MethylFlash™ m^{6}A DNA Methylation ELISA Kit (Colorimetric)

Base Catalog # P-9010

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The MethylFlash™ m^{6}A DNA Methylation ELISA Kit (Colorimetric) is suitable for detecting N6-methyladenosine (m^{6}A) DNA methylation status directly using DNA isolated from any species such as mammals, plants, fungi, bacteria, and viruses.

Input DNA: The amount of DNA for each assay can be 100 ng to 300 ng. For optimal quantification, the input DNA amount should be 200 ng, as the abundance of m^{6}A is generally less than 0.05% of total DNA.

Starting Material: Starting materials can include various tissue or cell samples such as cells from flask or microplate cultured cells, fresh and frozen tissues, paraffin-embedded tissues, blood, body fluid samples, etc.

Internal Control: Both negative and positive m6A controls are provided in this kit. A standard curve can be performed (range: 0.04 to 0.4 ng of m^{6}A) or a single quantity of m^{6}A can be used as a positive control. Because m^{6}A content can vary from tissue to tissue, and from normal and diseased states, or vary under treated and untreated conditions, it is advised to run replicate samples to ensure that the signal generated is validated. This kit will allow the user to quantify an absolute amount of m^{6}A and determine the relative m^{6}A DNA methylation states of two different DNA samples.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

KIT CONTENTS

Component	48 Assays Cat. #P-9010-48	96 Assays Cat. #P-9010-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
BS (Binding Solution)	5 ml	10 ml	RT
NDC (Negative DNA Control, 100 µg/ml)*	10 µl	20 µl	–20°C
PC (Positive Control, m^6A 2 µg/ml)*	10 µl	20 µl	–20°C
CA (Capture Antibody, 1000 X)*	5 µl	10 µl	4°C
DA (Detection Antibody, 1000 X)*	6 µl	12 µl	–20°C
ES (Enhancer Solution)*	5 µl	10 µl	–20°C
DS (Developer Solution)	5 ml	10 ml	4°C
SS (Stop Solution)	5 ml	10 ml	RT
8-Well Assay Strips (With Frame)	6	12	4°C

** Spin the solution down to the bottom prior to use.*

Note: *The* ***NC*** *(Negative Control) is DNA polynucleotides containing no* m^6A*.* m^6A *oligos and is normalized to have 100% of* m^6A*.*

SHIPPING & STORAGE

The kit is shipped in two parts: the first part at ambient room temperature and the second part on frozen ice packs at 4°C.

Upon receipt: (1) Store **NDC**, **PC**, **DA**, and **ES** at –20°C away from light; (2) Store **WB**, **CA**, **DS**, and **8-Well Assay Strips** at 4°C away from light; (3) Store remaining components (**BS** and **SS**) at room temperature away from light.

Note: Check if wash buffer, **WB**, contains salt precipitates before using. If so, warm (at room temperature or 37°C) and shake the buffer until the salts are re-dissolved.

All components of the kit are stable for 6 months from the date of shipment, when stored properly.

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Adjustable pipette
- ☐ Aerosol resistant pipette tips
- ☐ Microplate reader capable of reading absorbance at 450 nm
- ☐ 1.5 ml microcentrifuge tubes
- ☐ Incubator for 37°C incubation
- ☐ Plate seal or Parafilm M
- ☐ Distilled water

- ☐ 1X TE buffer pH 7.5 to 8.0
- ☐ Isolated DNA of interest

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the MethylFlash™ m^{6}A DNA Methylation ELISA Kit (Colorimetric) is tested against predetermined specifications to ensure consistent product quality. EpigenTek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply contact our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: EpigenTek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigentek.com/datasheet.

Usage Limitation: The MethylFlash™ m^{6}A DNA Methylation ELISA Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The MethylFlash™ m^{6}A DNA Methylation ELISA Kit (Colorimetric) and methods of use contain proprietary technologies by EpigenTek.

A BRIEF OVERVIEW

N^6-methyladenosine (m^{6}A) is the most common and abundant modification on RNA molecules present in eukaryotes. DNA m^{6}A is also identified in multicellular eukaryotes including Caenorhabditis elegans and Drosophila melanogaster, and furthermore identified in higher eukaryotes including plants, mouse and human cells. m6A plays crucial roles in regulating DNA replication, transposition, transcription, and cellular defense. In humans, the DNA m^{6}A modification is most likely catalyzed by a methyltransferase complex METTL3 and removed by the α-ketoglutarate (α-KG)- and Fe^{2+}-dependent dioxygenases such as ALKBH5 and TET-like enzymes. It was shown that METTL3 and α-KG /Fe^{2+}-dependent dioxygenases play important roles in many biological processes, ranging from development and metabolism to fertility. The dynamic and reversible chemical m^{6}A modification on DNA may also serve as a novel epigenetic marker of profound biological significance. Down-regulation of m^{6}A modification was first characterized in human cancer cells and tissues, relative to their normal controls. m^{6}A is found to be the most regulated DNA modification in cancers. In addition to the regulation in cancer cells, relative to the primary cell/tissues which contain quite low amounts of DNA m^{6}A (<0.001%), a hundreds-fold increase of m^{6}A modification was found for *in vitro* cultured human cells (0.03%-0.22%). Therefore, identifying m^{6}A DNA methylation levels and distribution on DNA could advance understanding of epigenetic regulation of biological process at the genomic level, and further provide useful information for improving diagnostics and therapeutics of disease.

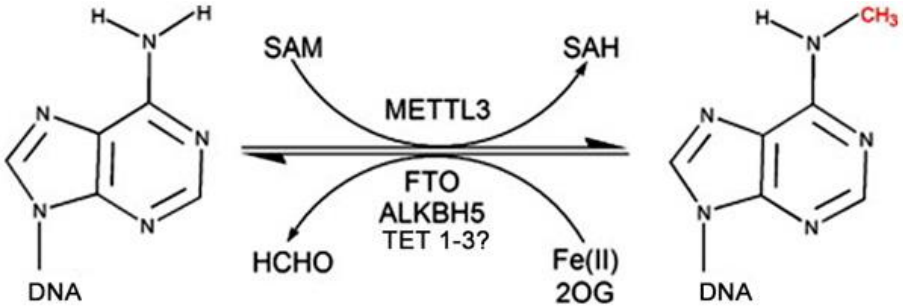


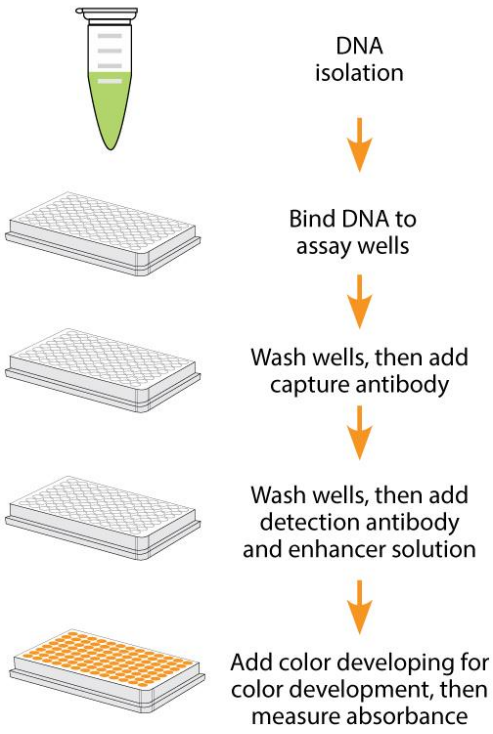
Fig 1. Reversible m^{6}A methylation in DNA

Several chromatography-based techniques such as HPLC-ECD and LC-MS are used for detecting DNA m^{6}A in tissues and cells. However, these methods are time consuming and have low throughput with high costs. To address these problems, EpigenTek offers the MethylFlash™ m^{6}A DNA Methylation ELISA Kit (Colorimetric) which uses a unique procedure to directly quantify m^{6}A DNA methylation status using DNA isolated from cells/tissues. As the first commercially available product used for quantification of m^{6}A DNA methylation, this kit has the following advantages and features:

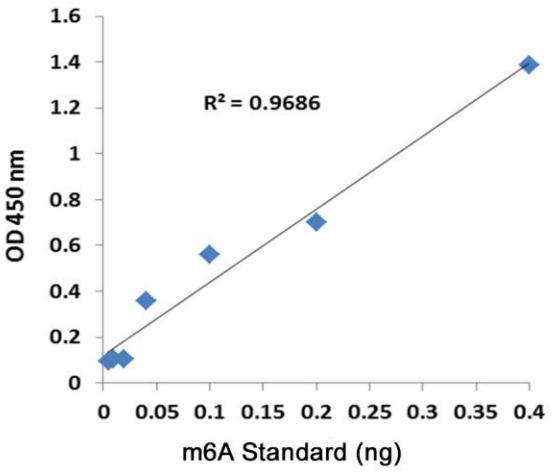
- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be completed within 3 hours and 45 minutes.
- High sensitivity, of which the detection limit can be as low as 5 pg of m^{6}A.
- Unique binding solution allows DNA >100 nts to be tightly bound to the wells, which enables quantification of m^{6}A from both intact DNA or fragmented DNA such as ChIPed DNA.
- Optimized antibody and enhancer solutions allow high specificity to m^{6}A, with no cross-reactivity to unmethylated adenosine within the indicated concentration range of the sample DNA.
- Universal positive and negative controls are included, which are suitable for quantifying m^{6}A from any species.
- Strip-well microplate format makes the assay flexible for manual or high throughput analysis.
- Simple, reliable, and consistent assay conditions.

PRINCIPLE & PROCEDURE

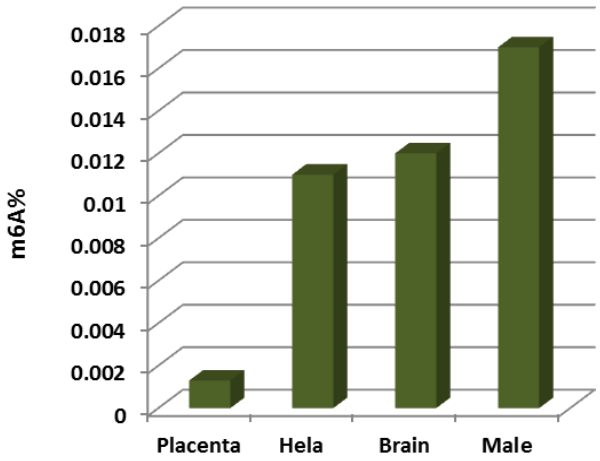
The MethylFlash™ m^{6}A DNA Methylation ELISA Kit (Colorimetric) contains all reagents necessary for the quantification of m^{6}A in DNA. In this assay, DNA is bound to strip wells using DNA high binding solution. m^{6}A is detected using capture and detection antibodies. The detected signal is enhanced and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of m^{6}A is proportional to the OD intensity measured.



Schematic procedure of the MethylFlash ™ m^{6}A DNA Methylation ELISA Kit (Colorimetric)



m^{6}A standard control was added into the assay wells at different concentrations and then measured with the EpiQuik™ m^{6}A DNA Methylation ELISA Kit (Colorimetric)



Quantification of m^{6}A content of various human DNA samples with the MethylFlash ™ m^{6}A DNA Methylation ELISA Kit (Colorimetric)

ASSAY PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

1. Starting Materials

Input DNA Amount: DNA amount can range from 100 ng to 300 ng per reaction. An optimal amount is 200 ng per reaction. Input DNA should be highly pure with 260/280 ratio >1.8 and relatively free of RNA. RNase A can be used to remove RNA. Starting DNA may be in water or in a buffer such as TE. You can use your method of choice for DNA isolation.

DNA Storage: Isolated DNA can be stored at -20°C (short term) or -80°C (long term) until use.

2. Buffer and Solution Preparation

a. Preparation of 1X Wash Buffer:

48-Assay Kit: Add 13 ml of **WB** (10X Wash Buffer) to 117 ml of distilled water (final pH 7.2-7.5).

96-Assay Kit: Add 26 ml of **WB** (10X Wash Buffer) to 234 ml of distilled water (final pH 7.2-7.5).

This **Diluted WB** 1X Wash Buffer can now be stored at 4°C for up to six months.

b. Prepare Diluted **CA** (Capture Antibody) Solution:

Dilute **CA** (Capture Antibody) with **Diluted WB** at a ratio of 1:1000 (i.e., add 1 µl of **CA** to 1000 µl of **Diluted WB**). About 50 µl of this **Diluted CA** will be required for each assay well.

c. Prepare Diluted **DA** (Detection Antibody) Solution:

Dilute **DA** (Detection Antibody) with **Diluted WB** at a ratio of 1:2000 (i.e., add 1 µl of **DA** to 2000 µl of **Diluted WB**). About 50 µl of this **Diluted DA** will be required for each assay well.

d. Prepare Diluted **ES** (Enhancer Solution):

Dilute **ES** (Enhancer Solution) with **Diluted WB** at a ratio of 1:5000 (i.e., add 1 µl of **ES** to 5000 µl of **Diluted WB**). About 50 µl of this **Diluted ES** will be required for each assay well.

e. Preparation of Diluted Positive Control:

Single Point Control Prep: Dilute **PC** (Positive Control) with 1X TE buffer to 0.5 ng/µl of m6A (ex: 1 µl **PC** + 3 µl TE).

Suggested Standard Curve Prep: First, dilute **PC** to 0.5 ng/µl (ex: 5 µl of **PC** + 15 µl of 1X TE) and 0.1 ng/ µl (ex; 1 µl of PC + 19 µl of 1 X TE). Then, further prepare 7 different concentrations with the 0.5 ng/µl and 0.1 ng/µl **PC,** and 1X TE into 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, and 0.2 ng/µl according to the following dilution chart:

Tube	PC (0.5 ng/µl)	1 X TE buffer	PC (0.1 ng/µl)	Resulting PC Concentration
1	0.0 µl	19.5 µl	0.5 µl	0.002 ng/ µl
2	0.0 µl	9.5 µl	0.5 µl	0.005 ng/ µl
3	0.0 µl	9 µl	1 µl	0.01 ng/µl
4	0.0 µl	4 µl	1 µl	0. 02 ng/µl
5	0.0 µl	3 µl	3 µl	0.05 ng/µl
6	1.0 µl	4 µl	0.0 µl	0.1 ng/µl
7	2 µl	3 µl	0.0 µl	0.2 ng/µl

Note: *Keep each of the diluted solutions (except **Diluted WB**) on ice until use. Any remaining diluted solutions, other than **Diluted WB**, should be discarded if not used within the same day.*

3. DNA Binding

a. 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).

b. Add 80 µl of **BS** (Binding Solution) to each well.

c. Add 2 µl of **NDC**, 2 µl of **Diluted PC** (see note below), and 200 ng of your sample DNA (1-8 µl) into the designated wells depicted in Table 1 or Table 2. 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: *(1) For a single point control, add 2 µl of **PC** at a concentration of 0.5 ng/µl as prepared in Step 2e; For the standard curve, add 2 µl of **Diluted PC** at concentrations of 0.002 to 0.2 ng/µl (see the chart in Step 2e). The final amounts should be 0.004, 0.01, 0.02, 0.04, 0.1, 0.2, and 0.4 per well. (2) For optimal binding, sample DNA volume added should not exceed 8 µl. (3) To ensure that **NDC**, **Diluted PC**, and sample DNA are completely added into the wells, the pipette tip should be placed into the **BS** solution in the well and aspirated in/out 1-2 times.*

d. Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 90 min.

e. Remove the **BS** (Binding Solution) from each well. Wash each well with 150 µl of **Diluted WB** by pipetting **Diluted WB** into the wells and then removing it using a pipette. Repeat the wash two times for a total of three washes.

4. m^{6}A DNA Capture

a. Add 50 µl of **Diluted CA** to each well, then cover and incubate at room temperature for 60 min.

b. Remove the **Diluted CA** solution from each well using a pipette.

c. Wash each well with 150 µl of **Diluted WB** each time for three times.

d. Add 50 µl of **Diluted DA** to each well, then cover and incubate at room temperature for 30 min.

e. Remove the **Diluted DA** solution from each well using a pipette.

f. Wash each well with 150 µl of **Diluted WB** each time for four times.

g. Add 50 µl of **Diluted ES** to each well, then cover and incubate at room temperature for 30 min.

h. Remove the **Diluted ES** solution from each well.

i. Wash each well with 150 µl of **Diluted WB** each time for five times.

5. Signal Detection

a. Add 100 µl of **DS** to each well and incubate at room temperature for 1 to 10 min away from light. Begin monitoring color change in the sample wells and control wells. The **DS** solution will turn blue in the presence of sufficient m^{6}A.

b. Add 100 µl of **SS** 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 **SS** and the absorbance should be read on a microplate reader at 450 nm within 2 to 15 min.

Note: *If the strip-well plate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

6. m^{6}A Calculation

Relative Quantification: To determine the relative m^{6}A DNA methylation status of two different DNA samples, a simple calculation for the percentage of m^{6}A in your DNA can be carried out using the following formula:

$$m^6A\ \% = \frac{(Sample\ OD - NDC\ OD) \div S}{(PC\ OD - NDC\ OD) \div P} \times 100\%$$

S is the amount of input sample DNA in ng.
P is the amount of input positive control (**PC**) in ng.

Example calculation:

Average OD450 of NDC is 0.1
Average OD450 of PC is 0.6
Average OD450 of Sample is 0.14
S is 200 ng
P is 1 ng

$$m^6A\ \% = \frac{(0.14 - 0.1) \div 200}{(0.6 - 0.1) \div 1} \times 100\% = \mathbf{0.04\%}$$

Absolute Quantification: To quantify the absolute amount of m^{6}A using an accurate calculation, first generate a standard curve and plot the OD values (background (NC)-subtracted) versus the amount of **PC** at each concentration point. Next, determine the slope (OD/ng) of the standard curve using linear regression (*Microsoft Excel's* linear regression functions are suitable for such calculation). Use the most linear part of the standard curve (include at least 4 concentration points) for optimal slope calculation. Now calculate the amount and percentage of m^{6}A in your DNA using the following formulas:

$$m^6A\ (ng) = \frac{Sample\ OD - NC\ OD}{Slope}$$

$$m^6A\ \% = \frac{m^6A\ Amount\ (ng)}{S} \times 100\%$$

S is the amount of input sample DNA in ng.

Example calculation:

Average OD450 of NC is 0.10
Average OD450 of sample is 0.14
Slope is 0.5 OD/ng
S is 200 ng

$$m^6A\ (ng) = \frac{0.14 - 0.1}{0.5} = \mathbf{0.08\ ng}$$

$$m^6A\ \% = \frac{0.08}{200} \times 100\% = \mathbf{0.04\%}$$

SUGGESTED STRIP WELL SETUP

Table 1. Review the configuration of the strip-well plate setup for standard curve preparation in a 48-assays format below (for a 96-reaction format, strips 7 through 12 can be configured as *Sample*). The controls and samples can be measured in duplicate, loaded vertically instead of horizontally.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	Sample3	Sample7	Sample11	Sample15	Sample19
B	NC	Sample3	Sample7	Sample11	Sample15	Sample19
C	PC	Sample4	Sample8	Sample12	Sample16	Sample20
D	PC	Sample4	Sample8	Sample12	Sample16	Sample20
E	Sample1	Sample5	Sample9	Sample13	Sample17	Sample21
F	Sample1	Sample5	Sample9	Sample13	Sample17	Sample21
G	Sample2	Sample6	Sample10	Sample14	Sample18	Sample22
H	Sample2	Sample6	Sample10	Sample14	Sample18	Sample22

Table 2. Review the configuration of the strip-well plate setup for standard curve preparation in a 48-assays format below (for a 96-reaction format, strips 7 through 12 can be configured as *Sample*). The controls and samples can be measured in duplicate, loaded vertically instead of horizontally.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	PC 0.04 ng/well	Sample1	Sample5	Sample9	Sample13
B	NC	PC 0.04 ng/well	Sample1	Sample5	Sample9	Sample13
C	PC 0.004 ng/well	PC 0. 1 ng/well	Sample2	Sample6	Sample10	Sample14
D	PC 0.004 ng/well	PC 0.1 ng/well	Sample2	Sample6	Sample10	Sample14
E	PC 0.01 ng/well	PC 0.2 ng/well	Sample3	Sample7	Sample11	Sample15
F	PC 0.01 ng/well	PC 0. 2 ng/well	Sample3	Sample7	Sample11	Sample15
G	PC 0.02 ng/well	PC 0. 4 ng/well	Sample4	Sample8	Sample12	Sample16
H	PC 0. 02 ng/well	PC 0.4 ng/well	Sample4	Sample8	Sample12	Sample16

SUGGESTED WORKING BUFFER AND SOLUTION SETUP

Table 3. Approximate amount of required buffers and solutions for defined assay wells based on the protocol.

Reagents	1 well	8 wells (1 strip)	16 wells (2 strips)	48 wells (6 strips)	96 wells (12 strips)
Diluted WB	2.5 ml	20 ml	40 ml	120 ml	240 ml
BS	80 µl	640 µl	1300 µl	3900 µl	8000 µl
Diluted CA	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted DA	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted ES	50 µl	400 µl	800 µl	2400 µl	4800 µl
DS	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml
SS	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml
NDC	N/A	0.5 µl – 1 µl	0.5 µl – 2 µl	1 µl – 4 µl	2 µl – 8 µl
PC	N/A	0.5 µl – 1 µl	0.5 µl – 2 µl	1 µl – 4 µl	2 µl – 8 µl

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal in both the positive control and sample wells	Reagents are added incorrectly.	Check if reagents are added in the proper order and if any steps in the protocol may have been omitted by mistake.
	The well is incorrectly washed before DNA binding.	Ensure the well is **NOT** washed prior to adding the positive control and sample.
	The bottom of the well is not completely covered by the **BS** (Binding Solution).	Ensure the solution coats the bottom of the well by gently tilting from side to side or shaking the plate several times.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol is followed correctly.
	Insufficient input materials.	Ensure that a sufficient amount of positive control (> 0.2 ng) and sample (200 ng) is added into the wells.
	Incorrect absorbance reading.	Check if the appropriate absorbance wavelength (450 nm) is used.
	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperature and the cap is tightly secured after each opening or use.
No signal or weak signal in only the **PC** (Positive control) wells	The **PC** (Positive Control) is insufficiently added to the well in Step 3c.	Ensure a sufficient amount of **PC** (Positive Control) is added.
	The **PC** (Positive Control) is degraded due to improper storage conditions.	Follow the Shipping & Storage guidelines of this User Guide for storage of **PC** (Positive Control).
High background present in the negative control wells	Insufficient washing of wells.	Check if washing recommendations at each step are performed according to the protocol.
	Contaminated by sample or positive control.	Ensure the well is not contaminated by the sample or positive control or from the use of contaminated tips.
	Incubation time is too long.	The incubation time at Step 3d should not exceed 2 h.
	Over development of color.	Decrease the development time in Step 5a before adding **SS** (Stop Solution) in Step 5b.
No signal or weak signal only in sample wells	DNA sample is not properly extracted or purified.	Ensure the DNA sample is good quality. The 260/280 ratio should be >1.8 with no or minimal RNA contamination.

	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of DNA is used as indicated in Step 3c.
	Little or no m^6A contained in the sample.	N/A
Uneven color development	Insufficient washing of the wells.	Ensure the wells are washed according to the washing guidelines. Make sure the residue of the washing buffer is removed as much as possible.
	Delayed color development or delayed stopping of color development in the wells.	Ensure color development solution or stop solution is added sequentially and is consistent with the order you added the other reagents (e.g., from well A to well H or from well 1 to well 12).

RELATED PRODUCTS

Methylated and Hydroxymethylated DNA Quantification

P-1030 MethylFlash™ Global DNA Methylation (5-mC) Easy Kit (Colorimetric)
P-1032 MethylFlash™ Global DNA Hydroxymethylation Easy Kit (Colorimetric)
P-1034 MethylFlash™ Methylated DNA Quantification Kit (Colorimetric)
P-1035 MethylFlash™ Methylated DNA Quantification Kit (Fluorometric)
P-1036 MethylFlash™ Hydroxymethylated DNA Quantification Kit (Colorimetric)
P-1037 MethylFlash™ Hydroxymethylated DNA Quantification Kit (Fluorometric)

RNA Methylation Detection

P-9003 Methylamp™ RNA Bisulfite Conversion Kit
P-9005 EpiQuik™ m6A RNA Methylation Quantification Kit (Colorimetric)
P-9008 EpiQuik™ m6A RNA Methylation Quantification Kit (Fluorometric)
P-9010 MethylFlash™ m6A DNA Methylation ELISA Kit (Colorimetric)
P-9015 MethylFlash™ Urine N6-methyladenosine (m6A) Quantification Kit (Colorimetric)
P-9016 EpiNext™ CUT&RUN RNA m6A-Seq Kit
P-9016 EpiQuik™ CUT&RUN M6A-Seq Kit
P-9018 EpiQuik™ CUT&RUN M6A Enrichment Kit