CytoGlow™

Histone H2B (Acetyl-Lys126)
Colorimetric Cell-Based ELISA Kit

Catalog #: CBA1014
Reactivity: Human, Mouse, Rat

Storage: 4°C
Expiration: 6 months from receipt

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

Manual Version: 45.7.1527

CONTENTS

Introduction.....3
Colorimetric Cell-Based ELISAs
Histone H2B (Acetyl-Lys126) Cell-Based Specificity

Assay Format.....4

Materials Included/ Storage and Stability.....5

Additional Materials Required.....5

Antibody Specificity.....6
Anti- Histone H2B (Acetyl-Lys126) Antibody WB
Anti-Histone H2B Antibody WB
Anti-GAPDH Antibody WB

Reagent Preparation.....7

Assay Protocol.....8

Data Analysis.....10

Short Protocol.....11

Health and Safety Precautions/Assay Restrictions.....12

Troubleshooting Guide.....13

ELISA Plate Template.....14

Technical Support.....15

ASSAY PRINCIPLES

Histone H2B (Acetyl-Lys126) Colorimetric Cell-Based ELISA

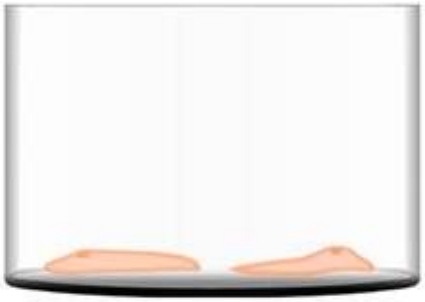
The Histone H2B (Acetyl-Lys126) Colorimetric Cell-Based ELISA Kit allows for the detection of various target proteins and the effects that certain stimulation conditions have on target protein expression in different cell lines. Qualitative determination of target protein concentration is achieved by 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.

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, allowing for generation of a standard curve and subsequent determination of protein concentration.

The Histone H2B (Acetyl-Lys126) Cell-Based ELISA Kit is a convenient, lysate-free, high throughput and sensitive assay kit that can monitor target protein Acetylation and expression profile in cells. The kit can be used for measuring the relative amounts of acetyled proteins in cultured cells as well as screening for the effects that various treatments, inhibitors (ie. siRNA or chemicals), or activators have on Histone H2B (Acetyl-Lys126).

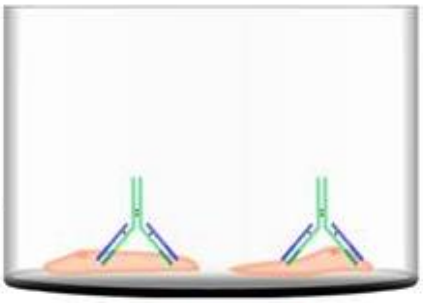
ASSAY FORMAT

α-Target
Primary Antibody



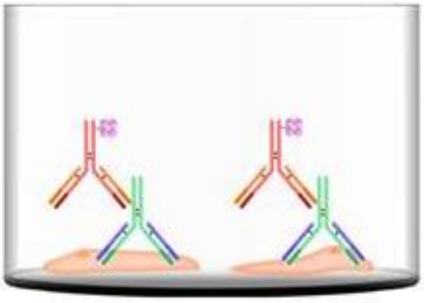
Cells are seeded onto the bottom of each well. The cells are quenched, fixed and the well is blocked.

α-Primary IgG
Secondary Antibody



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

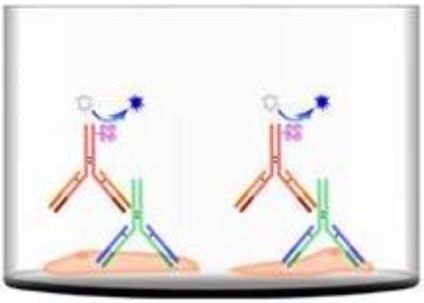
HRP Conjugate



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.

MATERIALS INCLUDED

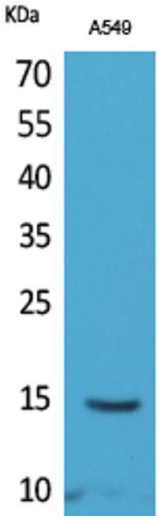
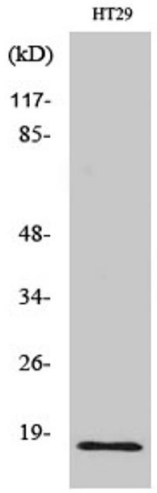
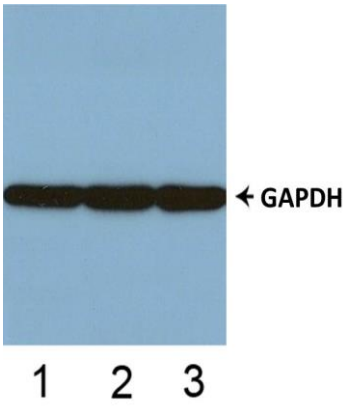
Reagent	Quantity	Storage/Stability after first use
96-Well Cell Culture Clear-Bottom Microplate	2 Plates	-
10x TBS	24 ml (10x)	1 month at 4°C
Quenching Buffer	24 ml (1x)	
Blocking Buffer	50 ml (1x)	
15x Wash Buffer	50 ml (15x)	
100x Anti-Histone H2B (Acetyl-Lys126) Antibody	60 µl (100x)	
100x Anti-Histone H2B Antibody	60 µl (100x)	
100x Anti-GAPDH Antibody	60 µl (100x)	
HRP-Conjugated Anti-Rabbit IgG Antibody	12 ml (1x)	
HRP-Conjugated Anti-Mouse IgG Antibody	12 ml (1x)	
Primary Antibody Diluent	12 ml (1x)	
Ready to Use Substrate	12 ml (1x)	
Stop Solution	12 ml (1x)	
Crystal Violet Solution	12 ml (1x)	
SDS Solution	24 ml (1x)	
Adhesive Plate Seals	2 Seals	-

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% paraformaldehyde stock concentration or 8% paraformaldehyde from other sources**
- Deionized or sterile water
- Squirt bottle, multichannel pipette and/or automated microplate washer
- 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, Incubator, and Stimulants
- **Poly-L-Lysine or Poly-D-Lysine for suspension or loosely attaching cells**

ANTIBODY SPECIFICITY

	**Figure 1.** ***Anti-Histone H2B (Acetyl-Lys126)*** Western Blot analysis of A549 cells using Acetyl-Histone H2B (K126) Polyclonal Antibody. Secondary antibody(catalogNo.:SA0002) was diluted at 1:20000
	**Figure 2.** ***Anti-Histone H2B Antibody WB*** Western Blot analysis of various cells using Histone H2B Polyclonal Antibody cells nucleus extracted by Minute TM Cytoplasmic and Nuclear Fractionation kit (SC-003,Inventbiotech,MN,USA).
	**Figure 3.** ***Anti-GAPDH Antibody WB*** Western blot analysis of tissue lysates from human (1), mouse (2) and rat (3).

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 (Acetylation Specific, Non-Acetylation Specific, and Anti-GAPDH) by using the Primary Antibody Diluent at a ratio of 1:100.

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-Histone H2B (Acetyl-Lys126) Antibody and 1x Anti-Histone H2B 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.

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-Histone H2B (Acetyl-Lys126)/OD595 Crystal Violet.

b. OD450 anti-Histone H2B/OD595 Crystal Violet.

By doing so, the expression levels of Histone H2B (Acetyl-Lys126) and Histone H2B are adjusted to account for the cell density present in each well.

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

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

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

OD450 (CV Normalized) (Histone H2B (Acetyl-Lys126))/

OD450 (CV Normalized) (Histone H2B)

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

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.

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.

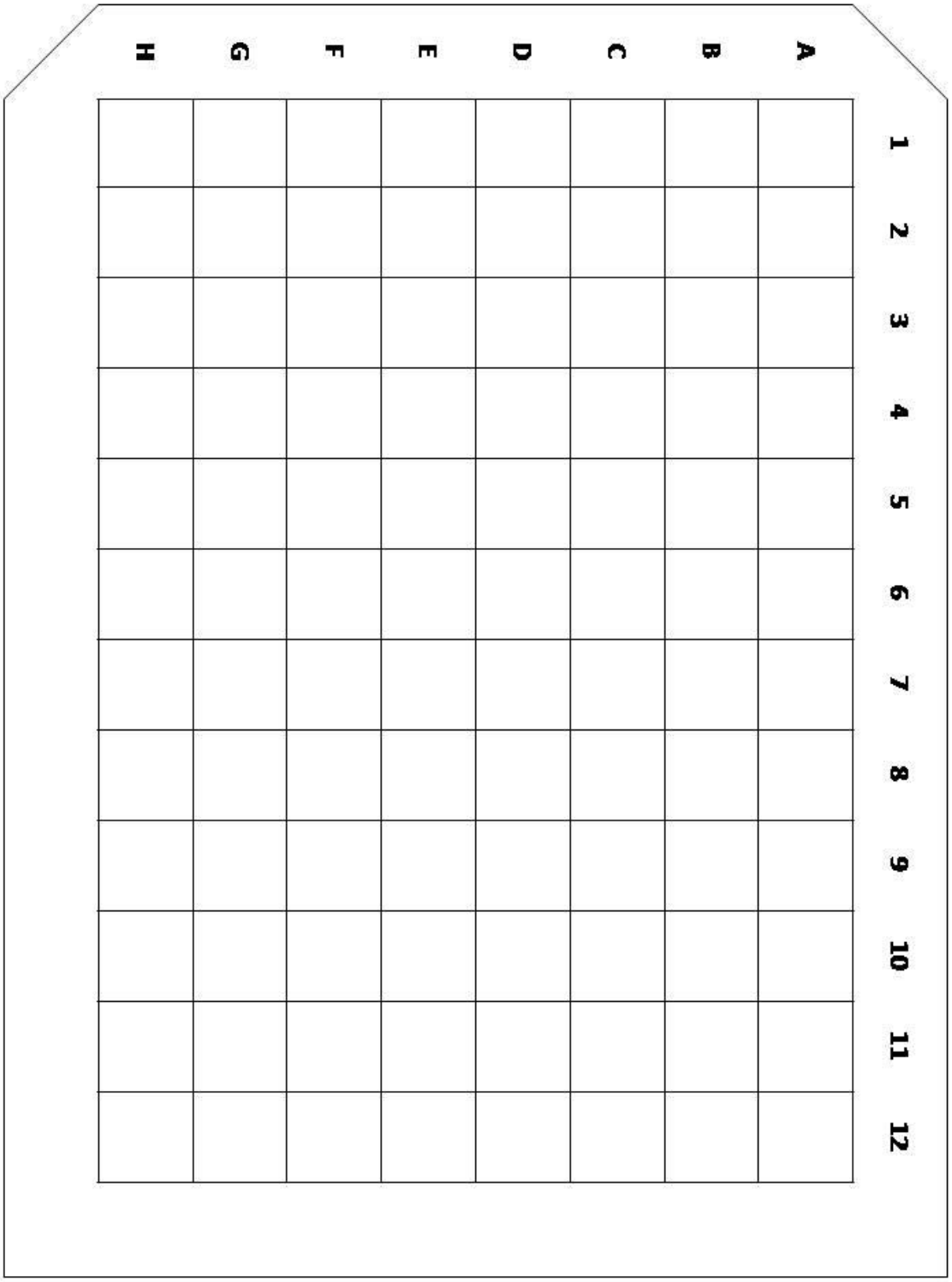
- Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.

- 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.

TROUBLESHOOTING GUIDE

Problem	Cause	Solution
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.

ELISA PLATE TEMPLATE



TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to www.assaybiotech.com or contact us at:

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