# LSBio™ Mouse/Human/Rat Phospho-c-Met (Tyr1234) Cell-Based Phosphorylation ELISA Kit

## Catalog No. LS-F1564

# User Manual

**Please Read the Manual Carefully**
**Before Starting your Experiment**




For research use only. Not approved for use in humans or for clinical diagnosis.

## CONTENTS PAGE

# 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.

## *Met (Phospho-Tyr1234) Colorimetric Cell-Based ELISA*

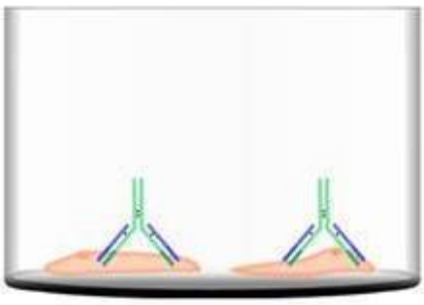
The Met (Phospho-Tyr1234) Cell-Based ELISA Kit is a convenient, lysate-free, high throughput and sensitive assay kit that can monitor Met protein phosphorylation and expression profile in cells. The kit can be used for measuring the relative amounts of phosphorylated Met in cultured cells as well as screening for the effects that various treatments, inhibitors (ie. siRNA or chemicals), or activators have on Met phosphorylation.

## ASSAY RESTRICTIONS

α-Target
Primary Antibody

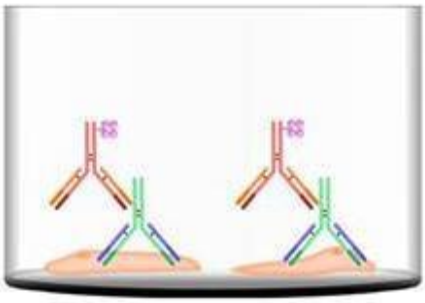
Cells are seeded onto the bottom of each well. The cells are quenched, fixed and the well is blocked.

α-Primary IgG
Secondary Antibody



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

HRP Conjugate

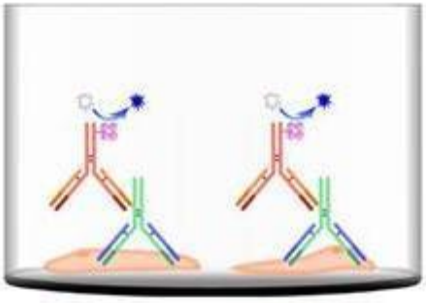


HRP-conjugated secondary antibodies specific for the primary antibody are added and allowed to bind to their respective epitopes.



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-Met (Phospho-Tyr1234) Antibody*

The Anti-Met (Phospho-Tyr1234) 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.

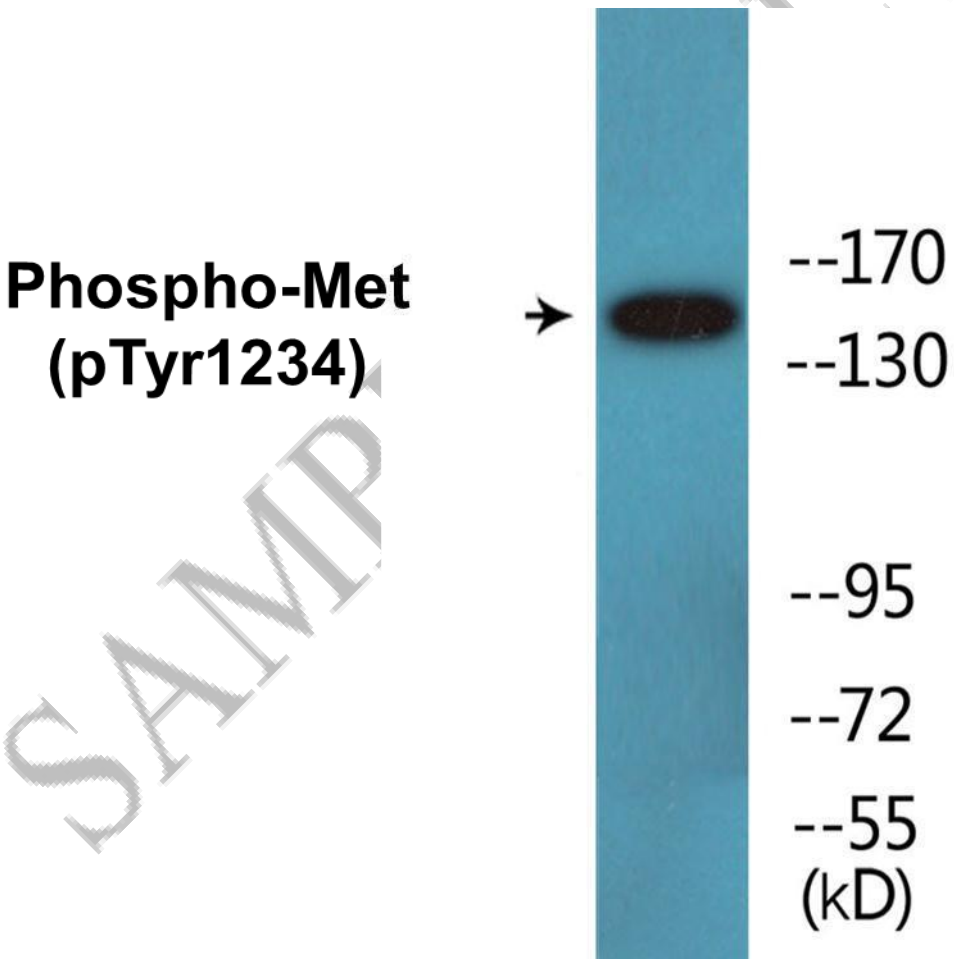



**Figure 2. Western blot analysis of extracts from HuvEc cells and COS7cells, using the Anti-Met (Phospho-Tyr1234) Antibody.**

The data in Figure 3 shows that the Anti-Met (Phospho-Tyr1234) Antibody is highly specific for the phospho-peptide in comparison to the non-phospho peptide counterpart, through an ELISA.

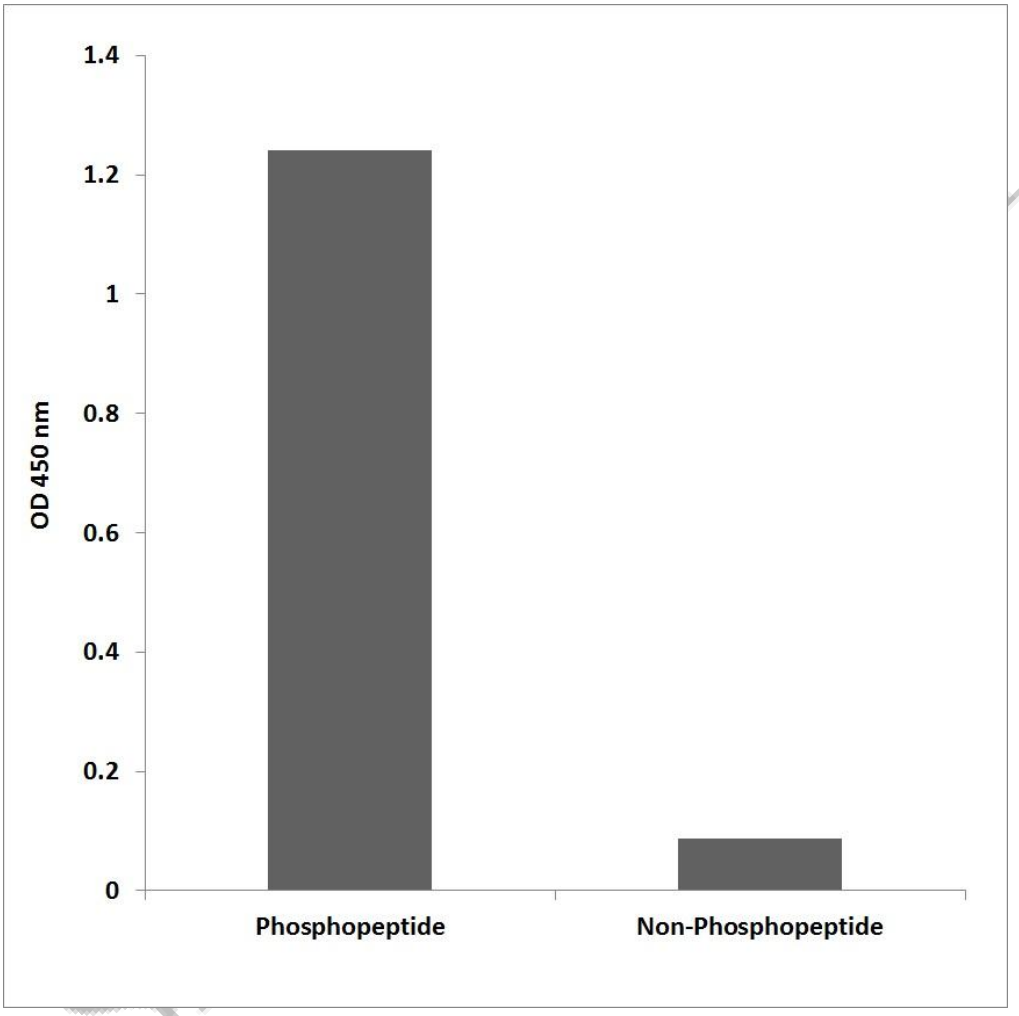



**Figure 3. Enzyme-Linked Immunosorbent Assay (ELISA) for immunogen phosphor-peptide (left) and non-phospho peptide (right), using Anti-Met (Phospho-Tyr1234) Antibody.**

***Anti-Met Antibody***

The Anti-Met 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.

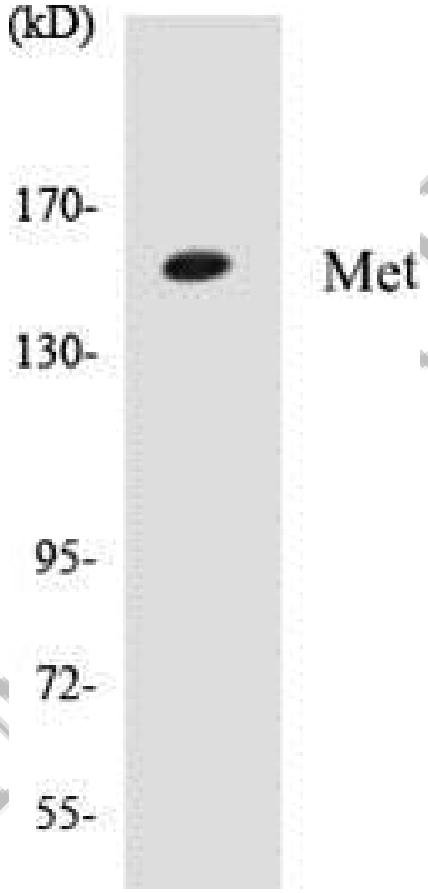





**Figure 4. Western blot analysis of extracts from HepG2 cells, using the Anti-Met Antibody.**

***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.

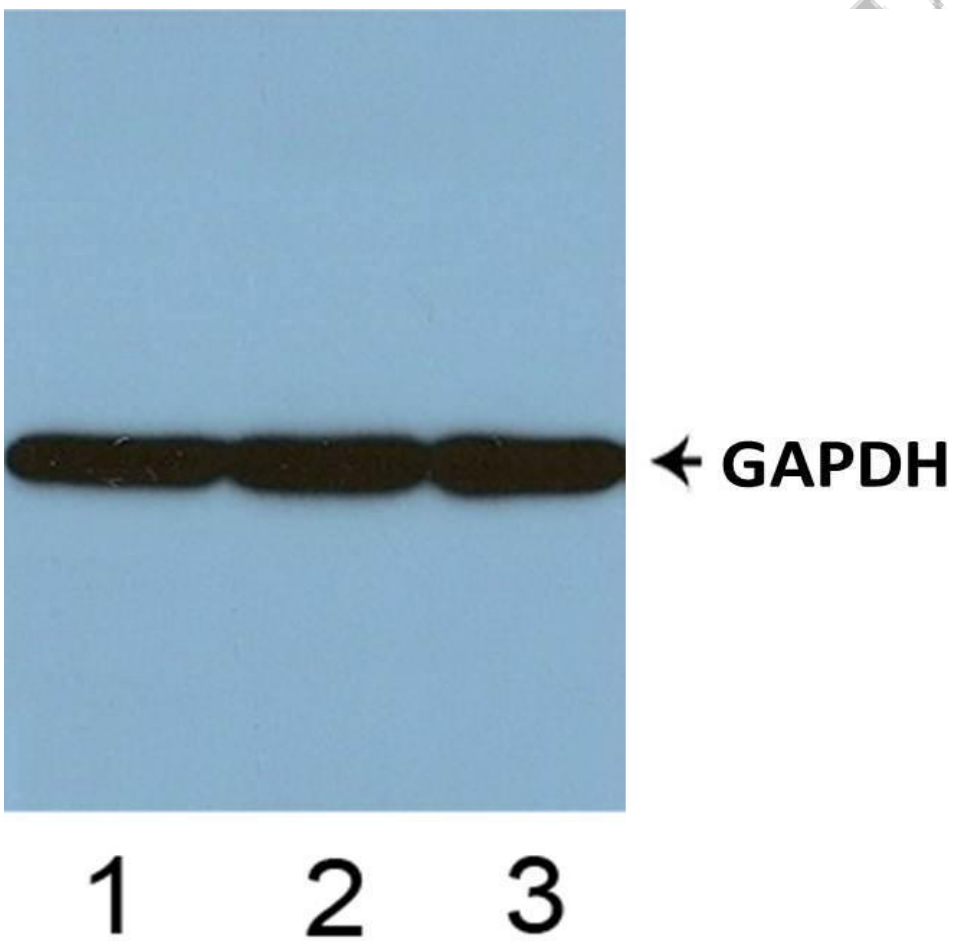



**Figure 5. Western blot analysis of tissue lysates from human (1), mouse (2) and rat (3).**

# 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 |
| 15x Wash Buffer | 50 ml (15x) | Clear |
| 100x Anti-Met (Phospho-Tyr1234) Antibody (Rabbit Polyclonal) | 60 µl (100x) | Red |
| 100x Anti-Met 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-Met (Phospho-Tyr1234) Antibody, Anti-Met 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.

# 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)

# REAGENT PREPARATION

Ensure that all supplied solutions are at ambient temperature before use. It is recommended to conduct assays for all controls and samples in duplicate. Sufficient reagents are provided to assay 192 wells; therefore, on the day of the experiment, prepare only the required amount. During incubation steps, use an orbital shaker set at 200 rpm to ensure proper equilibration of solutions in the cell culture plate wells. **All other provided solutions are ready to use.**

1. Dilute the 10x TBS to 1x TBS by combining 9 volumes of ddH2O and 1 volume of 10x TBS.

2. Dilute the 15x Wash Buffer to 1x Wash Buffer using 14 volumes of ddH2O and 1 volume of 15x Wash Buffer.

3. Prepare the Fixing solution to 4% paraformaldehyde (for adherent cell lines) or 8% paraformaldehyde (for suspension or loosely attaching cell lines). Dilute 37% paraformaldehyde with 1x PBS to desired paraformaldehyde percentage.

4. For suspension or loosely attached cells: Prepare Poly L Lysine to working concentration as stated in manual. (Not included, recommended Sigma Cat# P4832).

   *If the cell line is known to digest Poly-L-Lysine, Poly-D-Lysine may be an alternative for this step. Follow the manufacturer's instructions if using a different Poly-L-Lysine or Poly-D-Lysine.*

5. Prepare a 1x dilution of all three 100x primary antibodies (Phoshorylation Specific, Non-Phoshorylation Specific, and Anti-GAPDH) by using the Primary Antibody Diluent at a ratio of 1:100.

# 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 Met 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 Met (Phospho-Tyr1234) Colorimetric Cell-Based ELISA Kit can detect Phospho-Met 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-Met target experiments.

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

## ASSAY PROTOCOL

1. Seed 100 µl of 20,000 adherent cells into each well in a 96-well plate.
   **Note:** Optimal seeding concentrations should be determined by investigator and experimental goals.

   For suspension cells and loosely attached cells:

   a. Coat plates evenly with 20 uL of Poly-L-Lysine (Sigma Cat# P4832).
   b. After 5 minutes, remove solution by aspiration and rinse once with tissue culture grade water.
   c. Allow 2 hours to dry prior to seeding cells.

2. Incubate cells overnight or at least 6 hours at 37°C, 5% $CO_2$.

3. Treat the cells as desired.

4. Remove the cell culture medium *gently* and fix cells with 100 µl of 8% Paraformaldehyde per well. Incubate for 20 minutes at room temperature (RT).

   **Note:** 8% Paraformaldehyde solution is volatile. Wear appropriate personal protection equipment (mask, gloves and glasses) when using this chemical.

5. Aspirate the solution and add 300 µl of 1x Wash Buffer into each well being used and gently shake for 2-3 mins on an orbital shaker. Repeat this process 3 times. After the last wash ensure no liquid remains by inverting the plate and tapping it against clean paper towels.

   **Note:** The plate can be stored at 4°C for a week after this step.

6. Add 100 µl Quenching Buffer to each well and incubate for 20 mins at RT.

7. Repeat step 5.

8. Add 200 µl of Blocking Buffer and incubate for 1 hour at RT.

9. Add 50 µl of 1x Primary Antibody solutions into each well and incubate overnight at 4°C. If target concentration is known to be high, incubate for 2 hours at RT. Designate 2 wells for negative control by omitting the 1x Primary Antibodies.

10. Repeat step 5.

11. Add 50 µl of HRP-Conjugated Anti-Rabbit IgG Antibody into the wells incubated with 1x Anti-Met (Phospho-Tyr1234) Antibody and 1x Anti-Met Antibody.

12. Add 50 µl of HRP-Conjugated Anti-Mouse IgG Antibody into the wells incubated with 1x Anti-GAPDH Antibody.

13. Incubate the plate for 1.5 hours at RT.

14. Repeat step 5.

15. Add 50 µl of Ready to use Substrate into each well, cover plate from light, and incubate for 15-20 mins at RT.

16. Add 100 µl of Stop Solution into each well and read at 450 nm.

17. Repeat step 5 and allow plate to air dry for 5 mins at RT.

18. Add 50 µl of Crystal Violet Solution into each well and incubate for 30 mins at RT.

    **Note:** Crystal Violet is an intense stain. Avoid contact with skin and clothing.

19. Dip the plate into a bucket of water in the sink while keeping the water running, and carefully rinse the wells with ddH2O until no more color is visible. Let the plate to dry for 30 mins.

20. Add 100 µl of SDS Solution into each well and incubate for 1 hour at RT.

21. Read absorbance at 595 nm with microplate reader. If absorbance is too high, the solubilized Crystal Violet Solution may be diluted tenfold with ddH2O on a separate 96-well plate.

# 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.

## DATA ANALYSIS

**Background subtraction**
Average the duplicate or triplicate absorbance readings for each control and sample, subtracting them from the averaged absorbance for wells where primary antibodies have been omitted.

**Anti-GAPDH Internal positive control**
The OD450 values obtained ensure the assay is functioning, and cell densities between wells should be proportionate to seeding concentration. Stimulation of cells should impact target primary antibody absorbance values, with no effect on the anti-GAPDH absorbance.

**Crystal Violet Staining Normalization**
The Crystal Violet staining method enables intensity normalization within the same well. By using the ratio, the OD450 readings obtained can be normalized with the OD595 values. This constitutes a within-well method of analysis.

*If the absorbance was too high and a tenfold dilution was required. Multiply all values by 10 prior to normalization.*

a. OD450 anti-Met (Phospho-Tyr1234)/OD595 Crystal Violet.

b. OD450 anti-Met/OD595 Crystal Violet.

By doing so, the expression levels of Met (Phospho-Tyr1234) and Met are adjusted to account for the cell density present in each well.

**Phoshorylation to Non-Phoshorylation Comparison**
After normalization Crystal Violet staining, the resultant proportional values can be used to analyze the effects of stimulants in this assay.

A non-phosphorylated primary antibody is included to normalize the absorbance values between phosphorylated to non-phosphorylated values.

<u>Both antibodies utilize the proportion for analysis as follows:</u>

OD450 (CV Normalized) (Met (Phospho-Tyr1234))/

OD450 (CV Normalized) (Met)

## SHORT PROTOCOL

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

**Apply desired treatment conditions**

**Add 100ul of Fixing Solution and incubate 20 minutes at room temperature**

**Add 100ul of Quenching Buffer and incubate 20 minutes at room temperature**

**Add 200ul of Blocking Buffer and incubate for 1 hour at room temperature**

**Add 50ul of 1x Primary Antibodies and incubate overnight at 4°C**

**Add 50ul of HRP-Conjugated Secondary Antibodies and incubate for 1.5 hours at room temperature**

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

**Add 50ul of Stop Solution and read OD at 450nm**

**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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