



AlphaLISA[®] Di-Methyl-Histone H3 Lysine 36 (H3K36me2) Cellular Detection Kit

Product number: AL723 C/F

Research Use Only. Not for use in diagnostic procedures.

Material provided:

Format: AL723C: 500 assay points AL723F: 5 000 assay points

The number of assay points is based on an assay volume of 50 µL in 384-well assay plates using the kit components at the recommended concentrations.

Document version: 1

Product Information

Kit content: The kit contains 6 components: AlphaLISA Acceptor beads coated with an anti-epigenetic mark antibody, Streptavidin-coated Donor beads, Biotinylated anti-Histone H3 (C-terminus) Antibody, and Cell-Histone™ Lysis (1X), Extraction (1X) and Detection (10X) buffers.

Storage: Store kit in the dark at +4°C.

Stability: This product is stable for at least 12 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Application: This kit is designed for the detection of di-methylated Histone H3 Lysine 36 (H3K36me2) in cell lysates using a homogeneous AlphaLISA assay (no wash steps).

RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Precautions

- Only the Streptavidin (SA)-coated Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (Lee 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- The Biotinylated Anti-Histone H3 (C-ter) antibody contains sodium azide. Contact with skin or inhalation should be avoided.
- Sodium azide should not be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaLISA signal (0.0003% final in the assay).

Reagents and Materials

Reagents provided in the AlphaLISA kit are listed in the table below:

| Kit components | AL723C (500 assay points) | AL723F (5 000 assay points) |
|---|---|---|
| AlphaLISA anti-H3K36me2 Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2 | 100 µL @ 5 mg/mL (1 brown tube, <u>white cap</u>) | 1 mL @ 5 mg/mL (1 brown tube, <u>white cap</u>) |
| Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4 | 200 µL @ 5 mg/mL (1 brown tube, <u>black cap</u>) | 1 mL @ 5 mg/mL (1 brown tube, <u>black cap</u>) |
| Biotinylated Antibody anti-Histone H3 (C-ter) stored in PBS, 0.1% Tween-20, 0.05% NaN ₃ , pH 7.4 | 150 µL @ 500 nM (1 tube, <u>black cap</u>) | 1.5 mL @ 500 nM (1 tube, <u>black cap</u>) |
| Cell-Histone Lysis buffer | 20 mL | 200 mL |
| Cell-Histone Extraction buffer | 20 mL | 100 mL |
| Cell-Histone Detection (10X) buffer (see note) | 2.0 mL | 20 mL |

Note: The Cell-Histone Detection (10X) buffer may appear slightly cloudy. However, this will not affect assay performance.

Additional Reagents and Materials

The following items are recommended for the assays:

| Item | Supplier | Catalog number |
|---|----------|---------------------------------------|
| CulturPlate-384, white opaque with lid | Revvity | 6007680 (50/box) 6007688 (160/box) |
| TopSeal™-A Adhesive Sealing film | Revvity | 6050195 |
| EnSpire® or EnVision® Multilabel Alpha Reader | Revvity | Please consult our website |

The following reagents might be required for particular applications:

| Item | Supplier | Catalog number |
|---|-------------------|--|
| AlphaLISA Unmodified Histone H3 Lysine 4 Cellular Detection kit | Revvity | AL719C (500 assay points) AL719F (5 000 assay points) |
| AlphaScreen SureFire® GAPDH Assay Kit | Revvity | TGRGDS |
| ATPlite® Luminescence Assay System | Revvity | 6016941 (1 000 assay points) 6016947 (5 000 assay points) |
| AlphaLISA TruHits kit | Revvity | AL900D (1 000 assay points) AL900M (10 000 assay points) |
| OptiPlate-384, white opaque | Revvity | 6007290 (50/box) 6007299 (200/box) |
| CulturPlate-1536, white opaque with lid | Revvity | 6004680 (50/box) 6004688 (160/box) |
| ProxiPlate-384 TC | Revvity | 6008230 (50/box) 6008238 (160/box) |
| Histone H3 (K36me2) (31-41) peptide | AnaSpec | 64380 |
| Histone H3 (21-44) peptide | AnaSpec | 64454 |
| Halt Protease Inhibitor Cocktail (100X) | Thermo Scientific | 87786 (1 mL) |

Recommendations

General recommendations

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to prewet the tip.
- Centrifuge quickly all tubes before use to improve recovery of content (2 000 ×g, 10-15 sec). Resuspend all reagents by gentle mixing before use.
- Use Milli-Q® grade H₂O (18 MΩ•cm) to dilute AlphaLISA Cell-Histone Detection (10X) buffer.
- When reagents are added in the microplate, make sure the liquids are at the bottom of the well by tapping or swirling the plate gently on a smooth surface.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Sealing Film to reduce evaporation during incubation with the Alpha beads. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the ALPHA option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation time and temperature should be used for each plate.

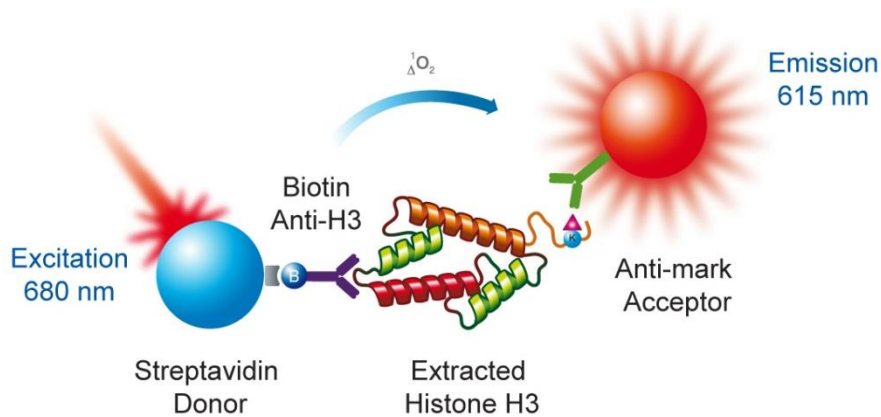
Specific recommendations

- Biotin present in culture medium may interfere with the binding of the biotinylated antibody to Streptavidin Donor beads. Use biotin-free culture medium when following the Universal Manual. Most cells will not be affected by overnight incubation in biotin-free medium. If longer incubation time is required, reduce biotin in culture medium by combining biotin-containing and biotin-free medium to find a condition not affecting cell growth or the Alpha signal. Alternatively, remove medium and follow the Wash Manual.
- For monitoring the determination of the H3K36me2 mark, keep DMSO under a maximal concentration of 3% when incubating compound with cells.
- It is recommended to confirm compound modulation by performing Western Blot analysis of the mark of interest.
- Evaporation can be problematic with cells cultured in microtiter plates. For overnight incubation, it is recommended to add warm PBS or sterile water to unused wells. Alternatively, cells can be cultured in larger wells, or in a larger volume of culture medium. The following modifications to the Universal Manual will then apply:
 1. Following compound treatment, transfer the desired number of cells resuspended in 15 µL PBS to an OptiPlate-384 microtiter plate. Continue with the Lysis step of the Universal Manual.
 2. Lyse adherent cells directly in the culture plate by upscaling the volume of Cell-Histone Lysis buffer to the volume of medium or PBS in the well. Transfer 20 µL of cell lysate to an OptiPlate-384 microtiter plate. Add the Cell-Histone Extraction buffer as indicated in the Universal Manual. See also the Wash Manual section for adherent cells.
- The Cell-Histone Lysis buffer does not include protease inhibitors. Protease inhibitors can be added to the lysis buffer if cellular lysates are not to be used immediately.
- Effects on the Alpha signal of fetal bovine serum (FBS) and phenol red present in the culture medium are minimal. However, medium without phenol red or with reduced FBS concentration can be used to maximize signal and increase assay window with the Universal Manual.

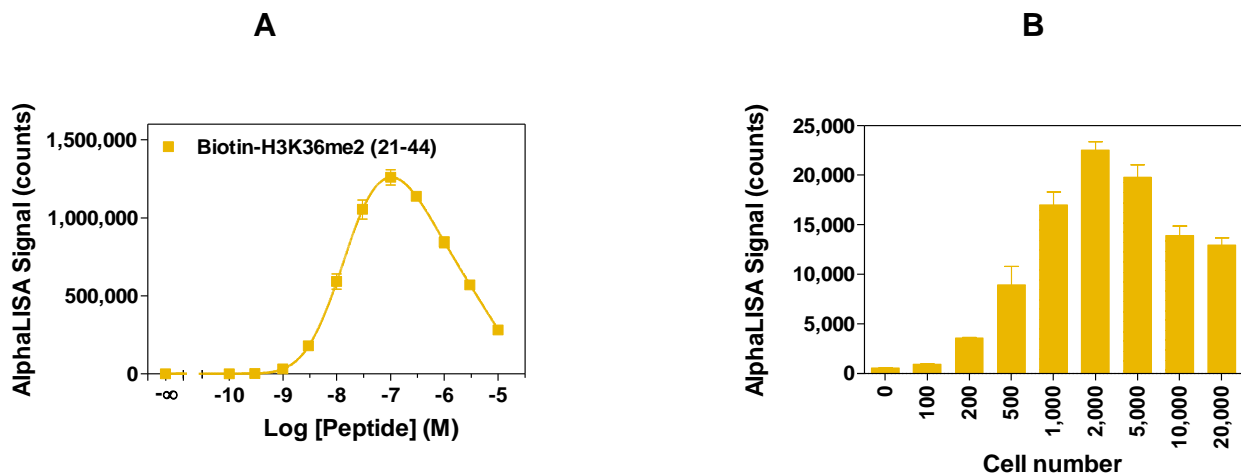
- It is recommended to include as controls cells or lysates treated with a known modulator.
- Assay specificity can be demonstrated by competing the binding of Acceptor beads to modified histones using peptides carrying marks of interest. See the Control Assays section for details.

AlphaLISA Detection of Epigenetic Marks in Cellular Extracts

The AlphaLISA detection of epigenetic marks in cellular extracts is performed as follows: cells cultured in the presence of compounds are lysed with the Cell-Histone Lysis buffer. Histones are then extracted from the nucleosomes by the addition of the Cell-Histone Extraction buffer. AlphaLISA anti-mark Acceptor beads and Biotinylated anti-Histone H3 (C-terminus) antibodies are then added for the capture of histone proteins carrying the mark of interest. After incubation, Streptavidin Donor beads are added for the capture of the biotinylated antibody. In the presence of histone proteins bearing the mark of interest, the beads come into proximity. Excitation of the Donor beads provokes the release of singlet oxygen molecules that trigger a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm.



Typical Data



Detection of the Histone H3K36me2 mark in peptide and cellular extracts. A) Representative QC data for Streptavidin Donor and anti-H3K36me2 Acceptor Beads. Results were generated using a biotinylated peptide titrated in QC buffer (50 mM Tris-HCl pH 8.0). **B)** AlphaLISA detection of H3K36me2 in cellular extracts. HeLa cells were seeded at densities ranging from 100 to 20 000 cells per well in 384-well culture plates. The cellular detection assay was performed as described in the Universal Manual. The hook effect observed at higher concentrations is typical of multi-component assays and occurs when the concentration of target molecules exceeds the binding capacity of the assay components. Signal was detected with an EnVision Alpha HTS instrument 2102.

A Technical Note presenting the optimization of a cellular detection assay of the H3K36me2 mark in HeLa cells is available on our website at www.revivity.com.

Universal Manual for Adherent and Suspension Cells (No Wash)

The manual described below is recommended for a 50 μ L assay volume in white opaque CulturPlate-384 microplates.

Please read the General and Specific Recommendation on page 4 before using this manual.

All reagent volumes should be adjusted for the number of assay points.

We recommend first titrating your cells, seeding them in triplicates at densities ranging from 200 to 20 000 cells per well (including a no-cell control). This initial titration can be performed with and without a compound modulating the epigenetic mark of interest, or using cell lines exhibiting different levels of the mark.

Cell Culture, Lysis and Histone Extraction

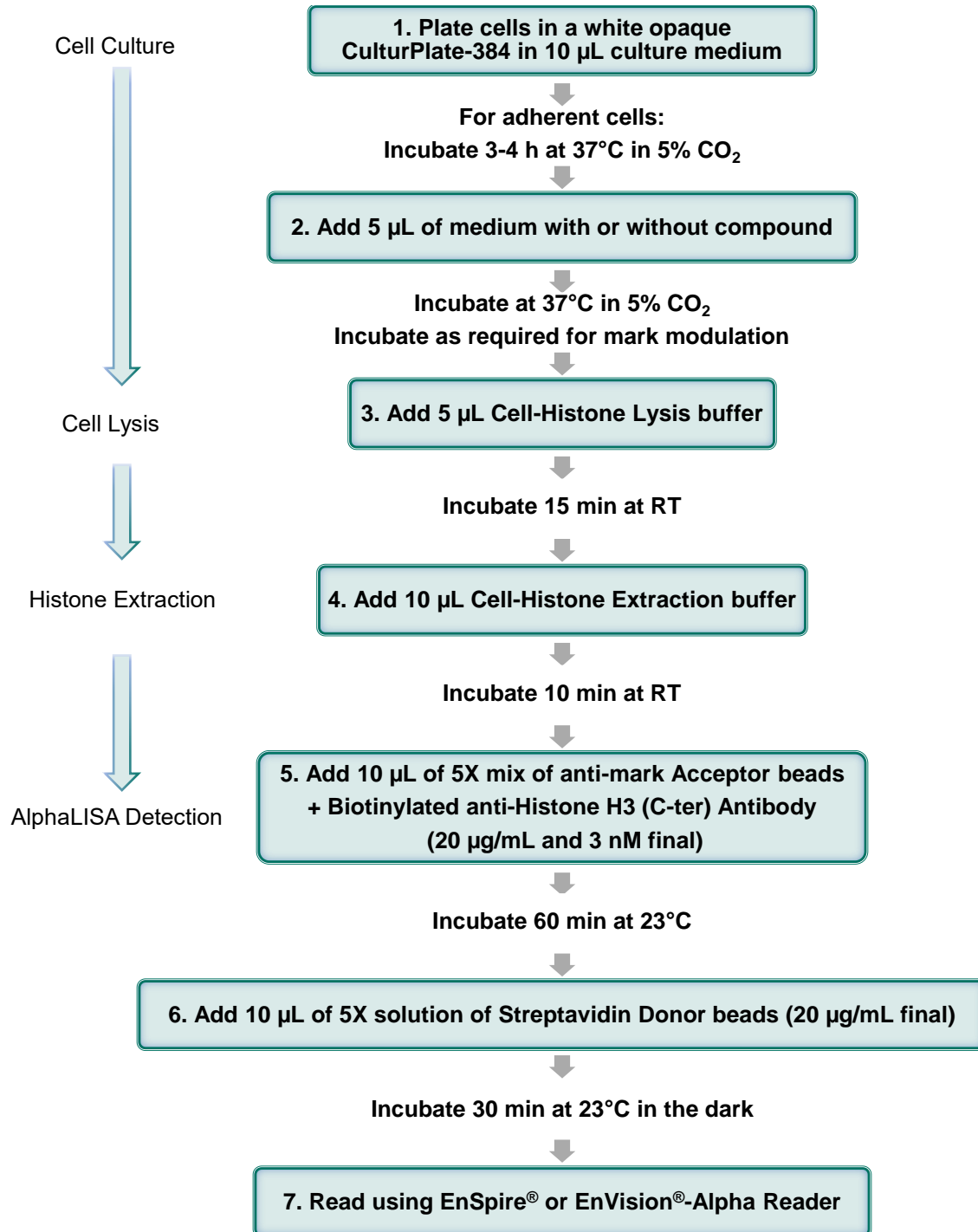
- Dispense 10 μ L of cells in culture medium in a white opaque CulturPlate-384 microplate.
- Incubate adherent cells for 3-4 h at 37°C in a 5% CO₂ atmosphere to allow cell adhesion. Skip this step for cells growing in suspension.
- Add 5 μ L of medium or compound prepared in medium at 3X its final concentration.
- Incubate cells for the time required for histone mark modulation.
- Add 5 μ L of Cell-Histone Lysis buffer.
- Incubate for 15 min at room temperature (RT).
- Add 10 μ L of Cell-Histone Extraction buffer.
- Incubate for 10 min at RT.

AlphaLISA Detection

- Dilute the 10X Cell-Histone Detection buffer to 1X with water.
- Prepare just before use, a 5X mix of AlphaLISA anti-mark Acceptor beads + Biotinylated anti-Histone H3 (C-ter) antibody in 1X Cell-Histone Detection buffer by diluting the anti-mark Acceptor beads to 100 μ g/mL and the biotinylated antibody to 15 nM. Final concentration of beads and antibody are 20 μ g/mL and 3 nM, respectively.
 - For example, prepare a 5X mix by adding 20 μ L Acceptor beads and 30 μ L biotinylated antibody to 950 μ L of 1X Cell-Histone Detection buffer. Adjust volumes according to the number of assay points.
- Add 10 μ L of the 5X mix containing AlphaLISA anti-mark Acceptor beads + Biotinylated anti-Histone H3 (C-ter) antibody.
- Cover with TopSeal-A film and incubate 60 min at 23°C.

- Working under subdued light, prepare a 5X solution of Streptavidin Donor beads in 1X Cell-Histone Detection buffer by diluting the beads to 100 µg/mL. Final concentration for Donor beads is 20 µg/mL.
 - For example, add 20 µL Donor beads to 980 µL of 1X Cell-Histone Detection buffer. Store dilution in the dark until use. Adjust volumes according to the number of assay points.
- Under subdued light, add 10 µL of the 5X solution of Streptavidin Donor beads.
- Cover with TopSeal-A film and incubate 30 min at 23°C in the dark.
- Read on an Envision® Multilabel or Enspire Alpha Reader.

Assay Diagram: Universal Manual (No Wash)



Wash Manual for Adherent Cells

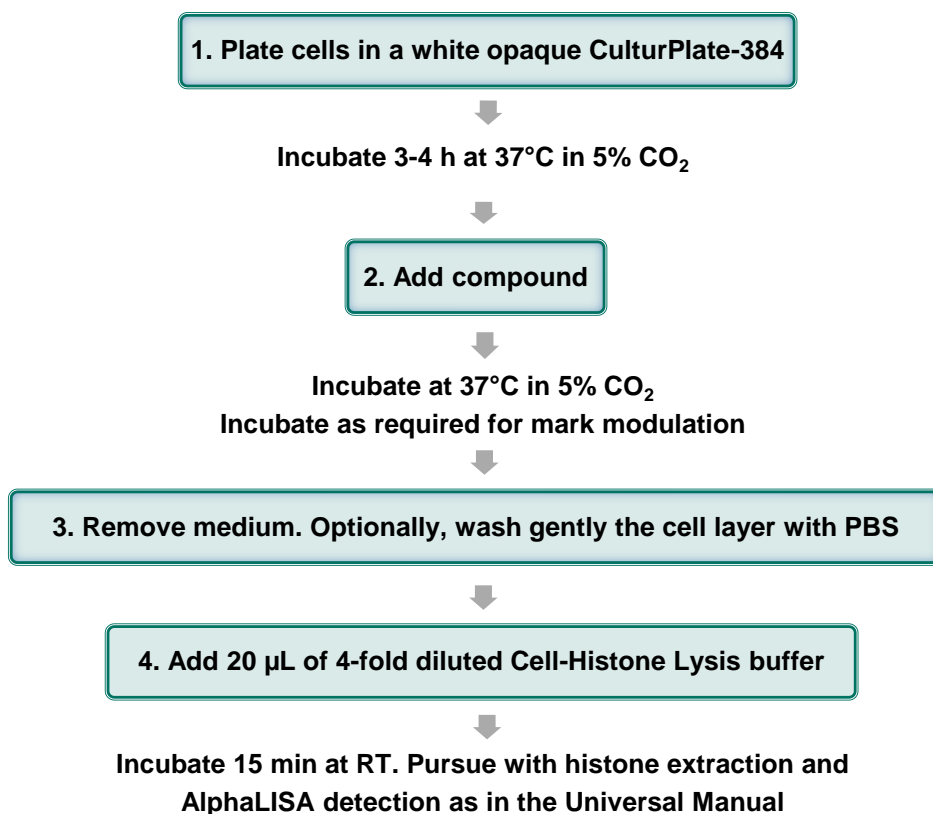
Removal of the original culture medium is recommended when cells are grown in medium containing biotin, high FBS or DMSO concentrations, or when compounds might interfere with the Alpha signal detection. Optionally, medium removal can be followed with a gentle wash of the cell layer with PBS.

Cell Culture, Lysis and Histone Extraction

- Dispense cells in culture medium in a white opaque CulturPlate-384 microplate.
- Incubate cells for 3-4 h at 37°C in a 5% CO₂ atmosphere to allow cell adhesion.
- Add medium, or compound prepared in culture medium at the desired concentration.
- Incubate for the time required for histone mark modulation.
- Dilute the Cell-Histone Lysis buffer 4-fold in H₂O.
 - For example, add 500 µL of Cell-Histone Lysis buffer to 1.5 mL of H₂O.
- Remove culture medium. Optionally, wash gently the cell layer with PBS, and then remove PBS from the wells.
- Add 20 µL of the 4-fold diluted Cell-Histone Lysis buffer.
- Incubate for 15 min at room temperature (RT).
- Add 10 µL of Cell-Histone Extraction buffer.
- Incubate for 10 min at RT.

Following histone extraction, proceed to the AlphaLISA detection as described in the Universal Assay Manual.

Assay Diagram: Wash Manual



Control Assays

Peptide Competitions

Specificity of the Alpha signal can be confirmed by performing peptide competition experiments, adding short peptides carrying the mark of interest to cellular extracts before the addition of Acceptor beads and biotinylated antibody.

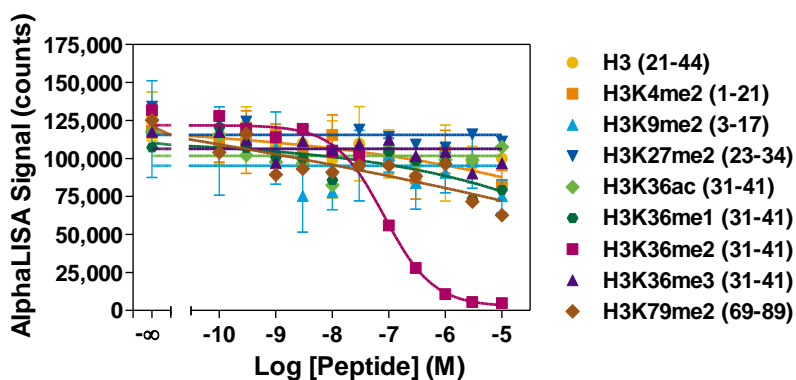
You will need to modify the Universal Manual as follows:

- Prepare 10X peptide solutions by diluting peptides serially in 1X Cell-Histone Detection buffer from 100 μM to 1 nM (final concentrations 10 μM to 100 pM).

| Tube | Volume of peptide | Volume of 1X Cell-Histone Detection Buffer (μL) | [peptide] (M) in 10X peptide dilutions |
|------|---|--|--|
| A | 30 μL of 500 μM stock | 120 | 1E-04 |
| B | 60 μL of tube A | 140 | 3E-05 |
| C | 60 μL of tube B | 120 | 1E-05 |
| D | 60 μL of tube C | 140 | 3E-06 |
| E | 60 μL of tube D | 120 | 1E-06 |
| F | 60 μL of tube E | 140 | 3E-07 |
| G | 60 μL of tube F | 120 | 1E-07 |
| H | 60 μL of tube G | 140 | 3E-08 |
| I | 60 μL of tube H | 120 | 1E-08 |
| J | 60 μL of tube I | 140 | 3E-09 |
| K | 60 μL of tube J | 120 | 1E-09 |
| L | 0 | 100 | 0 |

- Prepare just before use, a 10X mix of AlphaLISA anti-mark Acceptor beads + Biotinylated anti-Histone H3 (C-ter) antibody in 1X Cell-Histone Detection buffer by diluting Acceptor beads to 200 $\mu\text{g}/\text{mL}$ and biotinylated antibody to 30 nM.
 - For example, add 20 μL Acceptor beads and 30 μL biotinylated antibody to 450 μL of 1X Cell-Histone Detection buffer. Adjust volumes according to the number of assay points.
- Follow Universal Manual and stop at the end of the Extraction steps.
- Add 5 μL of 10X peptide dilutions to the wells
- Add 5 μL of 10X mix containing AlphaLISA anti-mark Acceptor beads + Biotinylated anti-Histone H3 (C-ter) antibody.
- Cover with TopSeal-A film and incubate 60 min at 23°C.
- Continue with the preparation and addition of the Donor beads as described in the Universal Manual.

Peptide Competition Data



HeLa cells were seeded at a density of 1,000 cells per well. Serial dilutions of histone H3-derived peptides bearing various epigenetic marks were added to the wells at concentrations ranging from 100 pM to 10 μ M just before the addition of the AlphaLISA detection reagents. The H3K36me2 peptide competed for the interaction between the Acceptor beads and histone proteins with an IC₅₀ value of 83 nM.

Assay Normalization

Results can be normalized and cell toxicity evaluated using one of the methods listed below:

- Estimation of total histone levels using the AlphaLISA Unmodified H3K4 Cellular Detection kit
 - i. This kit measures the unmodified H3K4 mark, which abundance is estimated at 92.6% in HeLa cells (Peach et al. Mol. Cell Proteomics 2012, **11**:128-37). More so, at equivalent seeding densities, H3K4 levels remain constant amongst cell lines. The detection of unmodified H3K4 appears to be a very robust method for assessing total histone levels and toxicity by modulators.
- ATPlite Luminescence Assay System (Revvity)
- AlphaScreen SureFire GAPDH Assay Kit (Revvity)
- Evaluation of cell density by Imaging Microscopy

Compound Interference

When using the no wash Universal Manual, compounds remain present for the AlphaLISA detection step. In some cases, these could interfere with the Alpha signal.

Compound Interference can be detected using different methods:

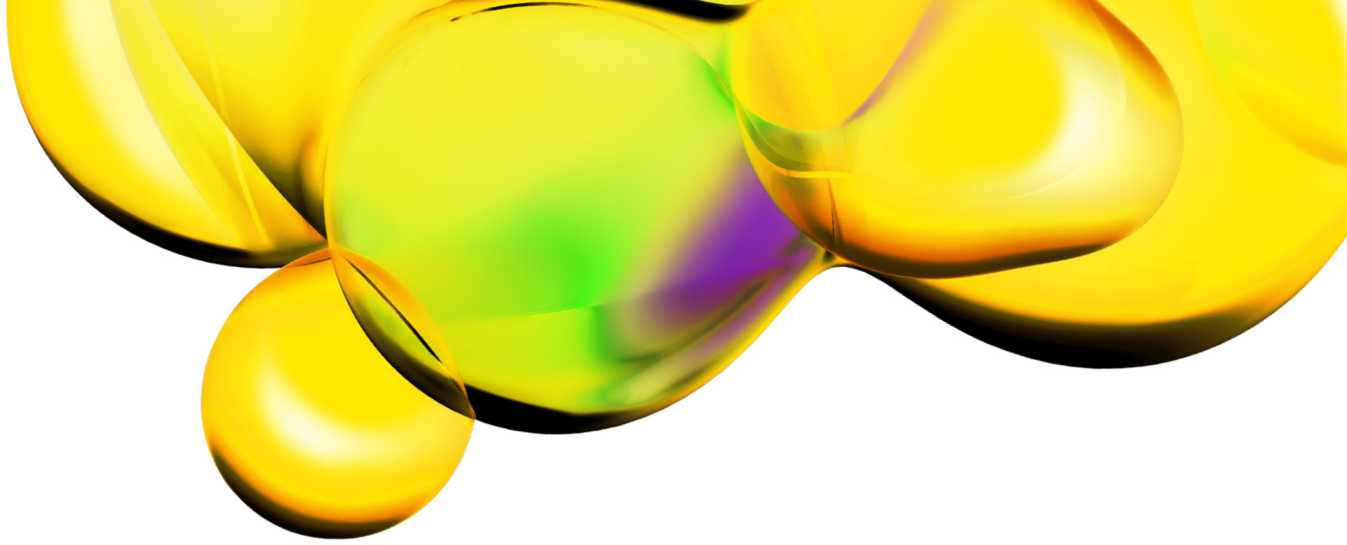
1. The AlphaScreen TruHits kit will allow you to determine if and how your compound interferes with the generation of the Alpha assay signal. Follow the instructions provided with the kit.
2. You may also include control wells in your culture plate where cells are plated without compound in a volume of 10 μ L of culture medium.
 - Grow cells as you normally would.
 - Proceed with cell Lysis and histone Extraction as described in the Universal Manual.
 - Following the Extraction step, add compound prepared in 5 μ L culture medium.
 - Proceed with the Alpha Detection as indicated in the Universal Manual.
 - Compare signal of the control wells with that of untreated wells.

If compound interference is detected, decrease compound concentration to reduce interference, or use the Wash Manual.

Troubleshooting Guide

You will find below recommendations for common situations that you might encounter with your AlphaLISA Epigenetics Cellular detection assay. If further assistance is needed, do not hesitate to contact our technical support team for assistance at www.revvity.com.

| Issue | Recommendations and Comments |
|---|---|
| No signal increase in cell titration experiment | <ul style="list-style-type: none"> • Endogenous mark levels might be too low for detection in your cell line. Try increasing mark level by compound treatment (e.g. 100 μM 5-carboxy-8-hydroxyquinoline for 48 h for the H3K9me2 mark). • It is recommended to include in your experiment a control for the assay. • Try one of the cell lines and assay conditions shown in the Typical Data section. • <u>Universal Manual</u>: use biotin-free culture medium, or culture medium with reduced biotin concentration. |
| No mark modulation upon compound treatment | <ul style="list-style-type: none"> • Verify by Western blot that the mark is actually modulated by compound. • Treat cells for different time periods and at different compound concentrations. • Try a different cell line or different compounds • Verify the Alpha signal specificity by peptide titration (see Control Assays section). |
| Modulation of mark in Alpha does not correlate with Western blot data | <ul style="list-style-type: none"> • Specificity of Western blot antibody could be different from that of Acceptor beads. • Verify the Alpha signal specificity by peptide titration (see Control Assays section). |
| Signal lower in the Universal Manual than in the Wash Manual | <ul style="list-style-type: none"> • Use biotin-free culture medium. • Use culture medium without phenol red and/or with lower FBS concentration. • Make sure treating compound is not interfering with the Alpha signal. (see Control Assays section) |
| High variation between replicates or low Z' values | <ul style="list-style-type: none"> • <u>Universal Manual</u>: make sure that reagents are at the bottom of the well by tapping or swirling the plate gently on a smooth surface after each addition. Be particularly careful when adding the 5 μL Cell-Histone Lysis buffer to the wells. • Rotate the plate to 120 rpm after Cell-Histone Lysis buffer addition to ensure efficient mixing of assay components. • <u>Wash Manual</u>: use if possible an automated plate washer, or remove medium carefully without disrupting the cell layer. Culture poorly adhering cells (e.g. HEK-293) on poly-lysine coated plates. • <u>All manuals</u>: lengthen incubation time with the Alpha Donor beads up to 18 hours before measuring the Alpha signal. |
| Insufficient assay window | <ul style="list-style-type: none"> • <u>Universal Manual</u>: use medium without phenol red or with lower FBS concentration to maximize signal. • <u>All manuals</u>: lengthen incubation time with the Alpha Donor beads up to 18 h with before measuring the Alpha signal. • See "Signal lower in the Universal Manual than in Wash Manual". |



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