Deionized or sterile water
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent papers or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 ml
- Orbital shaker
- Poly-L-Lysine (Sigma Cat# P4832 for suspension cells)

HEALTH AND SAFETY PRECAUTIONS

- Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin.

- Fixing Solution contains formaldehyde. Formaldehyde is known to be a highly toxic reagent. Personal protection is strongly recommended while working with this chemical.

- 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 or strips.

- Crystal Violet is an intense stain reagent. Avoid contact stain and clothing.

EXPERIMENT DESIGN

1) ***Cell Line:*** The cell line must express the target protein. This protocol can be used directly for adherent cells. For suspension cells and loosely attached cells, two steps are required: 1) Coat the plates with 100 µl of 10 µg/ml Poly-L-Lysine (Sigma Cat# P4832, not included) to each well of the 96-well plate for 30 minutes at 37°C before proceeding to Step 1 of Assay Protocol (on page 16). Use 8% formaldehyde to fix the cells on Step 5 of Assay Protocol.

2) ***Cell Number and Sensitivity:*** The number of cells plated onto the 96-well plates depends on the expression level of NPM protein in the cells, cell size, treatment conditions and incubation time. The cells used for testing should be around 75-90% confluent. Typically for HeLa cells, seed 30,000 cells per well overnight for treatment the following day. The NPM (Phospho-Thr234) Colorimetric Cell-Based ELISA Kit can detect Phospho-NPM expression in as little as 5,000 HeLa cells.

3) ***Cell Treatment:*** The cells can be treated with inhibitors, activators, stimulators (ie. chemicals, proteins/peptides) or a combination of the substances listed above. The cells can be treated with UV and serum starvation to meet the needs of the end-user.

4) ***Positive and Negative Controls:*** Mouse Anti-GAPDH Antibody (included) should be used to detect the internal positive controls for normalization of OD values of the target protein. The negative controls are HRP-Conjugated Anti-Rabbit IgG Antibody and HRP-Conjugated Anti-Mouse IgG Antibody alone in different wells (without the primary antibodies). Both positive and negative controls should be performed in the same plate with the Phospho-NPM target experiments.

5) ***Accuracy and Precision:*** Each condition should be performed in duplicate or in triplicate.

ASSAY PROTOCOL

Note: Please read the whole manual before performing the experiment.

1) Seed 200 µl of 20,000 adherent cells in culture medium in each well of a 96-well plate. The plates included in the kit are sterile and treated for cell culture. For suspension cells and loosely attached cells, coat the plates with 100 µl of 10 µg/ml Poly-L-Lysine (not included) to each well of a 96-well plate for 30 minutes at 37°C prior to adding cells.

2) Incubate the cells for overnight at 37°C, 5% CO_2.

3) Treat the cells as desired.

4) Remove the cell culture medium and rinse with 200 µl of 1x TBS, twice.

5) Fix the cells by incubating with 100 µl of Fixing Solution for 20 minutes at room temperature. The 4% formaldehyde is used for adherent cells and 8% formaldehyde is used for suspension cells and loosely attached cells. During the incubation, the plates should be sealed with Parafilm. **Note:** Fixing Solution is volatile. Wear appropriate personal protection equipment (mask, gloves and glasses) when using this chemical.

6) Remove the Fixing Solution and wash the plate 3 times with 200 µl 1x Wash Buffer for five minutes each time with gentle shaking on the orbital shaker. The plate can be stored at 4°C for a week. **Note:** For all wash steps, tap the plate **gently** on absorbent papers to remove the solution completely.

7) Add 100 µl Quenching Buffer and incubate for 20 minutes at room temperature.

8) Wash the plate 3 times with 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

9) Add 200 µl of Blocking Buffer and incubate for 1 hour at room temperature.

10) Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

11) Add 50 µl of 1x primary antibodies (Anti-NPM (Phospho-Thr234) Antibody, Anti-NPM Antibody and/or Anti-GAPDH Antibody) to the corresponding wells, cover with Parafilm and incubate for 16 hours (overnight) at 4°C. If the target expression is known to be high, incubate for 2 hours at room temperature with gentle shaking on the shaker.

12) Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

13) Add 50 µl of 1x secondary antibodies (HRP-Conjugated Anti-Rabbit IgG Antibody and/or HRP-Conjugated Anti-Mouse IgG Antibody) to corresponding wells and incubate for 1.5 hours at room temperature with gentle shaking on the shaker. **Note:** Add HRP-Conjugated Anti-Rabbit IgG Antibody to the wells incubated with Anti-NPM (Phospho-Thr234) Antibody (rabbit, polyclonal) and/or Anti-NPM (rabbit, polyclonal) and add HRP-Conjugated Anti-Mouse IgG Antibody to the wells incubated with Anti-GAPDH Antibody (mouse, monoclonal).

14) Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

15) Add 50 µl of Ready-to-Use Substrate to each well and incubate for 30 minutes at room temperature in the dark with gentle shaking on the shaker. **Note:** Ready-to-Use Substrate is a light-sensitive reagent. Keep away from light.

16) Add 50 µl of Stop Solution to each well and read OD at 450 nm immediately using the microplate reader.

Optional: Crystal Violet Cell Staining

Crystal Violet binds to cell nuclei and gives absorbance readings proportional to cell counts at 595 nm.

17) After finishing reading the absorbance at 450 nm, wash the plate twice with 200 µl of Wash Buffer and twice with 200 µl of 1x TBS for 5 minutes each. Tap the plates on paper towel to remove the excess liquid. Let plate air dry for 5 minutes at room temperature.

18) Add 50 µl of Crystal Violet Solution to each well, incubate for 30 minutes at room temperature on the shaker. **Note:** Crystal Violet is an intense stain. Avoid contact with skin and clothing.

19) Flick the plate to remove Crystal Violet Solution, rinse the plate by filling the wells with running tap water, and wash the plate with 200 µl of 1x TBS 3 times, 5 minutes each with gently shaking on the shaker.

20) Add 100 µl of SDS Solution into each well and incubate on the shaker at room temperature for 1 hour.

21) Read absorbance at 595 nm with microplate reader. If absorbance is too high, the solubilized Crystal Violet Solution can be diluted 10 times with H_2O on a separate 96-well plate.

SHORT PROTOCOL

Seed cells into wells and incubate overnight at 37°C, 5% CO_2.

⇩

Apply desired treatment conditions.

⇩

Add 100 µl of Fixing Solution and incubate 20 minutes at room temperature.

⇩

Add 100 µl of Quenching Buffer and incubate 20 minutes at room temperature.

⇩

Add 200 µl of Blocking Buffer and incubate for 1 hour at room temperature.

⇩

Add 50 µl of 1x primary antibodies and incubate overnight at 4°C. Add

⇩

50 µl of HRP-conjugated secondary antibodies and incubate for 1.5 hours at room temperature.

⇩

Add 50 µl of Ready-to-Use Substrate and incubate for 30 minutes at room temperature.

⇩

Add 50 µl of Stop Solution and read OD at 450 nm.

⇩

Crystal Violet Cell Staining Procedure (Optional)

DATA NORMALIZATION

Anti-NPM Antibody Normalization

The OD values obtained for the phosphorylated target protein can be normalized using the OD values obtained for the non-phosphorylated target protein via the proportion, OD_{450} (Anti-NPM P-Thr234 Antibody)/OD_{450} (Anti-NPM Antibody).

GAPDH Normalization

The OD_{450} values obtained for the target protein (phosphorylated and non-phosphorylated) can be normalized using the OD_{450} values obtained for GAPDH.

Crystal Violet Staining Normalization

The measured OD_{450} readings can be normalized using the OD_{595} values via the proportion, OD_{450}/OD_{595}.

Important Note: During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. We recommend briefly centrifuging the vial to dislodge any liquid in the container's cap prior to opening.

Warning: This reagent may contain sodium azide and sulfuric acid. The chemical, physical, and toxicological properties of these materials have not been thoroughly investigated. Standard Laboratory Practices should be followed. Avoid skin and eye contact, inhalation, and ingestion. Sodium azide forms hydrazoic acid under acidic conditions and may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent accumulation.

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