TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Research Use Only. Not for use in diagnostic procedures.

Di/Mono-Methyl-Histone H3 Lysine 27 (H3K27me2-1) Cellular Detection Kit

Product No.: AL721HV

Material Provided

Format: AL721HV: 100 assay points
When using the kit components at the recommended concentrations, the number of assay points for the HV format is based on an assay volume of 50 µL in 96-well assay half-area plates.

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/mono-methylated Histone H3 Lysine 27 (H3K27me2-1) in cell lysates using a homogeneous AlphaLISA assay (no wash steps).

Quality Control

Lot-to-lot consistency of Donor and Acceptor beads is confirmed by a Quality Control AlphaLISA titration assay read on an EnVision® Alpha HTS instrument. Maximum signal and EC_{50} value are determined using a biotin-H3K27me2 peptide. Minimum signal is measured in the absence of peptide.
Maximum counts may vary between bead lots. Maximum counts obtained in the QC assay are usually higher than those obtained in a cellular detection assay, which are dependent on epigenetic mark abundance and assay conditions (e.g. cell line, culture medium, incubation time, modulator concentration, etc.).

QC release specifications of the biotinylated antibody are based on spectrophotometric analysis of the labeled antibody.

We certify that these results meet our quality release criteria.

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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	**AL721HV (100 assay points)**
AlphaLISA anti-H3K27me2-1 Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4	20 µL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated Antibody anti-Histone H3 (C-ter) stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	30 µL @ 500 nM (1 tube, black cap)
Cell-Histone Lysis buffer	20 mL
Cell-Histone Extraction buffer	20 mL
Cell-Histone Detection (10X) buffer (see note)	2.0 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
1/2 AreaPlate-96, White	PerkinElmer	6005560 (50/box) 6005569 (200/box)
SpectraPlate-96 TC, Clear 96-well Microplate	PerkinElmer	6005650 (50/box) 6005658 (160/box)
TopSeal™-A Adhesive Sealing film	PerkinElmer	6050195
EnSpire® or EnVision® Multilabel Alpha Reader	PerkinElmer	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	PerkinElmer	AL719
AlphaScreen SureFire® GAPDH Assay Kit	PerkinElmer	TGRGDS
ATPlite® Luminescence Assay System	PerkinElmer	6016941 (1 000 assay points) 6016947 (5 000 assay points)
AlphaLISA TruHits kit	PerkinElmer	AL900D (1 000 assay points) AL900M (10 000 assay points)
OptiPlate-384, white opaque	PerkinElmer	6007290 (50/box) 6007299 (200/box)
CulturPlate-1536, white opaque with lid	PerkinElmer	6004680 (50/box) 6004688 (160/box)
ProxiPlate-384 TC	PerkinElmer	6008230 (50/box) 6008238 (160/box)
Histone H3 (K27me2) (23-34) peptide	AnaSpec	64377
Histone H3 (21-44) peptide	AnaSpec	64454
Halt Protease Inhibitor Cocktail (100X)	Thermo Scientific	87786 (1 mL)

Recommendations

General recommendations

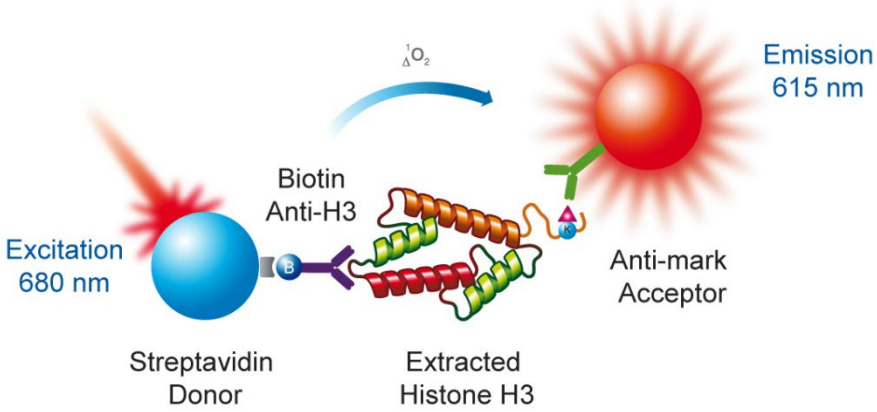
- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to prewet the tip.
- Centrifuge quickly all tubes before use to improve recovery of content (2 000 ×*g*, 10-15 sec). Resuspend all reagents by gentle mixing before use.
- Use Milli-Q® grade H_2O (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

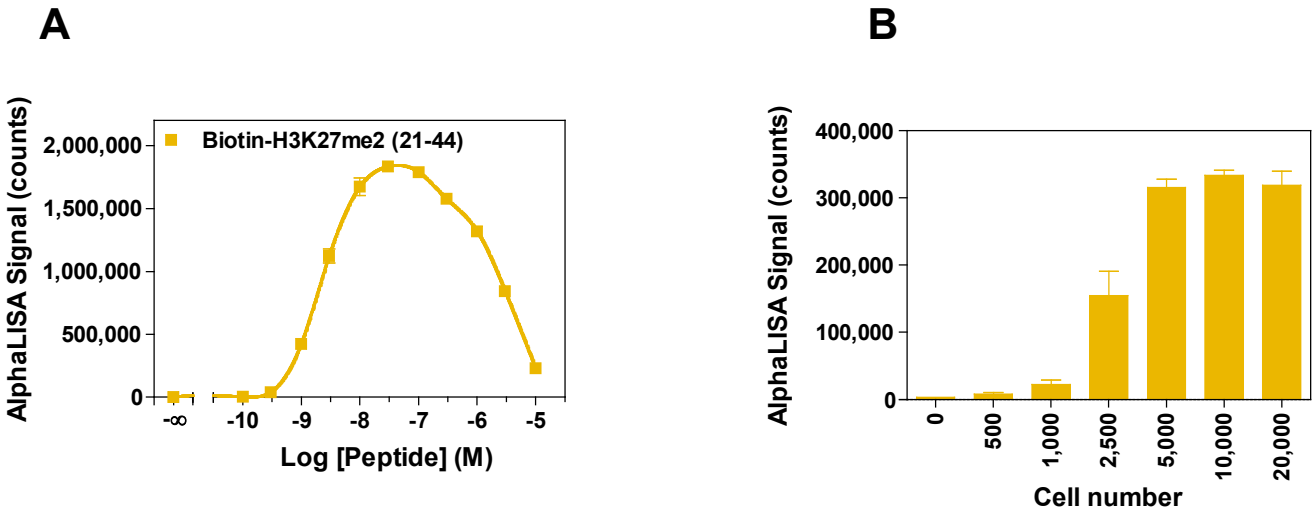
- Biotin present in culture medium may interfere with the binding of the biotinylated antibody to Streptavidin Donor beads. Use biotin-free culture medium if medium is not removed before cell lysis. Most cells will not be affected by 2-3 days incubation in biotin-free medium. You can also reduce biotin in culture medium by combining biotin-containing and biotin-free medium to find a condition not affecting cell growth or the Alpha signal.
- For monitoring the determination of the H3K27me2-1 mark, keep DMSO under a maximal concentration of 1% 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. It is recommended to add warm PBS or sterile water to outer and unused wells for long incubation periods. Alternatively, cells can be cultured in larger wells, or in a larger volume of culture medium. Adjust the volume of lysis buffer according to the volume of in which cells are
- 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 or are saved for Western blot analysis.
- 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 if medium is not removed before cell lysis.
- 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: cell 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 H3K27me2-1 mark in peptide and cellular extracts. A) Representative QC data for Streptavidin Donor and anti-H3K27me2-1 Acceptor beads. Results were generated using a biotinylated peptide titrated in QC buffer (50 mM Tris-HCl pH 8.0). **B)** AlphaLISA detection of H3K27me2-1 in cellular extracts. HeLa cells were seeded at densities ranging from 500 to 20 000 cells per well in 96-well culture plates. The cellular detection assay was performed in ½ AreaPlate-96 following the 96-well plate format Universal Protocol. 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 H3K27me2-1 mark is available on our website at www.perkinelmer.com/epigenetics.

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Universal Protocol for Adherent and Suspension Cells (No Wash)

The Universal Protocol is a transfer assay that measures histone mark modulation in cells seeded in 96-well format. Following lysis and histone extraction steps, cellular extracts are transferred to the wells of a ½ AreaPlate-96 microplate for the AlphaLISA detection steps. The final assay volume is 50 µL.

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

Cell Culture, Lysis and Histone Extraction

We recommend first titrating your cells, seeding them in triplicates at densities ranging from 500 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 of interest.

The transfer protocol allows preparing extracts that can be divided into separate wells for measuring different marks in parallel. A portion of the extracts can also be saved for Western blot analysis. We recommend adding protease inhibitors to the Cell-Histone lysis buffer if the lysates are not used immediately.

Cell Culture

- Seed cells in biotin-free (or low biotin) culture medium in a SpectraPlate-96 TC microplate.
    - For example, seed cells at various densities in triplicate in a volume of 40 µL.
- Incubate cells for 3-4 h at 37ºC in a 5% CO_2 atmosphere to allow cell adhesion (skip this step for cells growing in suspension)
- Add medium, or compound prepared in culture medium at the desired concentration.
    - For example, add 20 µL of a 3X compound solution prepared in culture medium.
- Incubate cells for the time required for histone mark modulation.

Cell Lysis and Histone Extraction

Volumes of Cell-Histone Lysis and Extraction buffers should be adjusted according to the volume of culture medium in the wells.

- Add the Cell-Histone Lysis buffer directly to the wells (1/3 of the cell culture volume).
    - For example, add 20 µL Cell-Histone Lysis buffer for cells in 60 µL culture medium
- Incubate 15 min at RT
- Add the Cell-Histone Extraction Buffer (2/3 of the cell culture volume)
    - For example, add 40 µL of Cell-Histone Extraction for cells in 60 µL culture medium
- Incubate 10 min at RT
- Transfer 30 µL of the histone extracts to wells of a ½ AreaPlate-96 (alternatively, a white OptiPlate-384 microplate can be used)
- Proceed to the AlphaLISA Detection as described below.

AlphaLISA Detection

Adjust the volume of detection reagents according to the number of assay points.

- 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 µg/mL and the biotinylated antibody to 15 nM. Final concentration of beads and antibody are 20 µg/mL and 3 nM, respectively.

- For example, prepare a 5X mix by adding 20 µL Acceptor beads and 30 µL biotinylated antibody to 950 µL of 1X Cell-Histone Detection buffer. Adjust volumes according to the number of assay points.

- Add 10 µL 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.

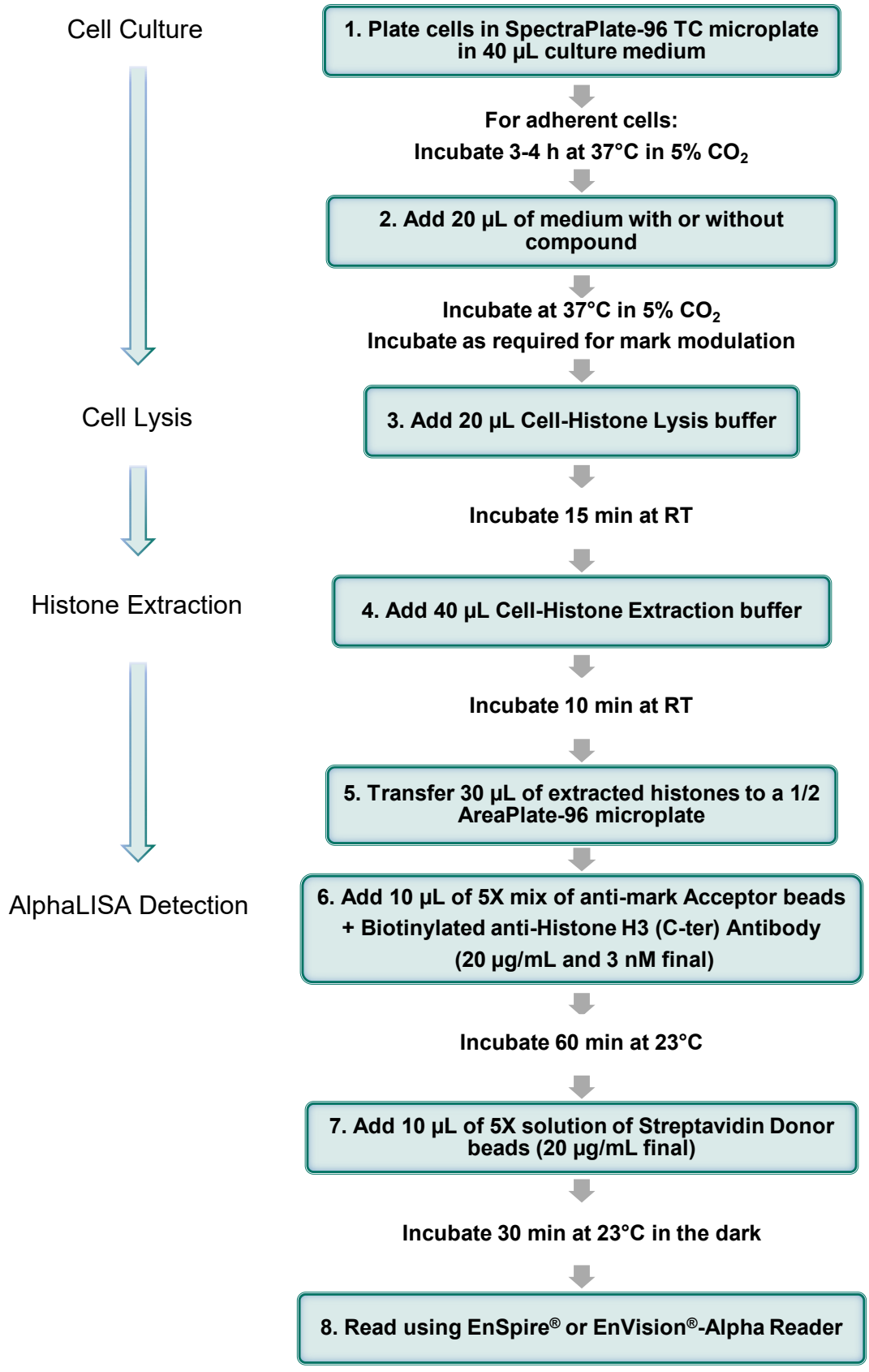
Wash Protocol for Adherent Cells

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

- Seed cells in a SpectraPlate-96 TC microplate.
- Incubate cells for 3-4 h at 37ºC in a 5% CO_2 atmosphere to allow cell adhesion.
- Add medium, or compound prepared in culture medium at the desired concentration.
- Incubate cells for the time required for histone mark modulation.
- Dilute the Cell-Histone Lysis buffer 4-fold in H_2O.
    - For example, add 500 µL of Cell-Histone Lysis buffer to 1.5 mL of H_2O.
- Remove culture medium. Optionally, wash gently the cell layer with PBS, and then remove PBS from the wells.
- Add 80 µL of the **4-fold diluted** Cell-Histone Lysis buffer.
- Incubate for 15 min at room temperature (RT).
- Add 40 µL of Cell-Histone Extraction buffer.
- Incubate for 10 min at RT.
- Transfer 30 µL of the histone extracts to wells of a ½ AreaPlate-96 (alternatively, a white OptiPlate-384 microplate can be used*)
- Proceed to the AlphaLISA detection as described in the Universal Assay Protocol.

Assay Diagram: Universal Protocol (No Wash)



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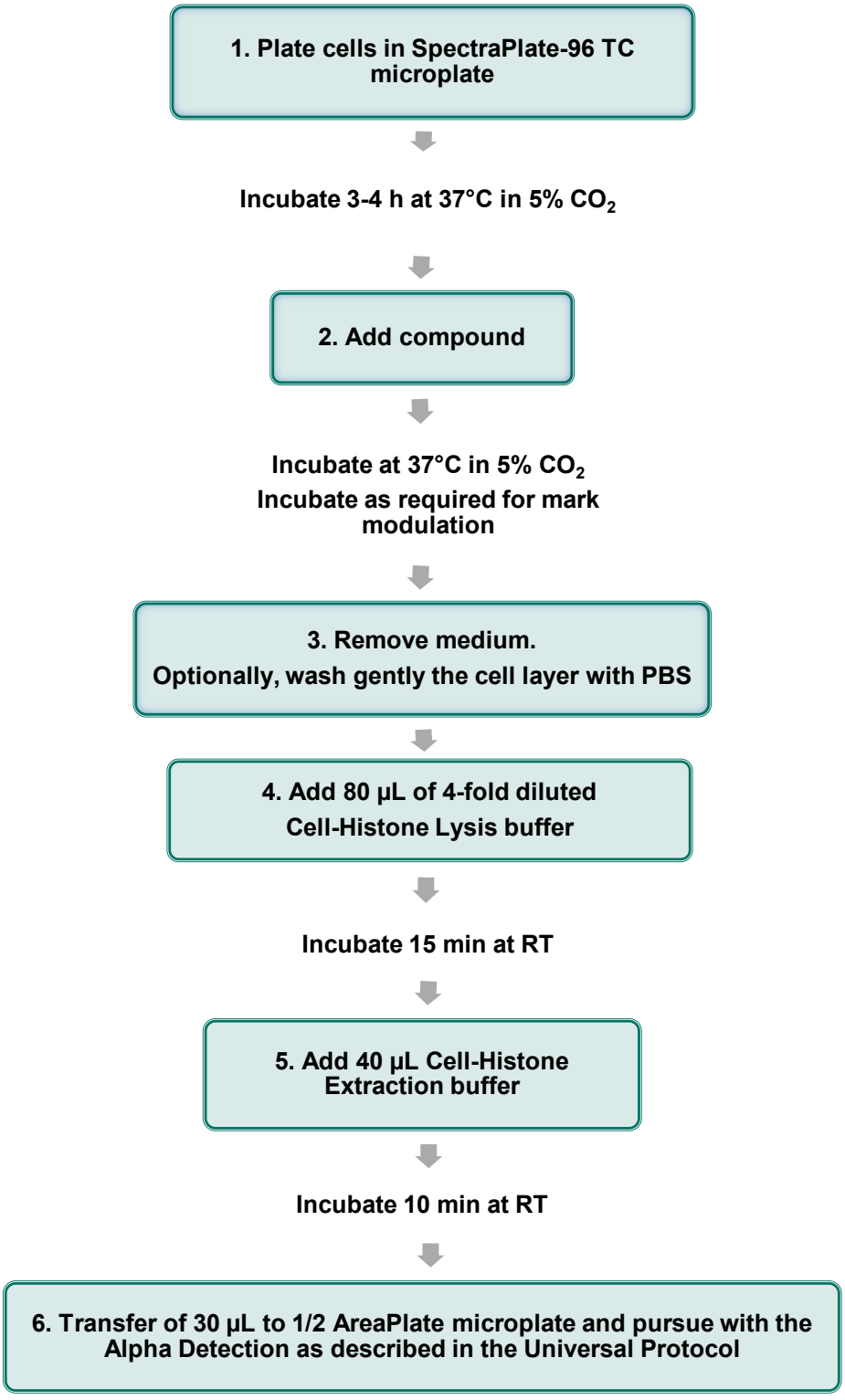
Assay Diagram: Wash Protocol



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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.

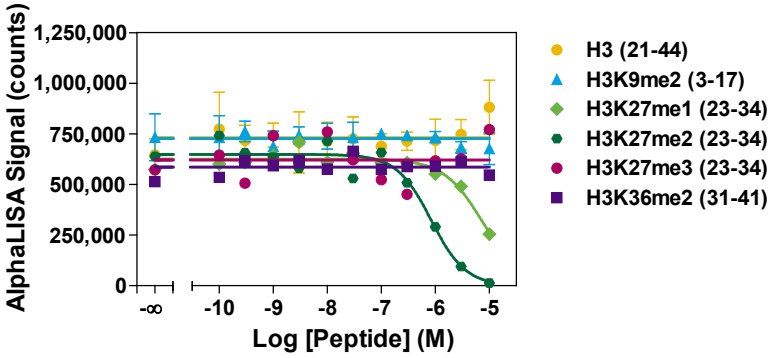
You will need to modify the Universal Protocol as follows:

- 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 µg/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 the Universal Protocol until after the transfer step to the ½ AreaPlate-96 microplate.
- 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 Protocol.

Peptide Competition Data



HeLa cells were seeded at a density of 2,000 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 µM just before the addition of the AlphaLISA detection reagents. Additional peptides tested but not shown include H3K4me2, H3K27ac, H3K36ac, H3K36me1, H3K36me3 and H3K79me2. The H3K27me2 peptide competed for the interaction between the Acceptor beads and histone H3 proteins with an IC_{50} value of 0.84 µM, while H3K27me1 displayed approximately 10-fold less potency.

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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
    - 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. Unmodified H3K4 appears to be extremely robust for assessing total histone levels and toxicity by modulators.
- ATPlite Luminescence Assay System (PerkinElmer)
- AlphaScreen SureFire GAPDH Assay Kit (PerkinElmer)
- Evaluation of cell density by Imaging Microscopy

Compound Interference

When using the No Wash Universal Protocol, compounds remain present for the AlphaLISA detection step. Some of them could interfere with the Alpha signal at high concentration.

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 µL of culture medium.
    - Grow cells as you normally would.
    - Proceed with cell Lysis and histone Extraction as described in the Universal Protocol.
    - Following the Extraction step, add compound prepared in 5 µL culture medium.
    - Proceed with the Alpha Detection as indicated in the Universal Protocol.
    - 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 Protocol.

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.perkinelmer.com/ask.

Issue	Recommendations and Comments
No signal increase in cell titration experiment	• 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. • Universal Protocol: use biotin-free culture medium, or culture medium with reduced biotin concentration.
No mark modulation upon compound treatment	• 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	• 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 Protocol than in the Wash Protocol	• 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	• Universal Protocol: 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. • Wash Protocol: 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. • All protocols: lengthen incubation time with the Alpha Donor beads up to 18 hours before measuring the Alpha signal.
Insufficient assay window	• Universal Protocol: use medium without phenol red or with lower FBS concentration to maximize signal. • All protocols: lengthen incubation time with the Alpha Donor beads up to 18 h with before measuring the Alpha signal. • See "Signal lower in the Universal Protocol than in Wash Protocol".

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