ab185914 – Histone H4 Modification Multiplex Assay Kit (Colorimetric)

Instructions for Use

For the measurement of histone H4 modifications in various samples

This product is for research use only and is not intended for diagnostic use.

Version 3 Last Updated 6 February 2019

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1. BACKGROUND

Histone modifications have been defined as epigenetic modifiers. Post-translational modifications of histones include the acetylation of specific lysine residues by histone acetyltransferases (HATs), deacetylation by histone deacetylase (HDACs), the methylation of lysine and arginine residues by histone methytransferases (HMTs), the demethylation of lysine residues by histone demethylases (HDMTs), and the phosphorylation of specific serine groups by histone kinases (HKs). Additional histone modifications include the attachment of ubiquitin (Ub), small ubiquitin-like modifiers (SUMOs), and poly ADP-ribose (PAR) units. Next to DNA methylation, histone acetylation and histone methylation are the most well characterized epigenetic marks. There are many known sites and types of post-translational modification on human histone H4 that include Ser-1 phosphorylation, Arg-3 methylation, lysine 5, 8, 12, and 16 acetylation and lysine 20 mono-, di-, and trimethylation. Generally, euchromatin is characterized by a high level of histone H4 acetylation, which is mediated by histone acetyltransferases. Histone deacetylases have the ability to remove this epigenetic mark, which leads to transcriptional repression. Condensed heterochromatin is enriched in methylation of H4K20. Lysine residues of histone H4 can be mono-, di-, or trimethylated, each of which can differentially regulate chromatin structure and transcription. Along with other histone modifications such as phosphorylation, this enormous variation leads to a multiplicity of possible combinations of different modifications. This may constitute a “histone code”, which can be read and interpreted by different cellular factors.

Abnormal histone modification patterns have been associated with many different diseases such as cancer, autoimmune disorders, and inflammatory and neurological diseases. Therefore, detection of histone H4 modifications would provide useful information for a better understanding of epigenetic regulation of gene activation and silencing, histone modification-associated pathological disease process, as well as for developing histone modification-targeted drugs.

As the first multiplex assay for detecting up to 10 modified histone H4 sites simultaneously, this kit has the following advantages:

- Simultaneously measure 10 different histone H4 modifications, which includes nearly all modified histone H4 sites.

H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4R3m2a	H4R3m2s
H4K20m1	H4K20m2	H4K20m3	H4ser1	Total H4	

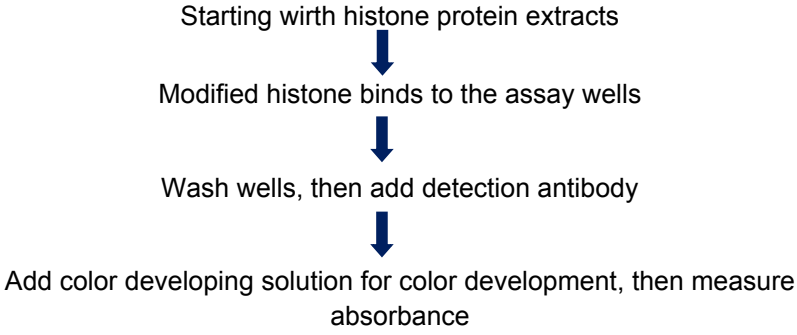
- Quick and efficient procedure, which can be finished within 2.5 hours.
- Innovative colorimetric assay without the need for radioactivity, electrophoresis, chromatography, or expensive equipment.
- High sensitivity with a detection limit as low as 0.5 ng/well for each modification pattern and a detection range from 20 ng to 500 ng/well of histone extracts.
- An assay control is conveniently included for quantification of each histone H4 modification.
- Total histone H4 sets are included, which can be used for normalizing total histone H4 levels for relative comparison of histone H4 content between different samples or different treatment conditions.
- Strip microplate format makes the assay flexible: manual or high throughput, which enables analysis of a single modification or a total of 10 modification patterns within the same samples.
- Two extra 8-well strips are included in the kit which can be used, if necessary, for sample amount pre-optimization to determine the input amount (ex: 50, 100, 200 ng/well) needed to fall within the detection limits of the assay. Extra strips may also be used as assay controls and total histone level controls if selective detection of some histone H4 modifications from the total 21 modification pattern is desired.
- Simple, reliable, and consistent assay conditions.

Abcam's Histone H4 Modification Multiplex Assay Kit (Colorimetric) ab185914 is suitable for specifically measuring nearly all histone H4 modifications (10 different types) simultaneously from human, mouse, rat,

and other species including most plants, fungi, and bacteria based on high sequence homology of histone H4. Starting materials can be in a variety of forms including cultured cells and fresh/frozen tissues. Histone extracts can be prepared by using your own successful method. The prepared histone extracts should not contain detergents. Each kit can be used for four different samples or two pairs of samples: control and treated, normal and diseased, and other paired comparisons.

This kit is designed for measuring multiple histone H4 modifications simultaneously. In an assay with this kit, each histone H4 modified at specific sites will be captured by an antibody that is coated on the strip wells and specifically targets the appropriate histone modification pattern. The captured histone modified at specific sites will be detected with a detection antibody, followed by a color development reagent. The ratio of modified histone is proportional to the intensity of absorbance measured by a microplate reader at a wavelength of 450 nm.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table upon receipt.

Observe the storage conditions for individual prepared components in sections 9 & 10.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

Check if Wash Buffer contain salt precipitates before use. If so, warm at room temperature or 37°C and shake the buffer until the salts are re-dissolved

5. MATERIALS SUPPLIED

Item	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	28 mL	4°C
Antibody Buffer	6 mL	4°C
Detection Antibody	12 µL	–20°C
Developer Solution	12 mL	4°C
Stop Solution	12 mL	RT
Assay Control Protein (100 µg/mL)	20 µL	–20°C
96-well strip plate (with frame)	1	4°C
Extra 8-well strips	2	RT
Adhesive covering film	1	RT

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Aerosol resistant pipette tips
- Microplate reader capable of reading absorbance at 450 nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Histone extracts or purified histone proteins
- Parafilm M or aluminum foil

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Prepare fresh reagents immediately prior to use.

9.1 1X Wash Buffer

Add 26 mL of 10X Wash Buffer to 234 mL of distilled water and adjust pH to 7.2-7.5. This Diluted 1X Wash Buffer can now be stored at 4°C for up to six months.

9.2 Detection Antibody Solution

Dilute Detection Antibody with Diluted Wash Buffer at a ratio of 1/1000 (i.e., add 1 µL of Detection Antibody to 1000 µL of Diluted Wash Buffer). 50 µL of Diluted Detection Antibody will be required for each assay well.

Note: *Keep solution on ice until used. Any remaining diluted solutions should be discarded if not used within the same day*.

10. STANDARD PREPARATION

Suggested Assay Control Preparation: Prepare 2 concentrations by combining the 100 ng/µL Assay Control Protein with Antibody Buffer into final concentrations of 5 and 25 ng/µL according to the following dilution chart. The high concentration (25 ng/µL) of the Assay Control Protein can be used for a simple amount quantification of histone H4 modification and total H4. The low concentration (5 ng/µL) along with high concentration is used to generate proportional concentration-signal intensity for determining if the assay control works properly.

Tube	Assay Control (µL)	Antibody Buffer (µL)	Final Conc (ng/ µL)
1	1	19	5
2	1	3	25

Note: *Keep solution on ice until used. Any remaining diluted solutions should be discarded if not used within the same day*.

11. SAMPLE PREPARATION

Input Amount: Input materials can be histone extracts or purified histone H4 proteins. The amount of histone extracts for each assay can be 20 ng to 500 ng with an optimal range of 50 to 100 ng depending on the purity of histone extracts. The amount of purified histone H4 proteins for each assay can be 1 ng to 25 ng with an optimal range of 4 to 5 ng.

Histone Extraction: You can use your method of choice for preparing histone extracts from treated or untreated samples. Abcam offers a Histone Extraction Kit (ab113476) optimized for use with this kit. The prepared histone extracts should not contain detergents such as SDS, Tween, Triton X-100, or NP-40. Histone extracts should be stored in aliquots at –80°C until use.

Use of Extra Strips: If necessary, the extra strips included in the kit can be used for input amount pre-optimization or used as controls if only a few histone H4 modifications are selected for detection. The strips can be set up as indicated in Table 2 and Table 3 under the 'Extra Strip Well Setup' section and carried out by using the same assay protocol.

12. ASSAY PROCEDURE

Internal Control: An assay control is provided in this kit. It can be used for signal intensity comparison between the assay control and the samples to indicate the amount of each histone H4 modification captured from the samples. Because the content of each histone H4 modification can vary from tissue to tissue, and from normal and diseased states, or from different treatments, it is advised to run duplicate for each sample to ensure that the signal generated is validated.

12.1 Histone Binding

12.1.1 Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (including blank and positive controls) to ensure that the signal generated is validated. Carefully remove un-needed strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).

***Note**: If removing strip wells, you must absolutely keep track of which wells have been removed, as each well represents a specific histone modification pattern according to Table 2.*

12.1.2 Blank Wells: Add 49 µL of Antibody Buffer to each blank well.

12.1.3 Control Wells: Add 49 µL of Antibody Buffer and 1 µL of Diluted Assay Control Protein to each standard well using 2 wells for each concentration point (5 and 25 ng/well) (based on the dilution chart in Assay Control preparation.

12.1.4 Sample Wells: Add 46 to 49 µL of Antibody Buffer and 1 to 4 µL of your histone extracts. Total volume should be 50 µL per well.
Note: (1) Follow the strip well setup diagrams (Table 1); (2) It is recommended to use 50-100 ng or a pre-optimized amount of histone extract per well.

12.1.5 Tightly cover strip-well microplate with Adhesive Covering Film to avoid evaporation and incubate at 37°C for 60 to 120 minutes.

***Note:** The Adhesive Covering Film can be cut to the required size to cover the strips based on the number of strips to be used.*

12.1.6 Remove the reaction solution from each well. Wash each well three times with 150 µL of the Diluted Wash Buffer each time.

12.2 **Antibody Binding**

12.2.1 Add 50 µL of the Diluted Detection Antibody to each well, then cover Parafilm M or aluminium foil and incubate at room temperature for 60 minutes.

12.2.2 Remove the Diluted Detection Antibody solution from each well.

12.2.3 Wash each well four times with 150 µL of the Diluted Wash Buffer each time.

Note: Ensure any residual wash buffer in the wells is removed as much as possible at each wash step.

12.3 **Signal Detection**

12.3.1 Add 100 µL of Detection Solution to each well and incubate at room temperature for 1 to 10 minutes away from light. Begin monitoring color changes in the sample wells and control wells. The Detection Solution will turn blue in the presence of sufficient modified products.

Note: *Average color development time is 2-5 minutes. Use control wells and blank wells as a reference for color development.*

12.3.2 Add 100 µL of Stop Solution to each well to stop enzyme reaction when color in the positive control wells turns medium blue. The color will change to yellow after adding Stop Solution and the absorbance should be read on a microplate reader within 2 to 10 minutes at 450 nm with an optional reference wavelength of 655 nm.

Note*: (1) Most microplate readers have the capability to carry out dual wavelength analysis and will automatically subtract reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, the plate can be read twice, once at 450 nm and once at 655 nm. Then, manually subtract the 655 nm ODs from 450 nm ODs; (2) If the strip-well microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay control	Total H4	H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4K20m1	H4K20m2	H4K20m3	H4R3m2a	H4R3m2s	H4ser1P
B	Assay control	Total H4	H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4K20m1	H4K20m2	H4K20m3	H4R3m2a	H4R3m2s	H4ser1P
C	Assay control	Total H4	H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4K20m1	H4K20m2	H4K20m3	H4R3m2a	H4R3m2s	H4ser1P
D	Assay control	Total H4	H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4K20m1	H4K20m2	H4K20m3	H4R3m2a	H4R3m2s	H4ser1P
E	Blank	Total H4	H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4K20m1	H4K20m2	H4K20m3	H4R3m2a	H4R3m2s	H4ser1P
F	Blank	Total H4	H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4K20m1	H4K20m2	H4K20m3	H4R3m2a	H4R3m2s	H4ser1P
G	Blank	Total H4	H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4K20m1	H4K20m2	H4K20m3	H4R3m2a	H4R3m2s	H4ser1P
H	Blank	Total H4	H4K5ac	H4K8ac	H4K12ac	H4K16ac	H4K20m1	H4K20m2	H4K20m3	H4R3m2a	H4R3m2s	H4ser1P

Table 1. An antibody for each H4 modification is coated onto the indicated wells accordingly.

Well	Strip 1	Strip 2
A	Assay Control 5 ng	Assay Control 5 ng
B	Assay Control 5 ng	Assay Control 5 ng
C	Assay Control 25 ng	Assay Control 25 ng
D	Assay Control 25 ng	Assay Control 25 ng
E	Total H4 Sample 1	Total H4 Sample 1
F	Total H4 Sample 2	Total H4 Sample 2
G	Total H4 Sample 3	Total H4 Sample 3
H	Total H4 Sample 4	Total H4 Sample 4

Table 2. Two extra strip wells can be set up for input amount pre-optimization. Different concentrations of samples can be added to wells C through H as shown.

Well	Strip 1	Strip 2
A	Blank	Blank
B	Assay Control 25 ng	Assay Control 25 ng
C	50 ng	50 ng
D	50 ng	50 ng
E	100 ng	100 ng
F	100 ng	100 ng
G	200 ng	200 ng
H	200 ng	200 ng

Table 3. Alternatively, the two extra strip wells can be set up as controls for detection of select H4 modifications (each strip can be used as an extra control for the assay)

13. ANALYSIS

13.1 Histone H4 Modification Calculation

Calculate the average duplicate readings for the sample wells, assay control wells and blank wells.

Simple amount quantification of each H4 modification or total H4 in the samples can be carried out using Formula 1 shown below:

Formula 1:

$$H4\ modification\ or\ total\ H4\left(\frac{ng}{\mu g} protein\right) = \left(\frac{Sample\ OD - Blank\ OD + S}{Assay\ Control\ OD - Blank\ OD + P}\right) \times 1000$$

S is the amount of input sample protein in ng.
P is the amount of input assay control in ng (use 25 ng).

Example calculation:

Average OD450 of blank is 0.115
Average OD450 of Assay control is 0.775
Average OD450 of Sample (H4 modification or total H4) is 0.575
S is 100 ng
P is 25 ng

$$4\ modification\ or\ total\ H4\left(\frac{ng}{\mu g} protein\right)$$

$$= \left(\frac{0.575 - 0.115\ \div 100}{0.775 - 0.115\ \div 25}\right) \times 1000$$

$$= 174.2\ ng/\mu g\ protein$$

Calculate the percentage of histone H4 modification in total H4 using Formula 2 shown below:

Formula 2:

$$4\ modification = \left(\frac{H4\ modification\ \%\ in\ sample\ 1\ or\ treated\ sample}{H4\ modification\ \%\ in\ sample\ 2\ or\ treated\ sample}\right) x\ 100\%$$

Calculate the relative change of each histone H4 modification between different samples using Formula 3 shown below:

Formula 3:

$$Relative\ change\ \% = \left(\frac{H4\ modification\ \%\ in\ sample\ 1\ or\ treated\ sample}{H4\ modification\ \%\ in\ sample\ 2\ or\ treated\ sample}\right) x\ 100\%$$

13.2 **Typical Results**

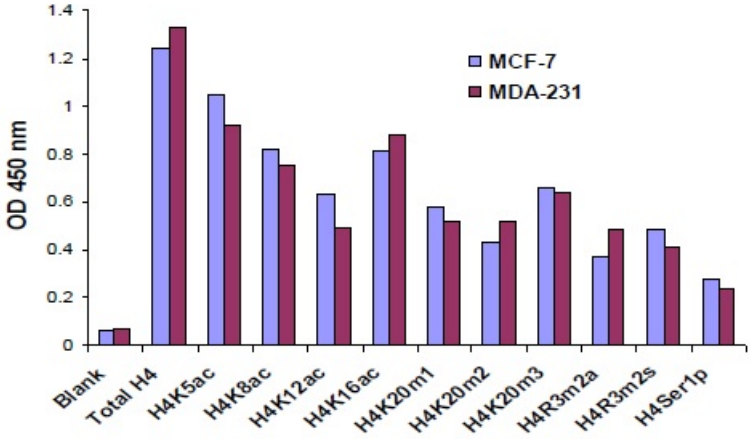


Figure 1. Histone extracts were prepared from MCF-7 and MDA-231 cells using a total Histone extraction kit and 10 histone H4 modifications were measured using ab185914. 100 ng of total histone proteins per well were used.

14. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in both the assay control and sample wells	Reagents are added incorrectly	Check if reagents are added in the proper order with the right amount, and if any steps in the protocol may have been omitted by mistake
	Incubation time and temperature are incorrect	Ensure the incubation time and temperature described in the protocol are followed correctly
	Incorrect absorbance reading	Check if the appropriate absorbance wavelength (450 nm filter) is used
	Kit was not stored or handled properly	Ensure all components of the kit were stored at the appropriate temperatures and the cap is tightly capped after each opening or use
No signal or weak signal in only the assay control wells	The Diluted Assay Control Protein amount is insufficiently added to the well	Ensure a sufficient amount of Diluted Assay Control Protein is added
	The Assay Control Protein is degraded due to improper storage conditions	Follow the Shipping & Storage guidance of this User Guide for storage of Assay Control Protein

High background present in the blank wells	Insufficient washing of wells.	Check if washing at each step is performed according to the protocol
	Contaminated by sample or Assay Control Protein.	Ensure the well is not contaminated from adding sample or Assay Control Protein accidentally or from using contaminated tips
	Incubation time with diluted DAH is too long.	The incubation time for DAH should not exceed 90 minutes
	Over development of color.	Decrease the development time before adding Stop Solution
No signal or weak signal only in sample wells or for some of H4 modification patterns	Protein sample is not properly extracted or purified	Ensure your protocol is suitable for histone protein extraction. For the best results, it is advised to use Histone Extraction Kit (ab113476)
	Sample amount added into the wells is insufficient	Ensure a sufficient amount of histone extracts are used as indicated. The sample can be titrated to determine the optimal amount to use in the assay
	Sample was not stored properly or has been stored for too long	Ensure sample is stored in aliquots at –80°C. Histone extracts should be stored for up to 6 months at –80°C

	Little or no modified H4 at specific sites in the sample	This problem may be a result of many factors. If the affecting factors cannot be determined, use new or re-prepared histone extracts
Uneven color development	Insufficient wash of the wells	Ensure the wells are washed according to the guidance of washing and residue washing buffer is removed as much as possible
	Delayed color development or delayed stopping of color development in the wells	Ensure color development and stop solutions are added sequentially and consistent with the order you added the other reagents (e.g., from well A to G or from well 1 to 12)

15. NOTES

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