ab185910 – Histone H3 Modification Multiplex Assay Kit (Colorimetric)

Instructions for Use

For the measurement of Histone H3 modifications in various samples

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

Version 8 Last Updated 13 June 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. Generally, tri-methylation at H3-K4, H3-K36, or H3-K79 results in an open chromatin configuration and is therefore characteristic of euchromatin. Euchromatin is also characterized by a high level of histone acetylation, which is mediated by histone acetyltransferases. Lysine residues can be mono-, di-, or tri-methylated, 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 H3 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.

This kit has the following advantages.

- Simultaneously measure 21 different Histone H3 modifications, which include all of the most important and the most well characterized patterns.

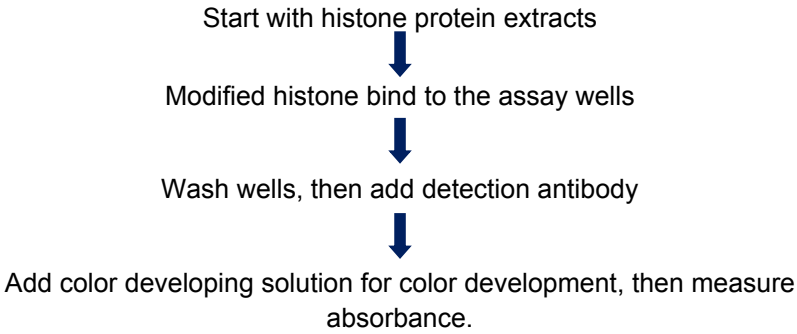
H3K4me1	H3K4me2	H3K4me3	H3K9me1	H3K9me2	H3K9me3
H3K27me1	H3K27me2	H3K27me3	H3K36me1	H3K36me2	H3K36me3
H3K79me1	H3K79me2	H3K79me3	H3K9ac	H3K14ac	H3K18ac
H3K56ac	H3ser10ph	H3ser28ph	Total H3		

- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be finished within 2.5 hours.
- Innovative kit composition enables background signals to be extremely low and allows the assay to be simple, accurate, reliable, and consistent.
- Total histone H3 sets are included, which can be used for normalizing total histone H3 levels for relative comparison of histone H3 content between different samples or different treatment conditions.
- High sensitivity with a detection limit as low as 0.5 ng/well for each modification pattern and detection range from 20 ng to 500 ng/well of histone extracts.
- An assay control is conveniently included for quantification of each Histone H3 modification.
- Strip microplate format makes the assay flexible: manual or high throughput analysis (96 assays).
- Two extra 8-well strips that are coated with anti-histone 3 antibody 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 H3 modifications from the total 21 modification pattern is desired.

The Histone H3 Modification Multiplex Assay Kit (Colorimetric) is suitable for measuring up to 21 Histone H3 modifications simultaneously from a broad range of species such as human, mouse, rat, and other species including most plants, fungi, and bacteria based on high sequence homology of Histone H3, in a variety of forms including, but not limited to cultured cells, fresh and 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 two different samples or a pair of samples: control and treated, normal and diseased, and other paired comparisons.

In this assay, each Histone H3 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.

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

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

5. MATERIALS SUPPLIED

Item	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	28 mL	4°C
Antibody Buffer	8 mL	4°C
Detection Antibody	12 µL	–20°C
Developer Solution	12 mL	4°C
Stop Solution	12 mL	RT
Assay Control Protein	20 µL	–20°C
96-Well Strip Plate (With Frame)	1	4°C
Extra 8-Well Strips	2	4°C
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

9.1 **1X Wash Buffer**

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

9.2 **Detection Antibody**

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

10. ASSAY CONTROL PROTEIN PREPARATION

Suggested preparation of Assay Control: Prepare 2 concentrations as given in the table below: Assay Control Protein supplied at 100 ng/ µL.
The high concentration (25 ng/µL) of the Assay Control Protein can be used for a simple amount quantification of Histone H3 modification and total H3. The low concentration (5 ng/uL) 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

11. SAMPLE PREPARATION

Input Amount: The amount of histone extracts for each assay can be 20 ng to 500 ng with an optimal range of 50 ng to 100 ng depending on the purity of histone extracts. The amount of purified histone H3 proteins for each assay can be 1 ng to 25 ng with an optimal range of 4 ng to 5 ng.

Histone Extraction: You can use your method of choice for preparing Histone extracts. 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 H3 modifications are selected for detection. The strips can be set up as indicated in Table 3 and Table 4 under the "Extra Strip Well Setup" section and carried out by using the same assay protocol described below.

12. ASSAY PROCEDURE

For the best results please read the entire protocol before starting your experiment.

Internal Control: An Assay Control Protein is provided in this kit for the quantification of Histone H3 modification and total H3. Because Histone H3 modification can vary from tissue to tissue, and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated is validated.

12.1 Enzymatic Reaction

12.1.1 Predetermine the number of strip wells required for your experiment. 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).

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 1µL of Diluted Assay Control Protein to each standard well using 2 wells for each concentration point (5 and 25 ng/well).

12.1.4 Sample Wells: Add 46-49 µL of Antibody Buffer and 1 to 4 µL of your histone extracts. Total volume should be 50 µL per well.

12.1.5 Tightly cover strip plate with Adhesive Covering Film to avoid evaporation and incubate at 37°C for 60 to 120 min.

12.1.6 Remove the Reaction Solution from each well.

12.1.7 Wash each well three times with 150 µL of the Diluted 1X Wash Buffer each time.

12.2 **Antibody Binding and Signal Enhancing**

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

12.2.2 Remove the Diluted Detection Antibody solution from each well.

12.2.3 Wash each well three times with 150 µL of the Diluted 1X Wash Buffer each time.

12.3 **Signal Detection**

12.3.1 Add 100 µL of Developer 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 Developer Solution will turn blue in the presence of sufficient demethylated 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 the 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 min at 450 nm with an optional reference wavelength of 655 nm.

Note: *(1) Most microplate readers have 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 stripwell microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.

The next Page shows Table 1 shows an antibody for each H3 modification is coated onto the indicated wells accordingly.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Control 5 ng	Blank	H3 K4me2	H3 K9me1	H3 K9me3	H3 K27me2	H3 K36me1	H3 K36me3	H3 K79me2	H3 K9ac	H3 K18ac	H3 ser10P
B	Assay Control 5 ng	Blank	H3 K4me2	H3 K9me1	H3 K9me3	H3 K27me2	H3 K36me1	H3 K36me3	H3 K79me2	H3 K9ac	H3 K18ac	H3 ser10P
C	Assay Control 25 ng	Blank	H3 K4me2	H3 K9me1	H3 K9me3	H3 K27me2	H3 K36me1	H3 K36me3	H3 K79me2	H3 K9ac	H3 K18ac	H3 ser10P
D	Assay Control 25 ng	Blank	H3 K4me2	H3 K9me1	H3 K9me3	H3 K27me2	H3 K36me1	H3 K36me3	H3 K79me2	H3 K9ac	H3 K18ac	H3 ser10P
E	Total H3	H3 K4me1	H3 K4me3	H3 K9me2	H3 K27me1	H3 K27me3	H3 K36me2	H3 K79me1	H3 K79me3	H3 K14ac	H3 K56ac	H3 ser28P
F	Total H3	H3 K4me1	H3 K4me3	H3 K9me2	H3 K27me1	H3 K27me3	H3 K36me2	H3 K79me1	H3 K79me3	H3 K14ac	H3 K56ac	H3 ser28P
G	Total H3	H3 K4me1	H3 K4me3	H3 K9me2	H3 K27me1	H3 K27me3	H3 K36me2	H3 K79me1	H3 K79me3	H3 K14ac	H3 K56ac	H3 ser28P
H	Total H3	H3 K4me1	H3 K4me3	H3 K9me2	H3 K27me1	H3 K27me3	H3 K36me2	H3 K79me1	H3 K79me3	H3 K14ac	H3 K56ac	H3 ser28P

Key to Table 1

Antibody	Location
Assay Control 5 ng	A1, B1
Assay Control 5 ng	C1, D1
Total H3	E1, F1, G1, H1
Blank	A2, B2, C2, D2
H3K4me1	E2,F2, G2, H2
H3K4me2	A3, B3, C3,D3
H3K4me3	E3, F3, G3, H3
H3K9me1	A4, B4, C4, D4
H3K9me2	E4, F4, G4, H4
H3K9me3	A5, B5, C5, D5
H3K27me1	E5, F5, G5, H5
H3K27me2	A6, B6, C6, D6
H3K27me3	E6, F6, G6, H6

Antibody	Location
H3K36me1	A7, B7, C7, D7
H3K36me2	E7, F7, G7, H7
H3K36me3	A8, B8, C8, D8
H3K79me1	E8, F8, G8, H8
H3K79me2	A9, B9, C9, D9
H3K79me3	E9, F9, G9, H9
H3K9ac	A10, B10, C10, D10
H3K14ac	E10, F10, G10, H10
H3K18ac	A11, B11, C11, D11
H3K56ac	E11, F11,G11, H11
H3ser10P	A12, B12, C12, D12
H3ser28P	E12, F12, G12, H12

Extra Strip well set-up

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	250 ng	250 ng
H	250 ng	250 ng

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 below.

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 H3 Sample 1	Total H3 Sample 1
F	Total H3 Sample 1	Total H3 Sample 1
G	Total H3 Sample 2	Total H3 Sample 2
H	Total H3 Sample 2	Total H3 Sample 2

Table 3. Alternatively, the two extra strip wells can be set up as controls for detection of select H3 modifications (each strip can be used as an extra control for the assay).

13. ANALYSIS

Histone H3 Modification Calculation

Calculate the average duplicate readings for sample wells assay control wells and blank wells. Calculate H3 Modification or total H3 or inhibition using the following formulas:

For simple calculation:

$$\frac{(Sample\ OD - Blank\ OD) \div S}{(Assay\ Control\ OD - Blank\ OD) \div P} = \times 1000$$

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 Assay control is 0.775

Average OD450 of blank is 0.115

Average OD450 of Sample (H3 modification or total H3) is 0.575

S is 100 ng

P is 25 ng

$$H3\ modification\ or\ total\ H3\ (ng/\mu g\ protein) = \frac{(0.575 - 0.155) \div 100}{(0.775 - 0.155) \div 25} \times 1000$$

For calculation of % of histone H3 modification in total H3:

$$H3\ modification\ \% = \frac{Amount\ of\ H3\ modification\ (ng/\mu g\ protein)}{Amount\ of\ total\ H3\ (ng/\mu g\ protein)} \times 100\%$$

For calculation of relative change of each histone H3 modification between different samples:

$$Relative\ Change\ \% = \frac{H3\ modification\ \%\ in\ sample\ 1\ or\ treated\ sample}{H3\ modification\ \%\ in\ sample\ 2\ or\ controlsample} \times 100\%$$

Typical Results

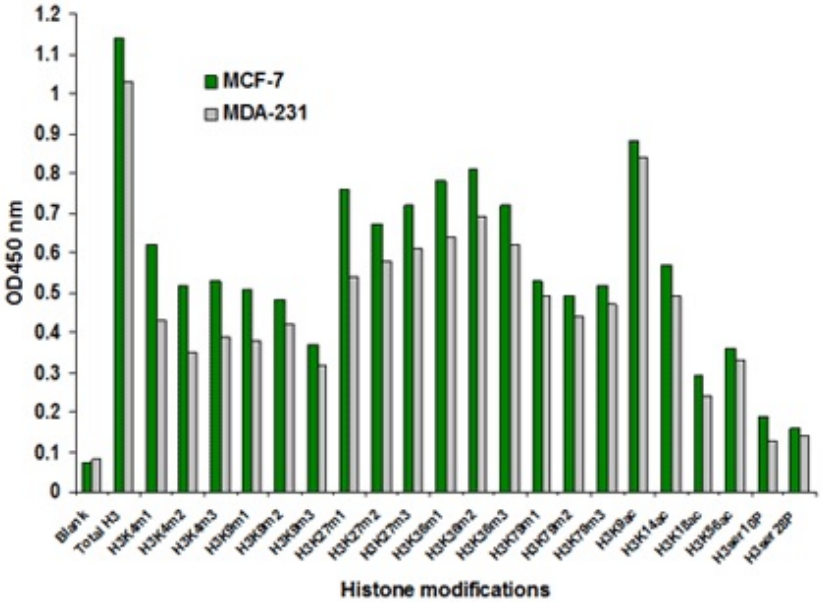


Fig. 1. Histone extracts were prepared from MCF-7 and MDA-231 cells and multiple Histone H3 modifications were screened and measured using the using the Histone H3 Modification Multiplex Assay Kit (Colorimetric). 100 ng of total histone proteins were used.

14. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in both the standard 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 standard amount is insufficiently added to the well in Step 12.2.	Ensure a sufficient amount of standard 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 Assay Control Protein	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips
	Incubation time with detection antibody is too long	The incubation time at Step 12.2 should not exceed 90 minutes
	Over development of color	Decrease the development time in Step 12.3.1 before adding Stop Solution in Step 12.3.2
No signal or weak signal only in sample wells	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 Nuclear Extraction Kit (ab113474). Also, use fresh cells or tissues for protein extraction, as frozen cells or tissues could lose enzyme activity
	Sample amount added into the wells is insufficient	Ensure a sufficient amount Histone is used as indicated in section 11. 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 more than 6 months
	Little or no modified H3 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 nuclear extracts or purified enzymes
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 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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