MethylFlash™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit (Colorimetric)

Base Catalog # P-9015

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The MethylFlash™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit (Colorimetric) is suitable for detecting total urinary m^{6}A levels, resulting from whole body turnover or degradation of DNA/RNA containing m^{6}A, using urine from humans and animals. The urine samples can be in fresh or frozen form.

Starting Material and Input Amount of Urine: The volume of urine for each assay can be between 1 and 20 µl. For optimal quantification, the input urine volume should be 5 µl.

Internal Control: Both a negative control and a positive m^{6}A standard are provided in this kit. A standard curve can be performed (range: 0.01 to 0.5 ng). Because m^{6}A content in urine can vary from individual to individual, and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated has been validated. This kit will allow the user to quantify an absolute amount of m^{6}A and to determine the relative turnover states of m^{6}A DNA/RNA of two different urine 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.

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KIT CONTENTS

Component	48 Assays Cat. #P-9015-48	96 Assays Cat. # P-9015-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
BS (Binding Solution)	5 ml	10 ml	RT
MS (m^6A Standard**,** 2 µg/ml)*****	10 µl	20 µl	–20°C
NC (Negative Control, 50 µg/ml)*	10 µl	20 µl	–20°C
MAS (m^6A Assay Solution, 500X)*	10 µl	20 µl	–20°C
CA (Capture Antibody, 1000X)*	4 µl	8 µl	4°C
DA (Detection Antibody, 2000X)*	4 µl	8 µl	–20°C
ES (Enhancer Solution)*	4 µl	8 µl	–20°C
DS (Developer Solution)	5 ml	10 ml	4°C
SS (Stop Solution)	5 ml	10 ml	RT
Plate 1 (Assay Plate, flat bottom)	1 (6 strips)	1 (12 strips)	4°C
Plate 2 (Sample Preparation Plate, round bottom)	1 (6 strips)	1 (12 strips)	RT

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

***Note**: The **NC** (Negative Control) is an RNA containing no* m^6A*. The **MAS** (m6A Assay Solution) is* 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 **MS**, **NC, MAS**, **DA** and **ES** at –20°C away from light; (2) Store **WB**, **CA**, **DS**, and **Plate 1** at 4°C away from light; (3) Store remaining components (**BS**, **SS**, and **Plate 2**) at room temperature away from light.

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

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

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

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- ☐ Incubator for 37°C incubation
- ☐ Plate seal or Parafilm M
- ☐ Distilled water
- ☐ 1X TE buffer pH 7.5 to 8.0
- ☐ Urine sample

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the MethylFlash™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit 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™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic applications.

Intellectual Property: The MethylFlash™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit (Colorimetric) and methods of use contain proprietary technologies by EpigenTek.

A BRIEF OVERVIEW

Nucleobase m^{6}A, a modified form of adenosine converted by adenosine methyltransferases is widespread in different cellular RNAs and also found in DNA. The biological importance of DNA/RNA m^{6}A-methylation as a major epigenetic modification in phenotype and gene expression has been recognized widely. m6A plays crucial roles in regulating DNA replication, DNA damage, RNA splicing, transposition, transcription, and cellular defense. In humans, the m^{6}A modification is probably catalyzed by a methyltransferase complex METTL3/METTL14 and removed by the α-ketoglutarate (α-KG)- and Fe^{2+}-dependent dioxygenases such as FTO, 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.

Urinary excretion of m^{6}A is an indication of the whole body turnover or the degradation of DNA/RNA, especially tRNA. The urinary m^{6}A level can be changed with a change of the bodies' turnover of m^{6}A DNA/RNA or alteration of cellular DNA/RNA m^{6}A status. A number of studies have indicated that m^{6}A excreted in urine has the potential to act as a cancer biomarker. For example, an elevated level of urinary m^{6}A was observed in colorectal cancer patients with active disease states.

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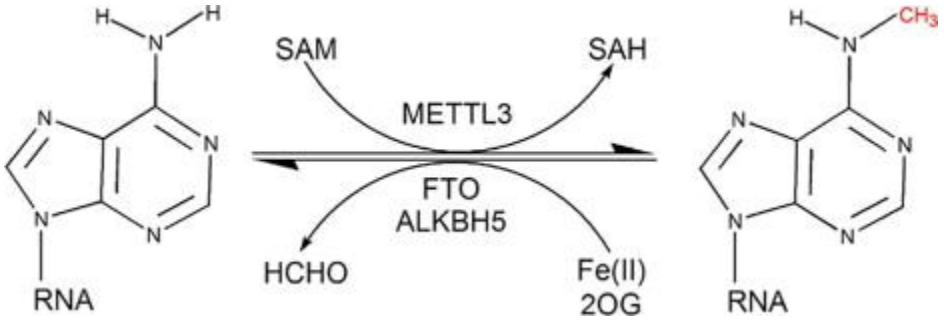

Fig 1. Reversible m^{6}A methylation in RNA

Chromatography-based techniques such as HPLC and TLC mass spectrometry are used for detecting m^{6}A in urine. These methods are accurate but are time consuming, less sensitive, and have low throughput with high costs. To address this problem, EpigenTek offers the MethylFlash™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit to quantify m^{6}A or body turnover status of m^{6}A DNA/RNA using urine samples. The kit has the following advantages and features:

- Innovative colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be finished within 4 hours.
- 96 strip-well microplate format makes the assay flexible: manual or high throughput analysis.
- Innovative kit composition enables background signals to be extremely low, which eliminates the need for plate blocking and allows the assay to be simple, accurate, reliable, and consistent.
- The level of m^{6}A measured in human urine samples using this kit is comparable to that detected by HPLC method.
- A novel assay principle allows high sensitivity to be achieved. The detection limit can be as low as 0.01 ng/assay well.
- Low input range of urine for each assay with a volume of 1 to 20 µl and an optimal volume of 5 µl.
- Optimized antibody and enhancer solutions allow for high specificity to m^{6}A, without cross-reactivity to unmethylated adenosine.
- Negative control and positive standard are included, which are suitable for quantification of m^{6}A in free form and m^{6}A contained in DNA/RNA fragments from different urine samples.

PRINCIPLE & PROCEDURE

In this ELISA-like inhibitory competitive immunoassay, urine samples and the m^{6}A standard are first incubated with a m^{6}A antibody solution and then transferred to strip wells coated with m^{6}A polynucleotide. The well is washed to remove any unbound reagents after incubation and then a detection antibody is added to generate a signal that can be measured colorimetrically by reading the absorbance in a microplate spectrophotometer. Because m^{6}A in the urine sample inhibits the binding of m^{6}A antibody to m^{6}A coated on the well, higher concentrations of m^{6}A in the urine sample lead to a reduced binding of the antibody to the m^{6}A on the well. Therefore the signal or OD intensity measured from the well will be inversely proportional to the amount of m^{6}A in the urine sample and

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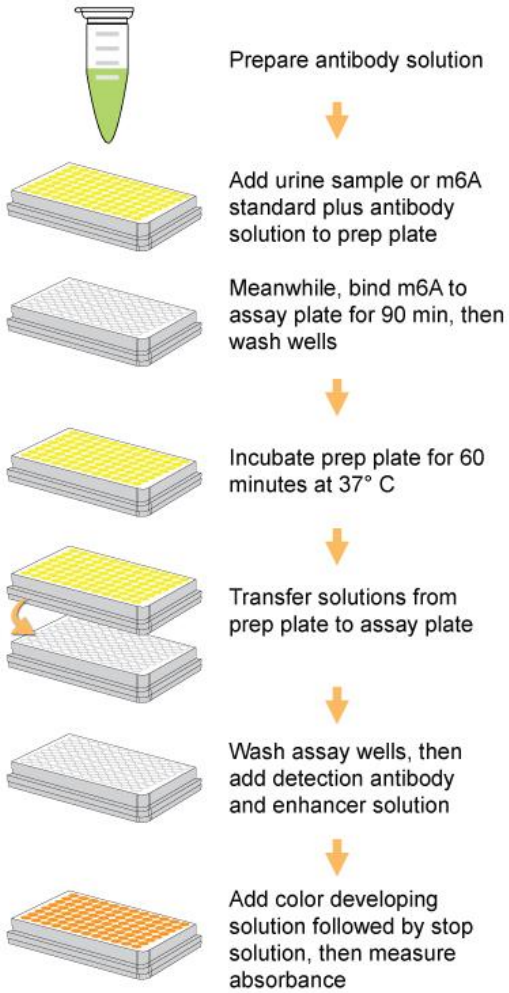
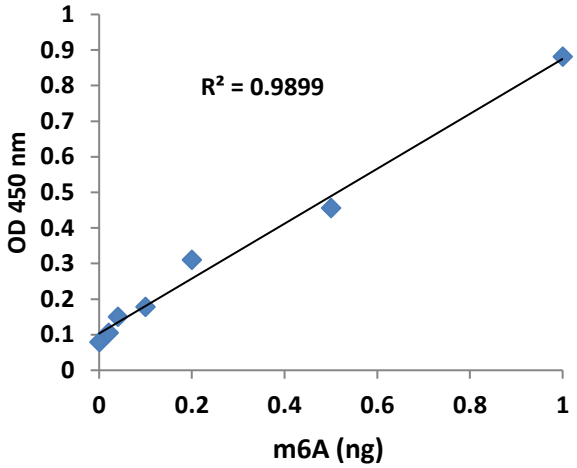
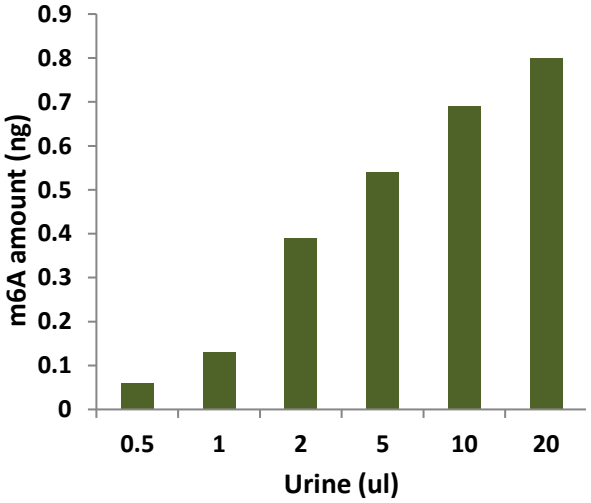
the amount of m^{6}A in the urine sample can be quantified by a comparison with a predetermined m^{6}A standard.



Schematic procedure of the MethylFlash™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit (Colorimetric).



m^{6}A standard was added into the assay wells at different concentrations and then measured with the MethylFlash™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit (Colorimetric).



m^{6}A level is quantified from different volumes of human urine using the MethylFlash™ Urine N^6-methyladenosine (m^{6}A) Quantification Kit (Colorimetric).

PROTOCOL

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

Starting Materials

Input Urine Volume: Urine amount can range from 1 to 20 µl per assay. An optimal amount is 5 µl per assay. Clear urine samples can be directly used for the assay. Centrifugation at 2500-3000 g for 10 min should be required for the samples containing precipitates.

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Storage: Urine sample should be stored at -20°C immediately after collection.

1. Buffer and Solution Preparation

a. Prepare **Diluted WB** 1X Wash Buffer:

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

96-Assay Kit: Add 26 ml of **WB** (10X Wash Buffer) to 234 ml of distilled water and adjust pH to 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 MAS** 1X m^6A Assay solution:

Dilute 500X **MAS at 1:500 ratio (**1 µl of **MAS** + 500 µl of **BS** Binding Solution). About 100 µl of **Diluted MAS** will be required for each assay well.

c. Prepare **Diluted CA** Capture Antibody Solution:

Dilute **CA** Capture Antibody with **Diluted WB** 1X Wash Buffer at a ratio of 1:1000 (ex: add 1 µl of **CA** to 1000 µl of **Diluted WB** 1X Wash Buffer). About 50 µl of **Diluted CA** will be required for each assay well.

d. Prepare **Diluted DA** Detection Antibody Solution:

Dilute **DA** Detection Antibody with **Diluted WB** 1X Wash Buffer at a ratio of 1:2000 (ex: add 1 µl of **DA** to 2000 µl of **Diluted WB**). About 50 µl of **Diluted ME6** will be required for each assay well.

e. Prepare **Diluted ES** Enhancer Solution:

Dilute **ES** Enhancer Solution with **Diluted WB** 1X Wash Buffer at a ratio of 1:3000 (ex: add 1 µl of **ES** to 3000 µl of **Diluted WB**). About 50 µl of **Diluted ES** will be required for each assay well.

f. Prepare **Diluted MS** m^6A Standard Solution:

Suggested Standard Curve Preparation: First, dilute **MS** to 1 ng/µl (4 µl of **MS** + 4 µl of 1X TE) and 0.1 ng/ µl (1 µl of **MS** + 19 µl of 1X TE). Then, further prepare five different concentrations with the diluted **MS** and 1X TE into 0.02, 0.05, 0.1, 0.2, 0.5, and 1 ng/µl, according to the following dilution chart:

Tube	MS (1 ng/µl)	MS (0.1 ng/µl)	1X TE	Resulting MS Concentration
1	0.0 µl	1.0 µl	4.0 µl	0.02 ng/µl
2	0.0 µl	2.0 µl	2.0 µl	0.05 ng/µl
3	0.0 µl	3.0 µl	0.0 µl	0.1 ng/µl
4	1.0 µl		4.0 µl	0.2 ng/µl
5	1.5 µl		1.5 µl	0.5 ng/µl
6	3.0 µl		0.0 µl	1.0 ng/µl

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

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2. m^{6}A Binding

a. Predetermine the number of assay strip wells from **Plate 1** to be 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. Negative Control Wells: Add 100 µl of **BS** to each negative control well.

c. No Sample Control Wells: Add 100 µl of **Diluted MAS** to each no sample control well.

d. Standard Wells: Add 100 µl of **BS** and 1 µl of **Diluted MS** at the different concentrations from 0.02 to 1 ng/µl (as shown in the chart in Step 1f) to each standard well

e. Sample Wells: Add 100 µl of **Diluted MAS** to each sample well.

f. Cover **Plate 1** with plate seal or Parafilm M and incubate at 37°C for 90 min.

g. Meanwhile, predetermine the number of wells required in **Plate 2** (Sample Preparation Plate). Cover remaining unused wells with plate seal and set up sample preparation in the subsequent steps (Steps 2h to 2k).

h. Negative Control Wells: Add 50 µl of **Diluted CA,** 4 µl **Diluted WB** and 1 µl **NC** to each negative control well.

i. No Sample Control Wells: Add 50 µl of **Diluted CA** and 5 µl **Diluted WB** to each control well.

j. Standard Wells: Add 50 µl of **Diluted CA** and 5 µl **Diluted WB** to each standard well.

k. Sample Wells: Add 50 µl of **Diluted CA** and 5 µl of urine sample to each sample well.

Note: *(1)* ***Plate 1*** *and* ***Plate 2*** *set up for the wells are depicted in Table 1 and Table 2 in the "Suggested Strip Well Setup" section of this user guide; (2) As the volume of the control and sample DNA is very small (1 µl), to ensure that the control and sample DNA are completely added into the wells, the pipette tip should be placed in the solution contained in the well and aspirated in/out 1-2 times.*

l. Mix solution by gently tilting from side to side or shaking **Plate 2** several times. Cover **Plate 2** with plate seal or Parafilm M and incubate at RT for 60 min.

m. Remove the solution from each well of **Plate 1** after its incubation period. Wash each well two times with 150 µl of the **Diluted WB** 1X Wash Buffer each time. This can be done by simply pipetting **Diluted WB** in and out of the wells.

3. m^{6}A Capture

a. Carefully transfer the solution from each well of **Plate 2** to the corresponding well of **Plate 1** (ex: from strip wells 1A through 1H of **Plate 2** to strip wells 1A through 1H of **Plate 1**. Also see the "Suggested Strip Well Setup" of this user guide). **Plate 2** will no longer be used from this point forward.

b. Cover **Plate 1** with a plate seal or Parafilm M and incubate at RT for 60 min.

c. Remove the solution from each well.

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d. Wash each well three times with 150 µl of the **Diluted WB** each time.

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

f. Remove the **Diluted DA** solution from each well.

g. Wash each well four times with 150 µl of the **Diluted WB** each time.

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

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

j. Wash each well five times with 150 µl of the **Diluted WB** each time.

4. 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 antibody-bound wells.

b. Add 100 µl of **SS** to each well to stop the enzyme reaction when color in the standard wells starts turning medium blue. Mix the solution by gently shaking the frame and wait 1-2 min to allow the color reaction to be completely stopped. 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.*

5. m^{6}A Calculation

m^{6}A Quantification: To quantify the amount of m^{6}A, first generate a standard curve and plot the OD values against the amount of **MS** 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) and the most linear part (include at least 4 concentration points) of the standard curve for optimal slope calculation. Now calculate the amount and concentration of m^{6}A in urine sample using the following formula:

$$m^6A\ (ng/ml) = \frac{(No\ Sample\ Control\ OD - NC\ OD) - (Sample\ OD - NC\ OD)}{Slope \times Urine\ Volume^*} \times 1000$$

***** *Volume of urine added at Step 2k, sample well.*

Example calculation:

Average OD450 of NC is 0.115
Average OD450 of No Sample control is 0.815
Average OD450 of sample is 0.615
Slope is 0.4 OD/ng
Urine volume is 5 µl

$$m^6A\ (ng/ml) = \frac{0.5 - 0.7}{-0.4 \times 5} \times 1000 = 100\ ng/ml$$

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Suggested Strip Well Setup

Table 1. Plate 2 Setup. The suggested sample preparation plate (U-shaped bottomed wells) setup in a 48-assay format depicted below (for a 96-assay format, Strips 7 to 12 can be configured as Sample Wells). The controls and samples can be measured in duplicate. **No sample:** 50 µl of **Diluted CA** and 5 µl **Diluted WB; Standard 1-6:** 50 µl of **Diluted CA** and 5 µl **Diluted WB.**

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	NC	Sample	Sample	Sample	Sample
B	No sample	No sample	Sample	Sample	Sample	Sample
C	Standard 1	Standard 1	Sample	Sample	Sample	Sample
D	Standard 2	Standard 2	Sample	Sample	Sample	Sample
E	Standard 3	Standard 3	Sample	Sample	Sample	Sample
F	Standard 4	Standard 4	Sample	Sample	Sample	Sample
G	Standard 5	Standard 5	Sample	Sample	Sample	Sample
H	Standard 6	Standard 6	Sample	Sample	Sample	Sample

Table 2. Plate 1 Setup After Transfer. The suggested assay strip-well plate (flat bottomed wells) setup for standard curve preparation in a 48-assay format depicted below (for a 96-assay format, Strips 7 to 12 can be configured as Sample Wells). The controls and samples can be measured in duplicate. **No sample: MAS; Standard 1: MS 0.02 ng; Standard 2: MS 0.05 ng; Standard 3: MS 0.1 ng; Standard 4: MS4 0.2 ng; Standard 5: MS 0.5 ng, and Standard 6: MS 1 ng.**

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	NC	Sample	Sample	Sample	Sample
B	No sample	No sample	Sample	Sample	Sample	Sample
C	Standard 1	Standard 1	Sample	Sample	Sample	Sample
D	Standard 2	Standard 2	Sample	Sample	Sample	Sample
E	Standard 3	Standard 3	Sample	Sample	Sample	Sample
F	Standard 4	Standard 4	Sample	Sample	Sample	Sample
G	Standard 5	Standard 5	Sample	Sample	Sample	Sample
H	Standard 6	Standard6	Sample	Sample	Sample	Sample

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal in the standard wells and the 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.
	Antibody was not correctly added into the designated wells of **Plate 2** (Sample Preparation Plate).	Ensure the antibody at a proper dilution is added into the designated wells of **Plate 2** (Sample Preparation Plate).
	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 by shaking the plate several times.

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	Incubation time and temperature are incorrect.	Ensure the incubation time and temperatures described in the protocol are followed correctly.
	Insufficient **MAS** (m6A Assay Solution) is added into the assay wells.	Ensure that a sufficient amount of **MAS** (m6A Assay Solution) is added into the wells.
	Incorrect absorbance reading.	Check if 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 sample wells	Too much urine sample is used.	Ensure the volume of urine samples added into the wells is within the recommended range. Optimal volume is 5 µl.
No signal or weak signal in only the standard wells	The **MS** solution is not properly diluted.	Properly dilute the **MS** solution to the different concentrations according to the Dilution Chart in Step 1f.
High background present in the negative control wells	Insufficient washing of wells.	Check if washing recommendations at each step is performed according to the protocol.
	Contaminated by **MAS** (m6A Assay Solution).	Ensure the well is not contaminated from adding by **MAS** (m6A Assay Solution) accidentally or from using contaminated tips.
	Over-development of color.	Decrease the development time in Step 4a before adding **SS** Stop Solution in Step 4b.
Large variation between replicate wells	Color reaction is not evenly stopped due to inconsistency in pipetting time.	Ensure **DS** (Developer Solution) and **SS** (Stop Solution) are added at the same time between replicates or otherwise maintain the consistent timing in between each addition of solutions.
	Color reaction is not evenly stopped due to an inconsistent order of adding solutions.	Ensure all solutions, particularly **DS** (Developer Solution) and **SS** (Stop Solution), are added in the same order each time as all other solutions.
	The solutions are not evenly added due to inconsistency in pipetting volume.	Ensure the solution in each pipette tip is equal in the multi-channel pipette. Equilibrate the pipette tip in any solutions before adding them. Ensure the solutions, especially those with small volume (ex: 1 ul) are completely added into the wells.

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	Solutions or antibodies are not actually added into the wells.	Do not allow pipette tip to touch the outer edges or inner sides of the wells to prevent solutions from sticking to the surface.
	Did not sufficiently shake the solutions in the wells evenly after adding **SS** Stop Solution in Step 4b.	Gently shake the plate frame across a flat surface so that the solutions in the wells are better distributed. Do not stir.
	Did not use the same pipette device throughout the experiment.	Use the same multi-channel pipette device throughout the entire experiment, as different pipette devices may have slight variations in performance.
Capture Antibody vial appears to be empty or insufficient in volume	Buffer evaporated due to the very small volumes, resulting in a higher concentrated antibody.	Add 1X PBS buffer into the Capture Antibody vial until you restore the correct, intended volume according to the Kit Contents described in this User Guide. Mix and centrifuge prior to use.

RELATED PRODUCTS

P-1040 MethylFlash™ Urine 5-Methylcytosine Quantification Kit (Fluorometric)
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)

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