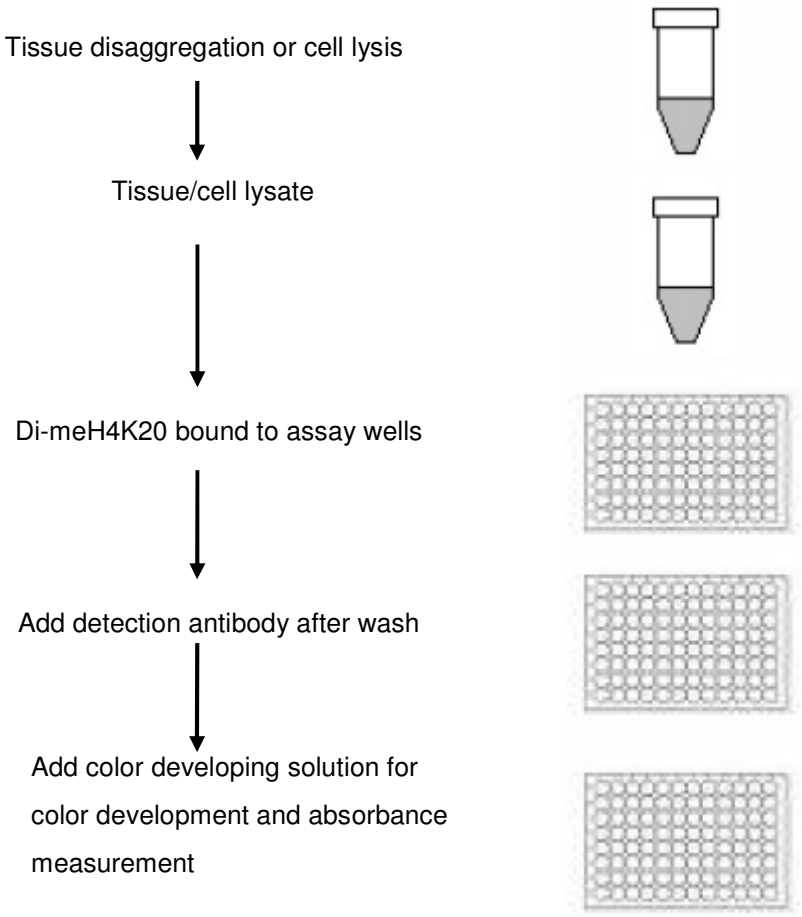
INTRODUCTION

Epigenetic activation or inactivation of genes play a critical role in many important human diseases, especially in cancer. A major mechanism for epigenetic inactivation of the genes is methylation of CpG islands in genome DNA caused by DNA methyltransferases. Histone methyltransferases (HMTs) control or regulate DNA methylation through chromatin-dependent transcriptional repression or activation. HMTs transfer 1-3 methyl groups from S-adenosyl-L-methionine to the lysine and arginine residues of histone proteins. PR-SET7, SET9, SUV4.20h, and ASH1are histone methyltransferases that catalyze methylation of histone H4 at lysine 20 (H4K20) in mammalian cells. Di-methylation of H4K20 has been de-scribed as another repressive chromatin domain and is involved in DNA damage response. The global H4K20 di-methylation can also be changed by inhibiton or activation of HMTs, making quantitative detection of global di-methyl histone H4K20 a useful tool for better understanding epigenetic regulation of gene activation/repression. This information is also useful for developing HMT-targeted drugs. Global Di-Methyl Histone H4K20 Quantification Kit (Colorimetric) provides a tool for measuring global di-methylation of histone H4K20. The kit has the following features:

- Quick and efficient procedure, which can be finished within 2.5 hours.
- Innovative colorimetric assay without the need for radioactivity, electrophoresis, or chromatography.
- Specifically captures di-methylated H4K20 with the detection limit as low as 5 ng/well and detection range from 20 ng-2 µg/well of histone extracts.
- The control is conveniently included for quantification of di-methylated H4K20.
- Strip microplate format makes the assay flexible: manual or high throughput.
- Simple, reliable, and consistent assay conditions.

PRINCIPLE AND PROCEDURE

Global Di-Methyl Histone H4K20 Quantification Kit (Colorimetric) is designed for measuring global histone H4K20 di-methylation. In an assay with this kit, the di-methylated histone H4 at lysine 20 is captured to the strip wells coated with an anti-dimethyl H4K20 antibody. The captured di-methylated histone H4K20 can then be detected with a detection antibody, followed by a color development reagent. The ratio of di-methylated H4K20 is proportional to the intensity of absorbance. The absolute amount of di-methylated H4K20 can be quantitated by comparing to the standard control.



PRODUCT USE INFORMATION

Global Di-Methyl Histone H4K20 Quantification Kit (Colorimetric) is suitable for specifically measuring global histone H4K20 di-methylation using a variety of mammalian cells (human, mouse, etc.) including fresh and frozen tissues, cultured adherent and suspension cells.

Global Di-Methyl Histone H4K20 Quantification Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application.

Suitable lab coat, disposable gloves, and eye protection are required when working with the kit.

Abnova guarantees the performance of all products in the manner described in our product instructions.

Abnova reserves the right to change or modify any product to enhance its performance and design.

Global Di-Methyl Histone H4K20 Quantification Kit (Colorimetric) and methods of use are covered by a pending US patent.

Material and Method

A. List of component

Components	48 assays
C1 (10X wash buffer)	10 ml
C2 (antibody buffer)	6 ml
C3 (detecting antibody, 1 mg/ml)*	5 µl
C4 (color developer)	5 ml
C5 (stop solution)	3 ml
Standard control (100 µg/ml)*	10 µl
Signal report solution*	5 µl
Signal enhancer*	120 µl
8 well sample strips (with frame)	4
8 well standard control strips	2

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

SHIPPING AND STORAGE

Upon receipt, store standard control at –20℃. Store all other components at 4℃ away from light. The components of the kit are stable for up to 6 months from date of shipment when stored properly.

Note: Check if buffers C1 and C2 contain salt precipitates before using. If so, warm (at room temperature or 37℃) and shake the buffers until the salts are re-dissolved.

MATERIALS REQUIRED BUT NOT SUPPLIED

Orbital shaker

Pipettes and pipette tips

Reagent reservoir

Microplate reader

PROTOCOL

1. **a)** Prepare histone extracts from cells/tissues treated or untreated by using your own successful method (acid extraction or high salt extraction).

   b) Preparation of histone extracts can also be performed using the attached procedure (See Histone Extraction Protocol). Histone extracts can be used immediately or stored at –80°C for future use.
2. Determine the number of strip wells required. Leave these strips in the plate frame (remaining unused strips can be placed back in the bag. Seal the bag tightly and store at 4°C). Dilute **C1** with distilled water (pH 7.2-7.5) at 1:9 ratio (1 ml of **C1**+ 9 ml of water).
3. Add 50 µl of **C2** into each well. For the sample, add 1-2 µg of the histone extract into the sample wells. For standard curve, dilute standard control with **C2** to 1 – 100 ng/µl for 5-7 points (e.g., 1.5, 3, 6, 12, 25, 50, and 100 ng/µl). Add 1 µl of standard control at the different concentrations into the standard wells. For the blank, do not add any nuclear extracts or standard control protein. Mix and cover the strip wells with Parafilm M and incubate at room temperature for 1 hour.

   Meanwhile, prepare detection solution: for each 1 ml of detection solution to be made, first add 1 µl of **C3** and 0.5 µl of signal report solution into 10 µl of diluted **C1**, mix and incubate at room temperature for 10 min; then add 20 µl of signal enhancer, mix and incubate at room temperature for 15 min; at last add 970 µl of diluted **C1** and mix.
4. Aspirate and wash the wells with 150 µl of diluted **C1** 3 times.
5. Add 50 µl of the detection solution to each well and incubate at room temperature for 60 min on an orbital shaker (100 rpm).
6. Aspirate and wash the wells with 150 µl of diluted **C1** 6 times.
7. Add 100 µl of **C4** into the wells and incubate at room temperature for 2-10 min away from light. Monitor color development in the sample and standard well (blue).
8. Add 50 µl of **C5** to each well to stop enzyme reaction when color in the standard wells containing the higher concentrations of standard control turns medium blue. The color should change to yellow and absorbance can be read on a microplate reader at 450 nm within 1-15 min.
9. Calculate % histone H4K20 di-methylation:

$$\text{Di - methylation}\,\% = \frac{\text{OD (treated (tested) sample blank)}}{\text{OD (untreated (control) sample blank)}} \times 100\%$$

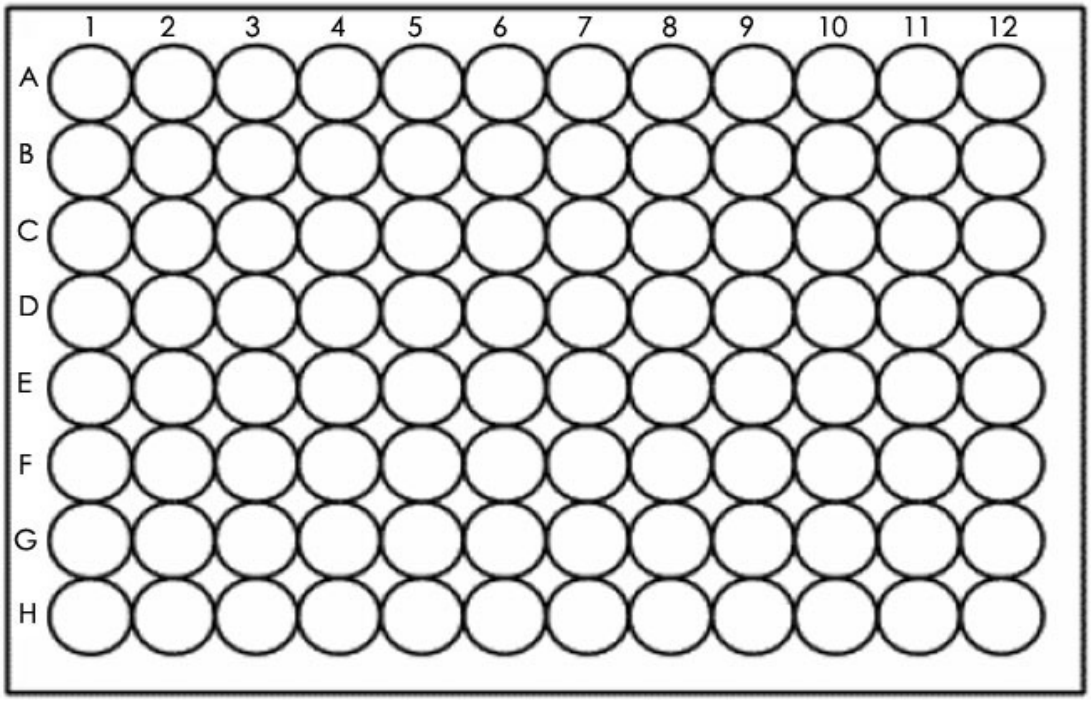
   For the amount quantification, plot OD versus amount of standard control and determine the slope as delta OD/ng.

   Calculate the amount of di-methylated H4K20 using the following formula:

$$\text{Amount (ng/mg protein)} = \frac{\text{OD (sample blank)}}{\text{Protein (ug) * x slope}} \text{x}\,1000$$

   * Histone extract amount added into the sample well at step 3.

Plate configuration



- Strip 1--2 (for 48 assays): standard wells (labeld as SC); the standard curve can be generated with 5-8 concentration points (includes blank).
- Example amount of standard control/well: **A1**: 100 ng; **B1**: 50 ng; **C1**: 25 ng; **D1**: 12 ng; **E1**: 6 ng; **F1:** 3 ng; **G1**: 1.5 ng; **H1**: 0 ng.
- Strip 3-6 (for 48 assays): sample wells (No label).
- Each sample or standard point can be assayed in the duplicates or triplicates.

Histone Extraction Protocol

1. For tissues (treated and untreated), weigh the sample and cut the sample into small pieces (1-2 mm^3) with a scalpel or scissors. Transfer tissue pieces to a Dounce homogenizer, add TEB buffer (PBS containing 0.5% Triton X 100, 2 mM PMSF and 0.02% NaN_3) at 200 mg/ml, and disaggregate tissue pieces by 50-60 strokes. Transfer homogenized mixture to a 15 ml conical tube and centrifuge at 3000 rpm for 5 min at 4℃. If total mixture volume is less than 2 ml, transfer mixture to a 2 ml vial and centrifuge at 10,000 rpm for 1 min at 4℃. Remove supernatant.
   For cells (treated and untreated), harvest cells and pellet the cells by centrifugation at 1,000 rpm for 5 min at 4℃. Resuspend cells in TEB buffer at 10^7 cells/ml and lyse cells on ice for 10 min with gentle stirring. Centrifuge at 3,000 rpm for 5 min at 4℃. If total volume is less than 2 ml, transfer cell lysates to a 2 ml vial and centrifuge at 10,000 rpm for 1 min at 4℃. Remove supernatant.
2. Resuspend cell/tissue pellet in 3 volumes (approx. 200 µl/10^7 cells or 200 mg tissues) of extraction buffer (0.5N HCl + 10% glycerol) and incubate on ice for 30 min.
3. Centrifuge at 12,000 rpm for 5 min at 4℃ and remove the supernatant fraction to new vial.
4. Add 8 volumes (approx. 0.6 ml/10^7 cells or 200 mg tissues) of acetone and leave overnight at –20℃.
5. Centrifuge at 12,000 rpm for 5 min and air-dry the pellet. Dissolve the pellet in distilled water (30-50 µl/10^7 cells or 200 mg tissues).
6. Quantify the protein concentration. Aliquot the extract and store the extract at –20℃ or –80℃.

TROUBLESHOOTING

<u>No Signal for Both the Standard Control and the Samples</u>	
Reagents are added incorrectly.	Check if reagents are added in order and if some steps of the procedure are omitted by mistake.
Incubation time and temperature is incorrect.	Ensure the incubation time and temperature described in the protocol are correctly followed.
<u>No Signal or Very Weak Signal for Only the Standard Control</u>	
The amount of standard control is not added into “standard control wells” or is added insufficiently.	Ensure sufficient amount of control is properly added to the standard control well.
<u>No Signal for Only the Sample</u>	
The protein sample is not properly extracted.	Ensure the procedure and reagents are correct for the nuclear protein ex-traction.
The protein amount is added into well insufficiently.	Ensure extract contains sufficient amounts of protein.
Protein extracts are incorrectly stored.	Ensure the nuclear extracts are stored at –20℃ or –80℃.
<u>High Background Present for the Blank</u>	
The well is not washed enough.	Check if wash at each step is per-formed according to the protocol.
Contaminated by the standard control.	Ensure the well is not contaminated by adding the control protein or by using control protein contaminated tips.
Overdevelopment.	Decrease development time in Step 7.