



Cell-Based ELISA Kit for Measuring Acetylation of Histones *in situ*

CycLex[®] Cellular Histone Acetylation

Assay Kit

Cat# CY-1140

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Intended Use

The CycLex Research Product **Cellular Histone Acetylation Assay Kit** is used for the semi-quantitative measurement of histone acetylation level *in situ* by means of cell-based ELISA.

Applications for this kit include:

- 1) Monitoring the effects of pharmacological agents on histone acetylation in cells.
- 2) Screening inhibitors of HDAC in cells.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

?? Upon receipt store all other components at 4°C; Do not expose reagents to excessive light



Introduction

Acetylation and deacetylation of nucleosomal histones play an important role in the modulation of chromatin structure, chromatin function and in the regulation of gene expression. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two opposing classes of enzymes, which tightly control the equilibrium of histone acetylation. An imbalance in the equilibrium of histone acetylation has been associated with carcinogenesis and cancer progression. So far, a number of structurally distinct classes of compounds have been identified as HDAC inhibitors including the short-chain fatty acids, hydroxamates, cyclic tetrapeptides and benzamides. These compounds lead to an accumulation of acetylated histone proteins both in tumor cells and in normal tissues. HDAC inhibitors are able to activate differentiation, to arrest the cell cycle in G1 and/or G2, and to induce apoptosis in transformed or cancer cells. Attention is currently being drawn to molecular mechanisms involving histone deacetylases. An induction of p21^{WAF1} and a suppression of angiogenic stimulating factors have been observed in tumor cells following exposure to HDAC inhibitors. In xenograft models, several HDAC inhibitors have demonstrated antitumor activity with only few side effects. Several clinical trials showed that HDAC inhibitors in well-tolerated doses have significant antitumoral activities. A combination of HDAC inhibitors with differentiation-inducing agents and cytotoxic drugs is an innovative therapeutic strategy that carries the potential for significant improvements in the treatment of cancer.

Principle of the Assay

The CycLex's Histone Acetylation Kit is a cell-based ELISA formatted for conventional chromometric detection of relative levels of acetylated histones in microplate cells cultures. Adherent cells are cultured in conventional 96-well microplates, treated with agents that induce histone acetylation, such as Trichostatin A, a specific HDAC inhibitor, and are then fixed and permeabilized. Several types of histones, including histone H3 and H4, which are acetylated at several lysine residues are detected by anti-acetylated histone/p53-K382 monoclonal antibody, clone TM-5C5 and an anti-mouse IgG-HRP (horseradish peroxidase) conjugate. The chromogenic HRP substrate TMB is then added, and signal is measured in a microplate reader.



CycLex's CycLex's **Histone Acetylation Kit** is designed to measure the relative levels of histone acetylation in situ. The summary of the assay is shown in below.

Summary of Procedure

Culture adherent cells in microplate at 40-60 % confluency

↓ **Incubate O/N at 37°C in CO₂ incubator**

Add appropriate amount of test compound for induction of histone acetylation

↓ **Incubate appropriate time at 37°C in CO₂ incubator**

Discard the culture medium and wash the microplate

↓

Add 150 µL of ice-cold 95 % methanol for fixation

↓ **Stand for 10 min at room temp. (20-25°C)**

Discard the methanol

↓

Add 200 µL of Blocking Reagent

↓ **Incubate for at least 2 hr at 37°C or O/N at 4°C**

Add 50 µL of Anti-acetylated histone/p53-K382 Monoclonal Antibody

↓ **Incubate 1 hr at room temp. (20-25°C)**

Wash the wells

Add 50 µL of HRP conjugated Anti-Mouse IgG

↓ **Incubate 1 hr at room temp. (20-25°C)**

Wash the wells

Add 50 µL of Substrate Reagent

↓ **Incubate 10-15 min at room temp. (20-25°C)**

Add 50 µL of Stop Solution

↓

Measure absorbance at 450 nm



Materials Provided

All compounds treatment and positive control (Trichostatin A treatment) should be assayed in duplicate. The following components are supplied and are sufficient for the two 96-well microplates.

Microplate: Two 96-well cell culture plates

100X Trichostatin A: One vial containing 50 μ L of 50 μ M Trichostatin A in DMSO

10X Wash Buffer: One 100 mL bottle of 10X buffer containing 2% Tween[®]-20

Blocking Reagent: Two bottles containing 20 mL of 1X Blocking Reagent. Ready to use.

Primary Antibody Solution (Anti-Acetylated Histone/p53-K382 Monoclonal Antibody TM-5C5): One vial containing 12 mL of Anti-Acetylated Histone/p53-K382 Monoclonal Antibody. Ready to use.

Secondary Antibody Solution (HRP conjugated Anti-Mouse IgG): One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-mouse IgG polyclonal antibody. Ready to use.

Substrate Reagent: 12 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle supplied ready to use, containing 12 mL of 0.5 N H₂SO₄.

Materials Required but not Provided

- Cell culture flasks for growing and splitting cells.
- Cell culture media.
- Ice-cold 95 % Methanol for 1st fixation of cells
- 1% paraformaldehyde in PBS for 2nd fixation of cells
- 1X PBS pH 7.2
- Pipettors: 2-20 μ L, 20-200 μ L and 200-1000 μ L precision pipettors with disposable tips.
- Precision repeating pipettor
- Orbital microplate shaker
- Microcentrifuge and tubes for sample preparation.
- Vortex mixer
- Microplate washer: optional (Manual washing is possible but not preferable)
- Software package facilitating data generation and analysis :optional
- 500 or 1000 mL graduated cylinder.
- Reagent reservoirs.
- Deionized water of the highest quality.
- Absorbent paper: disposable paper towels
- Plate reader capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.



Precautions and Recommendations

Safety Warnings and Precautions: The Cellular Histone Acetylation Assay Kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

Technical Notes

1. When performing washes manually, avoid introducing bubbles when dispensing liquids into the wells, and ensure each well is filled with buffer, but not overflowing to avoid cross-contamination between wells. Empty wells with a wrist-flick motion over an appropriate receptacle, and while still inverted, blot any remaining moisture onto clean absorbent paper.
2. Agitation of wells during incubation of Blocking Buffer and Antibody steps is recommended to reduce non-specific background. If microtiter plate agitator is not available, a platform vortex at a low setting can be used (e.g. level 1 of Fisher's Genie II platform vortex). If background problems occur, simply increase the number and/or duration of washes.
3. A brief 1X PBS rinse is recommended prior to the addition of the HRP substrate to remove any traces of the Tween-20™ which can interfere with the HRP activity.
4. Do not allow the wells to dry out during the protocol.
5. Incubation temperatures for Primary Antibody and Detection Antibody can be varied and should be empirically determined.

General Notes

- Allow all the components to come to room temperature before use.
- Do not use kit components beyond the indicated kit expiration date.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain NaN_3 as preservatives. Care should be taken to avoid direct contact with these reagents.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**



Detailed Protocol

The CycLex Research Product **Cellular Histone Acetylation Assay Kit** includes all reagents for detection of Histone acetylation in cultured cells, except cell fixative. Since experimental conditions may vary, treatment cells with Trichostatin A within the kit should be included in each experiment as a positive control for induction of histone acetylation. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Reagents

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of 10X Wash Buffer.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the 10X Wash Buffer (provided) to 900 mL of deionized (distilled) water. Mix well.
2. 95 % MetOH: For each 96-well plate, add 1 mL H₂O to 19 mL of methanol. Cool in -25°C freezer. This solution must be prepared fresh. Discard unused portion following assay completion.
3. 1% paraformaldehyde in PBS : For each 96-well plate, dissolve 0.2 g of paraformaldehyde in 20 mL of PBS pH7.2. This solution must be prepared fresh. Discard unused portion following assay completion.

Assay Procedure

A. Culture adherent cells in 96-well microplate and treatment with compounds

1. Plate adherent cells in 96-well microplate at 40-60 % confluency.
2. Incubate the microplate at 37°C over night in CO₂ incubator.
3. Add appropriate amount of test compounds to each well. Trichostatin A treatment* should be run in duplicate as a positive control for induction of histone acetylation. Please include vehicle control, e.g. DMSO in case of Trichostatin A.
4. Incubate the microplate at 37°C for appropriate time.

****Trichostatin A treatment: Treat the cells with 0.5 μM Trichostatin A for 0, 0.25, 0.5, 1, 2, 3, 4 and 5 hours.***



B. Fixing cells to 96-well micro plate and blocking (Single Fixation protocol)

Fixing of the cells in the 96-well plates should be done as soon as the desired treatment has completed.

1. Remove media from wells with a wrist-flick. Avoid touching the bottom of the well and removing cells.
2. Immediately add **150 μ L/well** of **95 % MetOH** as a fixative. Add the fixing solution slowly to insure cells are not detached from the plastic. Let stand for 10 minutes at room temperature.
3. Remove fixing agent from wells with a wrist-flick. While still inverted, tap the plate gently onto absorbent paper to remove any excess fixing agent still within the wells.
4. Add **200 μ L/well** Wash Buffer. Let stand for 1 minute at room temperature.
5. Remove wash buffer with a wrist flick. While still inverted, gently tap the plate onto absorbent paper to remove any excess liquid.
6. Add **200 μ L/well Blocking Reagent** and incubate for 2 hour at 37°C or overnight at 4°C.

Alternatively: In cases where the cells are easy to detach from 96-well plates even after 1st fixation.

B'. Fixing cells to 96-well microplate and blocking (Double Fixation protocol)

- 1'. Remove media from wells with a wrist-flick. Avoid touching the bottom of the well and removing cells.
- 2'. Immediately add **150 μ L/well** of **95 % MetOH** as a fixative. Add the fixing solution slowly to insure cells are not detached from the plastic. Let stand for 10 minutes at room temperature.
- 3'. Remove fixing agent from wells with a wrist-flick. While still inverted, tap the plate gently onto absorbent paper to remove any excess fixing agent still within the wells.
- 4'. Add **150 μ L/well** of **1 % paraformaldehyde** in PBS. Add solution slowly to ensure cells are not dislodged from the wells. Let stand for 5 minutes at room temperature
- 5'. Remove paraformaldehyde solution from wells with a wrist flick. While still inverted, gently tap the plate onto absorbent paper to remove any excess liquid still in the wells.
- 6'. Add **200 μ L/well** Wash Buffer. Let stand for 1 minute at room temperature.
- 7'. Remove wash buffer with a wrist flick. While still inverted, gently tap the plate onto absorbent paper to remove any excess liquid.
- 8'. Add **200 μ L/well Blocking Reagent** and incubate for 2 hour at 37°C or overnight at 4°C.



C. Detection of Signals (Addition of Primary and Secondary Antibodies and Substrate Reagent)

1. Remove **Blocking Reagent** with a wrist flick.
2. Rinse the wells once with **200 μ L/well** of **Wash Buffer**. This can be achieved either by using a multichannel pipette or a manifold.
3. Remove Wash Buffer with a wrist flick. While the plate is still inverted, tap onto absorbent paper to remove any excess buffer within the wells.
4. Add **50 μ L/well** of **Primary Antibody Solution** and incubate for 1 hour at room temperature.
5. Remove Primary Antibody with a wrist flick.
6. Rinse the wells **once** with **200 μ L/well** of **Wash Buffer**.
7. Remove Wash Buffer with a wrist flick. While still inverted, tap the plate onto absorbent paper.
8. Wash wells **4 times** with **200 μ L/well Wash Buffer** for 2 minutes each with shaking at ca. 200 rpm on an orbital microplate shaker. Remove Wash Buffer in-between each wash with a wrist flick.
9. Add **50 μ L/well** of **Secondary Antibody Solution** and incubate for 1 hour at room temperature.
10. Remove Secondary Antibody with a wrist flick.
11. Rinse wells **once** with **200 μ L/well Wash Buffer**.
12. Remove Wash Buffer with wrist flick and tap plate onto absorbent paper.
13. Wash wells **4 times** with **200 μ L/well Wash Buffer** for 2 minutes each with shaking at ca. 200 rpm on an orbital microplate shaker. Remove Wash Buffer in-between each wash with a wrist flick.
14. After last wash with Wash Buffer, rinse wells **once** with **300 μ L/well 1X PBS**. Remove with a wrist flick and tap onto absorbent paper. Ensure that that no liquid remains in the well.
15. **Add 50 μ L/well** of **Substrate Reagent**. (Avoid exposing the microplate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended). Return Substrate Reagent to 2-8°C immediately after the necessary volume is removed.
16. Incubate the plate for 10-15 minutes at room temperature. (The incubation time may be extended up to 20 minutes if the reaction temperature is below than 20°C).
17. Add **50 μ L** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.
18. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution*.



Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine of histone acetylation level of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Troubleshooting

1. The signals are influenced a great deal by cell line and cell number that you plated, please ensure the appropriate cell number for your experiment. See “Example of Test Results Fig.2 and 3”.
2. All treatments including treatment of Trichostatin A should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.
3. Poor duplicates, accompanied by elevated values for wells containing non-treated cells (vehicle control), indicate insufficient washing or vigorous washing. **Wash the plate thoroughly and gently.**
4. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the CycLex Research Product **Cellular Histone Acetylation Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C.

For research use only, not for use in diagnostic or therapeutic procedures.

Example of Test Results

Fig. 1. Typical result of time course experiment using MCF7, a breast cancer cell line, treated with 0.25 μ M Trichostatin A.

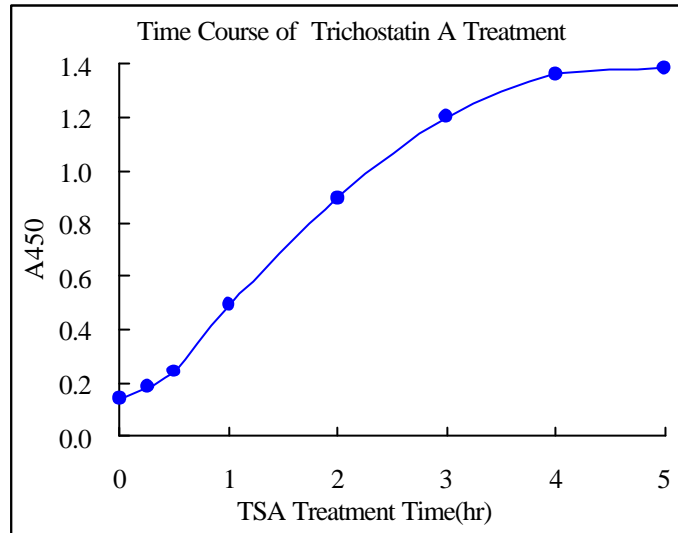


Fig. 2. Typical result of time course experiment using MCF7, HeLa and BALB/c 3T3 cell lines treated with 0.25 μ M Trichostatin A.

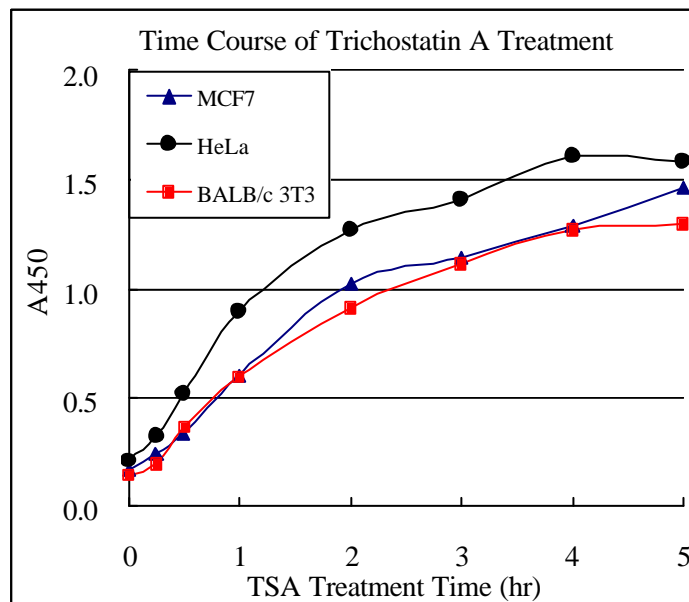
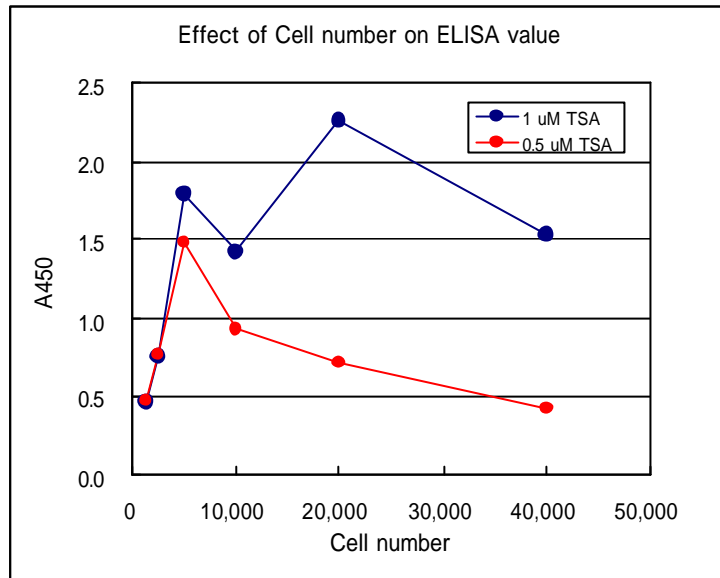


Fig. 3. Effect of cell number on ELISA value when MCF7 cell were treated with different concentration of Trichostatin A.





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Related Products

- *Cat# CY1150: CycLex HDAC Assay kit
- *Cat# CY1151: CycLex Sir2 Assay kit
- *Cat# CYM1029: Acetylated Histone/p53-K382 Monoclonal Antibody TM-5C5

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