ab156914 – Histone H4 (di-methyl R3) Quantification Kit (Colorimetric)

Instructions for Use

For the measurement of global histone H4 arginine 3 di-methylation from a broad range of species such as mammals, plants, fungi, and bacteria, in a variety of forms including cultured cells and fresh tissues

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

Version 1 Last Updated 6 February 2019

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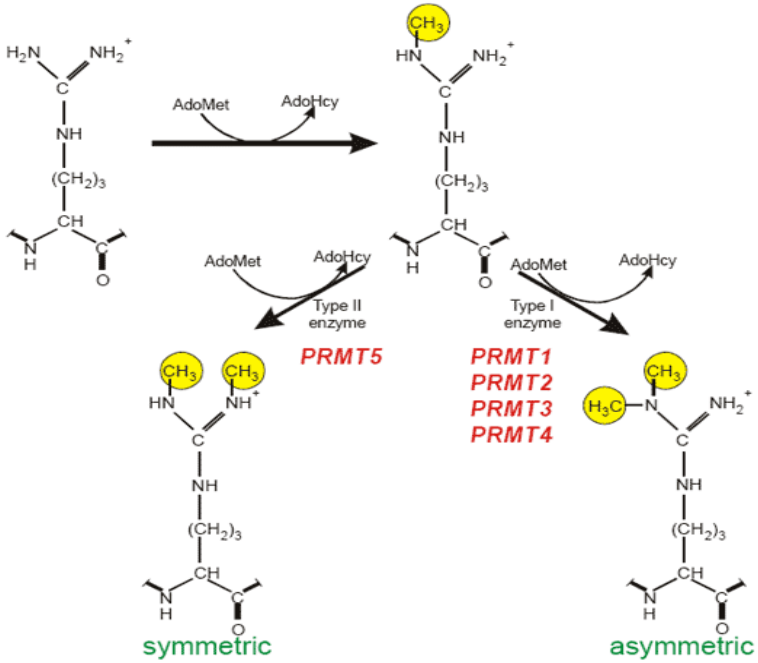
1. BACKGROUND

Arginine histone methylation is one of the many important epigenetic marks, and is essential for the regulation of multiple cellular processes. Arginine methylation of histones H3 (Arg2, 8, 17, 26) and H4 (Arg3) promotes transcriptional activation and is mediated by a family of protein arginine methyltransferases (PRMTs). There are 9 types of PRMTs found in humans but only 7 members are reported to methylate histones. They can mediate mono or dimethylation of arginine residues. These enzymes use S-adenosyl-methionine (SAM) as a methyl donor and transfer it to the guanidinium side chain of arginine. Based on the position of methyl group addition, the PRMTs can be classified into type I (CARM1, PRMT1, PRMT2, PRMT3, PRMT6, and PRMT8) and type II (PRMT5 and PRMT7).



Symmetric di-methylation of histone H4 arg3 (H4R3) are catalyzed by type II PRMTs, which are found to be strongly implicated in diseases like cancer. For example, PRMT5 plays a role in the repression of certain tumor suppressor genes such as RB tumor suppressors while PRMT7 overexpression is observed in breast cancer. The global H4R3 di-methylation can be changed by inhibition or activation of type II PRMTs. Therefore, quantitative detection of global symmetric di-methyl histone H4R3 would provide useful information for better understanding epigenetic regulation of gene activation and silencing, as well as for developing PRMT-targeted drugs.

Abcam's Histone H4 (di-methyl R3) Quantification Kit (Colorimetric) is designed to quantitatively detect global histone H4 (di-methyl R3). This kit has the following advantages:

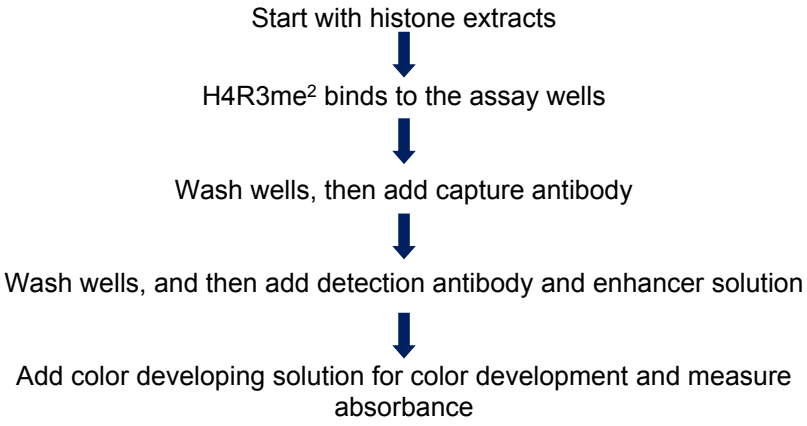
- Quick and efficient procedure, which can be finished within 3.5 hours
- Innovative colorimetric assay without the need for radioactivity, electrophoresis, or chromatography
- Specifically captures symmetric H4R3me^2 with the detection limit as low as 0.5 ng/well and detection range from 100 ng to 2 µg/well of histone extracts
- The control is conveniently included for the quantification of H4R3me^2
- Strip microplate format makes the assay flexible: manual or high throughput
- Simple, reliable, and consistent assay conditions

Abcam's Histone H4 (di-methyl R3) Quantification Kit (Colorimetric) is suitable for specifically measuring global histone H4 arginine 3 di-methylation from a broad range of species such as mammals, plants, fungi, and bacteria, in a variety of forms including cultured cells and fresh tissues.

Histone extracts can be prepared by using your own successful method. For your convenience and the best results, Abcam offers the Histone Extraction Kit (ab113476) optimized for use with this kit. Histone extracts can be used immediately or stored at -80°C for future use.

In this assay histone proteins are stably spotted on the strip wells. H4R3me^2 can be recognized with a high-affinity antibody and detected with a detection antibody, followed by a color development reagent. The ratio of H4R3me^2 is proportional to the intensity of absorbance. The absolute amount of H4R3me^2 can be quantitated by comparing to the standard control.

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 and away from light upon receipt.

Observe the storage conditions for individual prepared components in sections 9, 10 & 11.

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

Check if the 10X Wash Buffer contains salt precipitates before use. If so, warm at room temperature or 37°C and shake the buffer until the salts are re-dissolved.

Check if a blue color is present in the Developer Solution, which would indicate contamination of the solution and should not be used. To avoid contamination, transfer the amount of Developer Solution required into a secondary container (tube or vial) before adding the Developer Solution into the assay wells.

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	14 mL	28 mL	4°C
Histone Buffer	4 mL	8 mL	4°C
Blocking Buffer	10 mL	20 mL	4°C
1000X Capture Antibody	5 µL	10 µL	4°C
2000X Detection Antibody	6 µL	12 µL	–20°C
Enhancer solution	6 µL	12 µL	–20°C
Developer Solution	5 mL	10 mL	4°C
Stop Solution	5 mL	10 mL	RT
H4R3me2 Control, 50 µg/mL	10 µL	20 µL	–20°C
8-Well Assay Strips (With Frame)	6	12	4°C
Adhesive Covering Film	1	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
- 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 the volume specified in the table below of 10X Wash Buffer to distilled water and adjust to pH 7.2-7.5.

	Volume to Dilute (mL)	Volume distilled water (mL)	Total Volume (mL)
48 Tests	13	117	130
96 Tests	26	234	260

The 1X Wash Buffer can now be stored at 4°C for up to six months.

9.2 1X Capture Antibody

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

9.3 1X Detection Antibody

Dilute 2000X Detection Antibody with 1X Wash Buffer at a ratio of 1:2000 (e.g. add 1 µL of 2000X Detection Antibody to 2000 µL of 1X Wash Buffer). About 50 µL of 1X Detection Antibody will be required for each assay well.

9.4 Diluted Enhancer Solution

Dilute the Enhancer Solution with 1X Wash Buffer at a ratio of 1:5000 (e.g. add 1 µL of the Enhancer Solution to 5000 µL of 1X Wash Buffer). About 50 µL of Diluted Enhancer Solution will be required for each assay well.

Note: *Keep each of diluted solutions except 1X Wash Buffer on ice until use. Any remaining diluted solutions other than 1X Wash Buffer should be discarded if not used within the same day.*

Approximate amount of required buffers and solutions for defined assay wells based on the protocol:

Reagents	1 Well	1 Strip (8 Wells)	2 Strips (16 Wells)	6 Strips (48 Wells)	12 Strips (96 Wells)
1X Wash Buffer	2.5 mL	20 mL	40 mL	120 mL	240 mL
Histone Buffer	50 µL	400 µL	800 µL	2400 µL	4800 µL
Blocking Buffer	0.15 mL	1.2 mL	2.5 mL	7.5 mL	14.5 mL
H4R3me2 Control, 10 ng/µL	N/A	N/A	4 µL (optional)	8 µL	8 µL
1X Capture Antibody	50 µL	400 µL	800 µL	2400 µL	4800 µL
1X Detection Antibody	50 µL	400 µL	800 µL	2400 µL	4800 µL
Diluted Enhancer solution	50 µL	400 µL	800 µL	2400 µL	4800 µL
Developer Solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL	9.6 mL
Stop Solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL	9.6 mL

10. SAMPLE PREPARATION

Input Amount: The amount of histone extracts for each assay can be between 0.1 µg and 2 µg with an optimal range of 0.2-0.5 µg.

Histone Extraction: You can use your method of choice for preparing nuclear extracts. Abcam offers a Histone Extraction Kit (ab113476) optimized for use with this kit. Histone extracts can be used immediately or stored at -80°C for future use.

11. STANDARD PREPARATION

Suggested Standard Curve Preparation:

11.1 Dilute H4R3me^2 Control with Histone Buffer to 10 ng/µL by adding 2 µL of H4R3me^2 Control to 8 µL of Histone Buffer.

11.2 Then, further prepare five concentrations by combining the 10 ng/µL H4R3me^2 Control with Histone Buffer into final concentrations of 0.5, 1, 2, 5, and 10 ng/µL according to the following dilution chart:

Tube	H4R3me2 Control (10 ng/µL) (µL)	Histone Buffer (µL)	Resulting H4R3me2 Control Concentration (ng/µL)
1	1.0	19.0	0.5
2	1.0	9.0	1.0
3	1.0	4.0	2.0
4	2.0	2.0	5.0
5	4.0	0.0	10.0

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

12. PLATE PREPARATION

The suggested strip-well plate setup for standard curve preparation in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	H4R3me2 Control 0.5 ng/well	H4R3me2 Control 0.5 ng/well	Sample	Sample	Sample	Sample
C	H4R3me2 Control 1 ng/well	H4R3me2 Control 1 ng/well	Sample	Sample	Sample	Sample
D	H4R3me2 Control 2 ng/well	H4R3me2 Control 2 ng/well	Sample	Sample	Sample	Sample
E	H4R3me2 Control 5 ng/well	H4R3me2 Control 5 ng/well	Sample	Sample	Sample	Sample
F	H4R3me2 Control 10 ng/well	H4R3me2 Control 10 ng/well	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

13. ASSAY PROCEDURE

- Internal Control: The assay control (methylated histone H4-Arg3) is provided in this kit for the quantification of global di-methyl histone H4R3. Because content of histone H4R3me2 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

13.1 Histone Binding

13.1.1 Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (include 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).

13.1.2 Blank wells: Add 49 µL of Histone Buffer to each blank well.

13.1.3 Standard wells: Add 49 µL of Histone Buffer and 1 µL of Diluted H4R3me2 Control into the standard curve wells (see the designated wells depicted in Section 12 – Plate Preparation). Mix solution by gently tilting from side to side or shaking the plate several times. Ensure the solution coats the bottom of the well evenly.

Note: *For the standard curve, add 1 µL of Diluted H4R3me2 Control at concentrations between 0.5-10 ng/µL (see Section 11 - Standard Preparation).*

13.1.4 Sample wells: Add 46-49 µL of Histone Buffer and 1-4 µL of your histone extracts. Total volume should be 50 µL per well. ***Note****: (1) Follow the suggested well setup diagrams as per Section 12 – Plate Preparation; (2) It is recommended to use 0.2-0.5 µg of histone extract per well.*

13.1.5 Tightly cover strip-well microplate with plate seal or Parafilm M to avoid evaporation and incubate at 37°C for 90-120 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.*

13.1.6 Remove the reaction solution from each well. Add 150 µL of Blocking Buffer to each well, then cover with Parafilm M or aluminum foil and incubate at 37°C for 30 minutes.

13.1.7 Remove the reaction solution from each well. Wash each well three times with 150 µL of the 1X Wash Buffer each time.

13.2 **Antibody Binding and Signal Enhancing**

13.2.1 Add 50 µL of the 1X Capture Antibody to each well, then cover Parafilm M or aluminum foil and incubate at room temperature for 60 minutes.

13.2.2 Remove the 1X Capture Antibody solution from each well.

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

13.2.4 Add 50 µL of the 1X Detection Antibody to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 minutes.

13.2.5 Remove the 1X Detection Antibody solution from each well.

13.2.6 Wash each well four times with 150 µL of the 1X Wash Buffer each time.

13.2.7 Add 50 µL of the Diluted Enhancer Solution to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 minutes.

13.2.8 Remove the Diluted Enhancer Solution from each well.

13.2.9 Wash each well five times with 150 µL of the 1X Wash Buffer.

Note: *Ensure any residual wash buffer in the wells is thoroughly removed at each wash step.*

13.3 **Signal Detection**

13.3.1 Add 100 µL of the Developer Solution to each well and incubate at room temperature for 1-10 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 DNA.

13.3.2 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 the Stop Solution and the absorbance should be read on a microplate reader within 2-10 minutes 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.*

13.3.3 Calculate % H4R3me2 using the formulae provided in Section 14 - Data Analysis.

14. ANALYSIS

Calculate the average duplicate readings for sample wells and blank wells. Calculate the % H4R3me2 using the following formula:

$$\text{H4R3me2 \%} = \frac{\text{Treated (tested) Sample OD} - \text{Blank OD}}{\text{Untreated (control) Sample OD} - \text{Blank OD}} \times 100\%$$

Example calculation:

Average OD450 of treated sample is 0.5
Average OD450 of untreated control is 0.9
Average OD450 of blank is 0.1

$$\text{H4R3me2 (\%)} = \frac{(0.5 - 0.1)}{(0.9 - 0.1)} \times 100\% = 50\%$$

For an accurate calculation, generate a standard curve and plot OD versus amount of H4R3me2 Control at each concentration and determine the slope as delta OD/ng. Calculate the amount of H4R3me2 using the following formula:

$$\text{Amount (ng/mg protein)} = \frac{\text{Sample OD} - \text{Blank OD}}{\text{Protein amount (µg)*} \times \text{Slope}} \times 1000$$

*Histone extract amount added into the sample well at step 13.1.4.

Typical Results

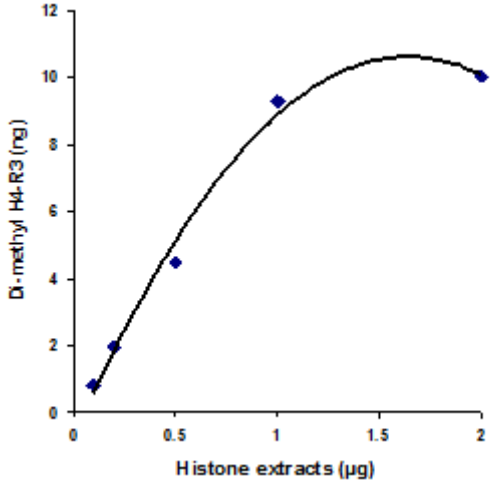


Figure 1. Histone extracts were prepared from MDA-231 cells using the (ab113476) Histone Extraction Kit and the amount of H4R3me2 was measured using Abcam's Histone H4 (di-methyl R3) Quantification Kit (Colorimetric) (ab156914).

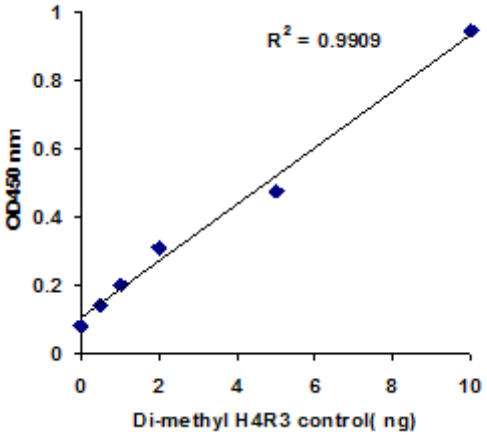


Figure 2. Illustrated standard curve generated with H4K3me2 Control.

15. 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 standard curve wells	The standard amount is insufficiently added to the well in step 13.1.3	Ensure a sufficient amount of standard is added
	The standard is degraded due to improper storage conditions	Follow the Storage and Stability guidance of this User Guide for storage of H4R3me2 Control
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 standard	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips
	Incubation time with diluted 1X Detection Antibody is too long	The incubation time at step 13.2.4 should not exceed 90 minutes
	Over development of color	Decrease the development time in step 13.3.1 before adding Stop Solution in step 13.3.2
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 Abcam's Histone Extraction Kit (ab113476)
	Sample amount added into the wells is insufficient	Ensure a sufficient amount of histone extracts is used as indicated in step 13.1. 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, with no more than 6 months for histone extracts
	Little or no activity of H4R3me2 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 extract

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)

16. NOTES

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 400 921 0189 / +86 21 2070 0500

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp