Technical Manual

Histone H1 (Phospho-Thr17) Fluorometric Cell-Based ELISA Kit

- Catalogue Code: FBCAB00051
- Indirect ELISA Kit
- Research Use Only

1. Description and Principle

Fluorometric Cell-Based ELISAs

The Fluorometric 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 Dye 1-conjugated and Dye 2-conjugated secondary (2°) antibodies bind the Fc region of the 1° antibody. Through this binding, the dye conjugated to the 2° antibody can emit light at a certain wavelength given proper excitation, hence allowing for a fluorometric detection method.

A monoclonal antibody specific for human GAPDH is included to serve as an internal positive control in normalizing the target RFU values. If a phosphorylated target is being detected, an antibody against the non-phosphorylated counterpart will be provided for normalization purposes. The RFU values obtained for the non-phosphorylated target can be used to normalize the RFU value for the phosphorylated target.

Histone H1 (Phospho-Thr17) Fluorometric Cell-Based ELISA

The Histone H1 (Phospho-Thr17) Cell-Based ELISA Kit is a convenient, lysate- free, high throughput and sensitive assay kit that can monitor Histone H1 protein phosphorylation and expression profile in cells. The kit can be used for measuring the relative amounts of phosphorylated Histone H1 in cultured cells as well as screening for the effects that various treatments, inhibitors (ie. siRNA or chemicals), or activators have on Histone H1 phosphorylation.

2. Assay Format

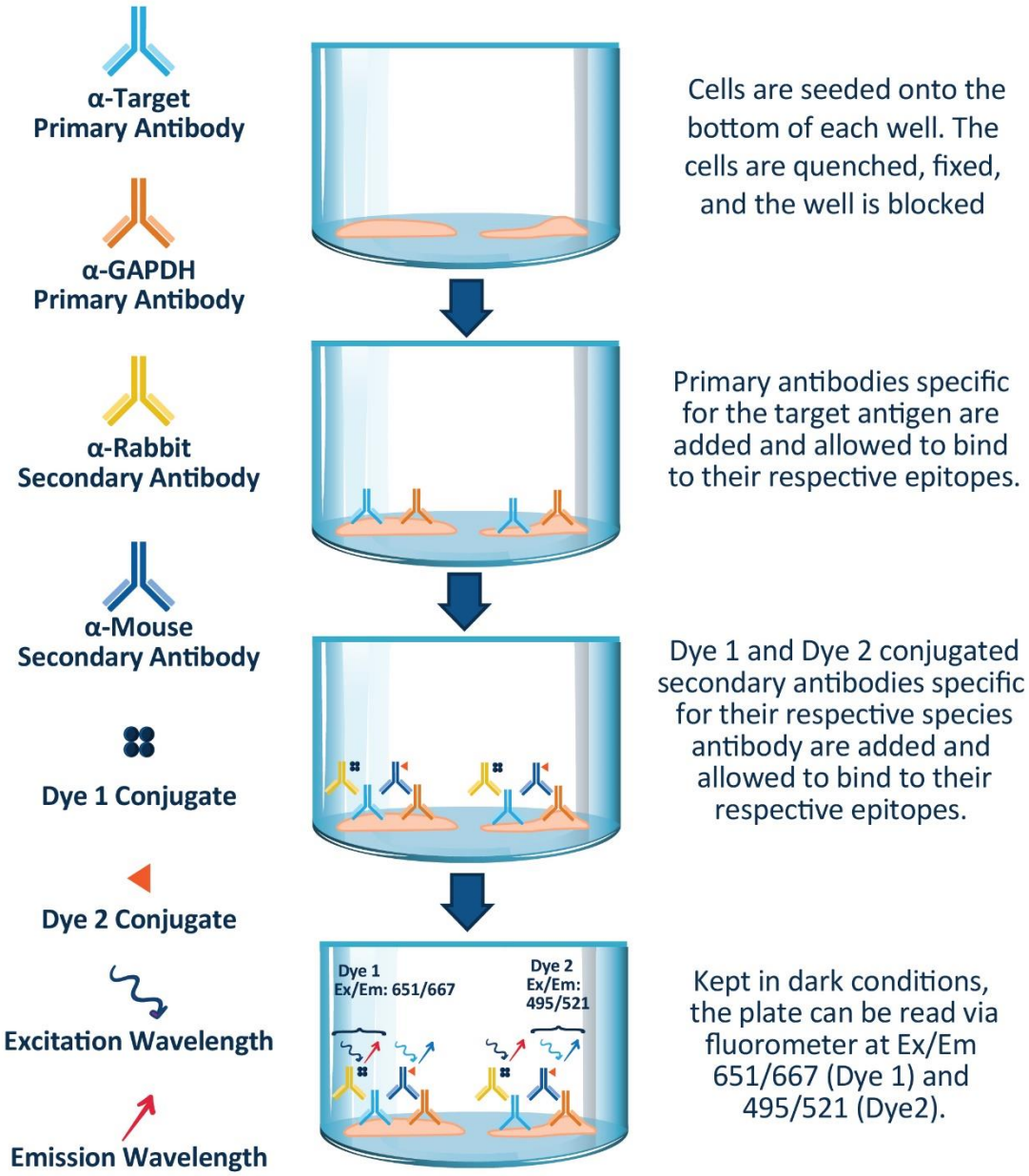


Figure 1: Fluorometric Cell-Based ELISA principle

3. Short Protocol

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

Apply desired treatment conditions

Add 100µl of Fixing Solution per well and incubate for 20 minutes at room temperature

Add 100µl per well of Quenching Buffer and incubate for 20 minutes at room temperature

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

Add 50µl of "Primary Antibody Mixture P" and/or "Primary Antibody Mixture NP" per well and incubate overnight at 4°C

Add 50µl of "Secondary Antibody Mixture" per well and incubate for 1.5 hours at room temperature

Read the plate at Ex/Em: 651/667 (Dye 1) and 495/521 (Dye 2)

4. 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.

5. 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 highly recommended while working with this chemical.

6. Antibody Specificity

Anti-Histone H1 (Phospho-Thr17) Antibody

The Anti-Histone H1 (Phospho-Thr17) 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.

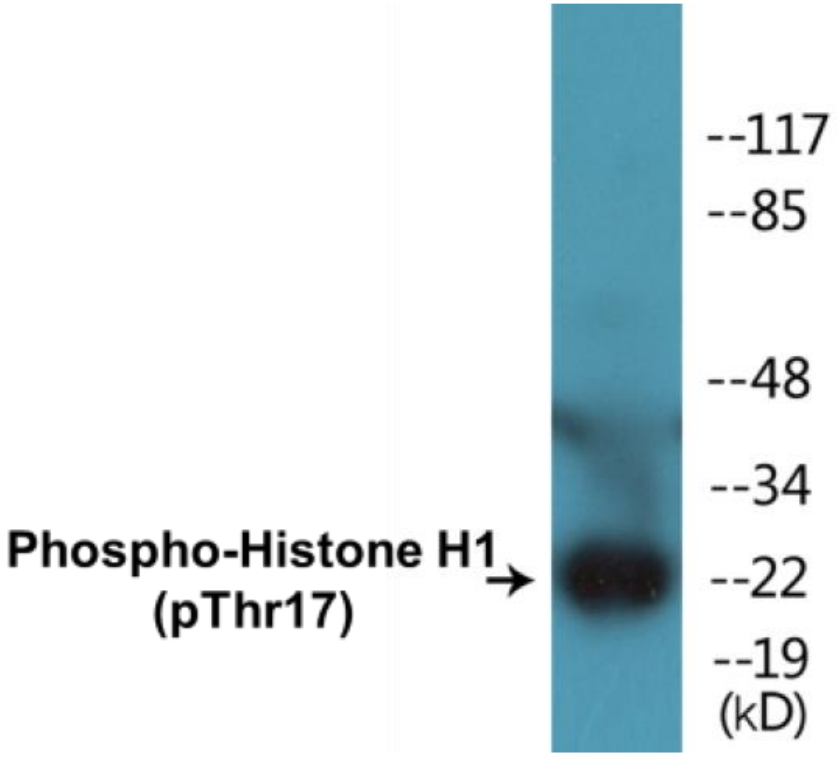


Figure 2: Western blot analysis of extracts from Jurkat cells treated with UV 15', using Histone H1 (Phospho-Thr17) Antibody.

The data in Figure 3 shows that the Anti-Histone H1 (Phospho-Thr17) Antibody is highly specific for the phospho-peptide in comparison to the non-phospho peptide counterpart, through an ELISA.

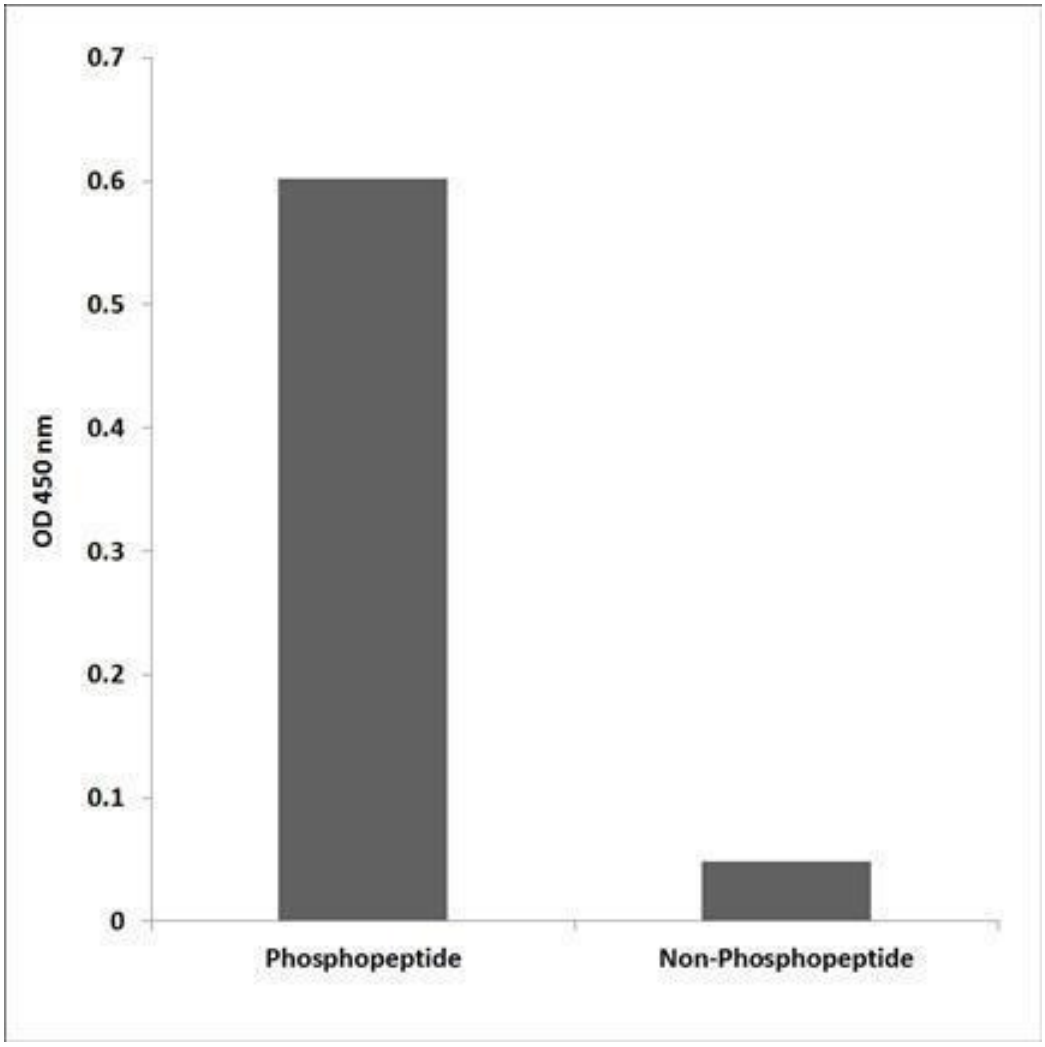


Figure 3. Enzyme-Linked Immunosorbent Assay (ELISA) Colorimetric for the immunogen phospho-peptide (left) and non-phospho peptide (right), using Anti-Histone H1 (Phospho-Thr17) Antibody.

Anti-Histone H1 Antibody

The Anti-Histone H1 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.

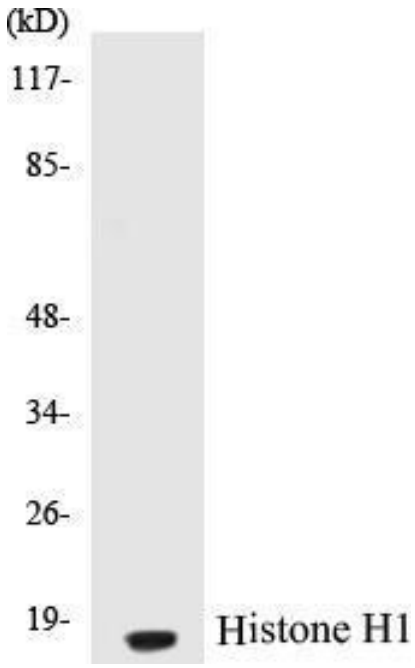
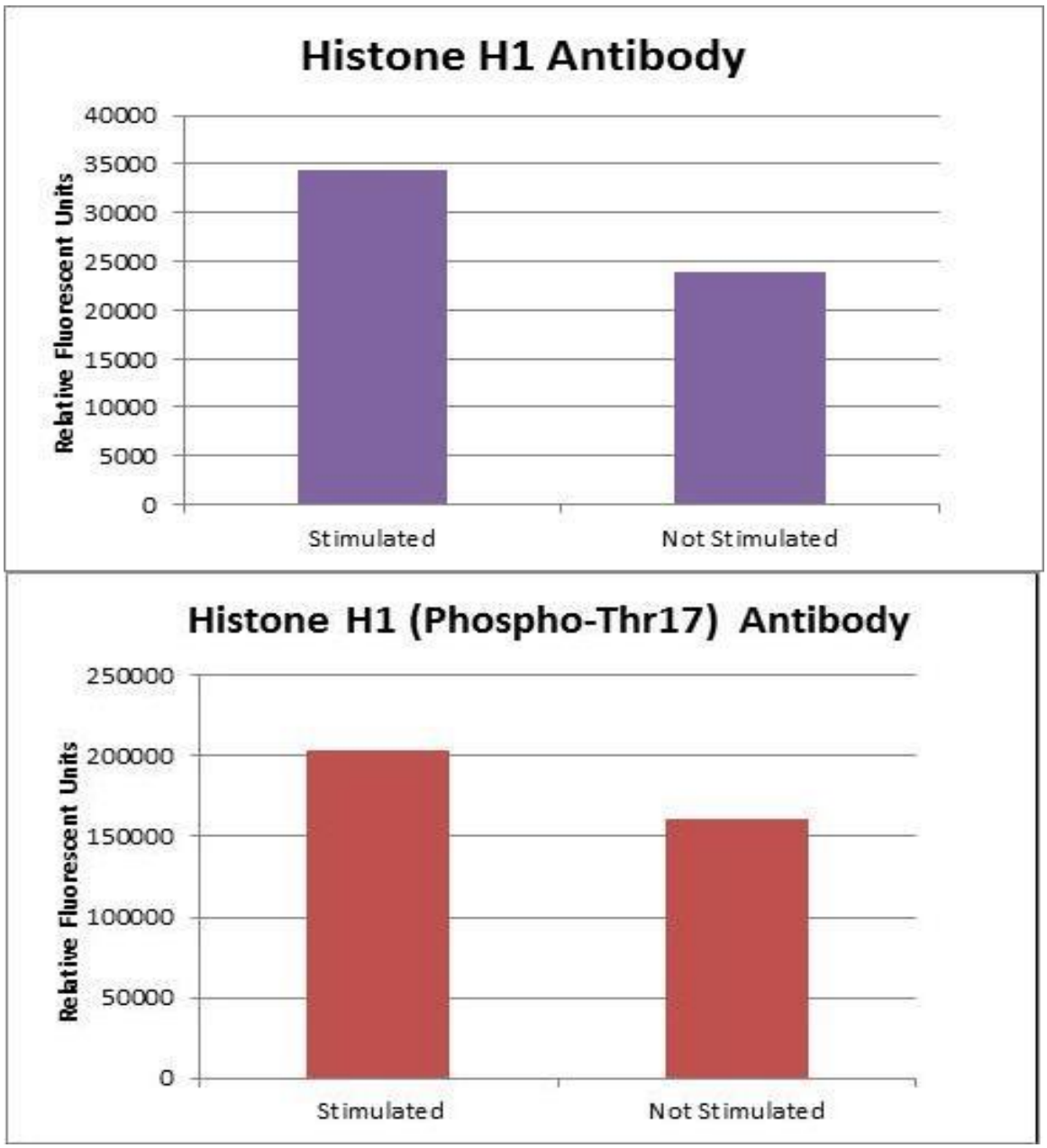


Figure 4. Western blot analysis of extracts from COLO cells

Fluorometric detection

The Anti-Histone H1 (Phospho-Thr17) and Anti-Histone H1 Antibody were used to measure the effects of stimulation. Data in Figure 5 shows upregulation of the phosphorylated target protein detected via fluorometric reaction.



**** Results may vary based on stimulations and fluorometer used****

Figure 5. Hek 293 cells were starved for 24 hrs. and stimulated with 20% Serum for 1 hr.

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 6 shows that a single protein band was detected from all three lysates.

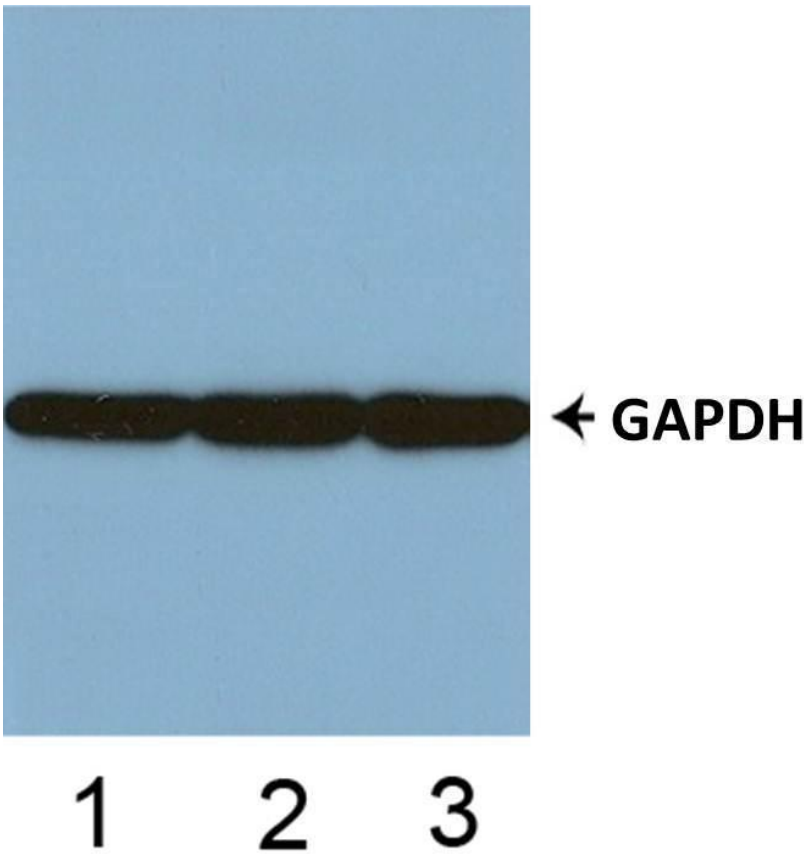


Figure 6. Western blot analysis of tissue lysates from human (1), mouse (2) and rat (3).

7. Kit Contents

Component	Quantity	Container
10x TBS	24 ml	Clear
Quenching Buffer	24 ml	Clear
Blocking Buffer	50 ml	Clear
15x Wash Buffer	50 ml	Clear
Primary Antibody Diluent	12 ml	Clear
100x Anti-Histone H1 (Phospho-Thr17) Antibody	60 µl	Red
100x Anti-Histone H1 Antibody	60 µl	Purple
100x Anti-GAPDH Antibody	110 µl	Green
Dye-1 Conjugated Anti-Rabbit IgG Antibody	6 ml	Glass
Dye-2 Conjugated Anti-Mouse IgG Antibody	6 ml	Glass
96-Well Black Cell Culture Clear-Bottom Microplate	2 Plates	-
Adhesive Plate Seals	2 seals	-

8. Shipping and Storage

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-Histone H1 (Phospho-Thr17) Antibody, Anti-Histone H1 Antibody and diluted Anti- GAPDH Antibody can each be stored at 4°C for up to two weeks. Dye-1 Conjugated Anti-Rabbit IgG Antibody and Dye-2 Conjugated Anti-Mouse IgG Antibody will be stable at 4°C for up to six months.

9. Buffer Preparation and Recommendations

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

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

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.

15x Wash Buffer – This buffer is provided as a 15x solution. 1x Wash Buffer can be prepared by adding 1 volume of 15x Wash Buffer provided in the kit to 14 volumes of ddH_2O.

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

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.

100x Anti-Histone H1 (Phospho-Thr17) Antibody – This antibody is a rabbit polyclonal antibody. This antibody was tested to be specific for the Histone H1 protein phosphorylated at Thr17. 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-Histone H1 Antibody – This antibody is a rabbit polyclonal antibody. This antibody was tested to be specific for the Histone H1 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.

Dye-1 Anti-Rabbit IgG Secondary Antibody – Dye-1 Conjugated Anti-Rabbit IgG antibody is the secondary antibody to detect the target bound, primary rabbit antibodies. The solution is light sensitive. Please store and handle in the dark.

Dye-2 Conjugated Anti-Mouse IgG Secondary Antibody – Dye-Conjugated Anti-Mouse IgG Antibody is used as the secondary antibody to detect the target bound, primary mouse antibodies. The solution is light sensitive. Please store and handle in the dark.

Primary Antibody Mixture P – Immediately before use, add 50 µl of 100x Anti-Histone H1 (Phospho-Thr17) Antibody and 50 µl of 100x Anti-GAPDH Antibody to 4,900 µl of Primary Antibody Diluent (for one plate, 96 wells). Gently mix and label the tube as “Primary Antibody Mixture P”.

Primary Antibody Mixture NP – Immediately before use, add 50 µl of 100x Anti-Histone H1 Antibody and 50 µl of 100x Anti-GAPDH Antibody to 4,900 µl of Primary Antibody Diluent (for one plate, 96 wells). Gently mix and label the tube as “Primary Antibody Mixture NP”.

Secondary Antibody Mixture – Immediately before use, for one plate, mix 3 ml of Dye-1 Conjugated Anti-Rabbit IgG Antibody and 3 ml of Dye-2 Conjugated Anti-Mouse IgG Antibody. Gently mix and label tube as “Secondary Antibody Mixture”. This solution is light sensitive. Please store and handle in the dark.

10. Additional Materials Required

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

- Fluorescent plate reader with two channels at Ex/Em: 651/667 and 495/521
- 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)

11. 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 adjustments are required.

a. 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 prior to cell seeding.

b. 8% Formaldehyde (not included) instead of 4% is required.

2) *Cell Number and Sensitivity:* The number of cells plated onto the 96-well plates depends on the expression level of Histone H1 protein in the cells, cell size, treatment conditions and incubation time. The cells used for testing should be around 75-90% confluency.

3) *Cell Treatment:* The cells can be treated with inhibitors, activators, stimulators (ie. chemicals, proteins/peptides) or a combination of the substances listed above. Stimulation of cells should be controlled. Non-stimulated wells of cells should be included within the same plate.

Note: Plate cover must be off during treatment of cells.

Note: Cell death via various stimulations may occur and should be factored into experimental design.

4) Positive, Negative and Blank Controls:

a. Positive Control: Mouse Anti-GAPDH Antibody is an internal positive control used to normalize the RFU values of the target protein in each well.

b. Negative Control: Incubation of only Secondary Antibody Mixture. 50 µl of Primary Antibody Diluent is incubated instead of Primary Antibody Mixture in these wells.

c. Blank Control: Incubation of 50 µl Primary Antibody Diluent instead of any Antibody Mixture.

The Positive, Negative and Blank controls should be performed in the same plate with the Phospho-Histone H1 target experiments.

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

12. Assay Protocol

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

1) Seed 200 µl of desired cell concentration in culture medium into each well of the 96-well plates. 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.

Note: Treatment of cells may result in cell death should be considered prior to treatment.

Note: Vigorous pipetting may knock cells off the plate. Utilize the well walls to dispense/aspirate gently at each step.

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.

a. 4% formaldehyde is used for adherent cells.

b. 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 3 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 3 minutes each time with gentle shaking on the shaker.

9) Dispense 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 3 minutes at a time with gentle shaking on the shaker.

11) Add 50 µl of “Primary Antibody Mixture P” to corresponding wells for Histone H1 (Phospho-Thr17) detection. Add 50 µl of “Primary Antibody Mixture NP” to the corresponding wells for total Histone H1 detection. Cover the plate 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.

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

13) Add 50 µl of “Secondary Antibody Mixture” to corresponding wells and incubate for 1.5 hours at room temperature with gentle shaking.

Note: The plates should be kept in the dark for each step after addition of “Secondary Antibody Mixture”.

14) Wash 3 times with 200 µl of 1x Wash Buffer for 3 minutes at a time, with gentle shaking on the shaker. Afterwards, rinse once with 200 µl of 1x TBS. Keep the plate(s) in the dark during wash.

15) Read the plate(s) at Ex/Em: 651/667 (Dye 1) and 495/521 (Dye 2). Shield plates from direct light exposure.

13. Data Normalization

Anti-Histone H1 Antibody Normalization

The RFU values obtained for the phosphorylated target protein can be normalized using the RFU values obtained for the non-phosphorylated target protein via the proportion, RFU (Anti-Histone H1 (Phospho-Thr17) Antibody)/RFU (Anti-Histone H1 Antibody). This assumes that both RFUs included in the equation are derived from the same read under the same excitation and emission wavelengths.

GAPDH Normalization

The RFU values obtained for the target protein (phosphorylated and non- phosphorylated) can be normalized using the RFU values obtained for GAPDH.

Anti-GAPDH Stimulated RFU/ Anti-GAPDH Non-Stimulated may be used as a ratio in determining Cell Density. This ratio may be used as a multiplier for Anti-Target Phospho Stimulated RFU values in comparison to Anti-Target Non-Stimulated RFU values. The calculated ratios of RFU values are meant to be a reference point for qualitative measurement of levels of expression between wells.

14. Troubleshooting Guide

Problem	Causes	Solutions
Low signal	Storage and Expiration	Read Manual for storage condition and expiration
	Antibody Dilution	Dilute antibody 1:100 with Antibody Diluent.
	Low Cell Number	Seed more cells.
	Cells Detach from Well-Bottoms	Add solutions slowly from the side wall of the wells. Use Poly-L-Lysine to coat the plate period seeding the cells, if necessary.
High Background	Washing	Remove Wash Buffer completely.
	High Cell Number	Reduce the amount of seeded cells.
Variation	Pipetting	Check and/or calibrate pipettes
	Washing	Remove Wash Buffer completely
	Cells Detach from Well-Bottoms	Add solutions slowly from the side wall of the wells. Use Poly-L-Lysine to coat the plate period seeding the cells, if necessary.

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Contact Details

Email: info@assaygenie.com

Web: www.assayenie.com