EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Fluorometric)

Base Catalog # P-9008

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

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

Input RNA: The amount of RNA for each assay can be 100 ng to 300 ng. For optimal quantification, the input RNA amount should be 200 ng, as the abundance of m^{6}A is generally less than 0.1% of total RNA.

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 RNA controls are provided in this kit. A standard curve can be performed (range: 0.02 to 1 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 RNA methylation states of two different RNA 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-9008-48	96 Assays Cat. #P-9008-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
BS (Binding Solution)	5 ml	10 ml	RT
NC (Negative 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
FD (Fluoro Developer)*	8 µl	16 µl	–20°C
FE (Fluoro Enhancer)*	8 µl	16 µl	4°C
DB (Dilution Buffer)	4 ml	8 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 an RNA containing no* m^6A*. The **PC** (Positive Control) 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 **NC**, **PC**, **DA**, **ES,** and **FD** at –20°C away from light; (2) Store **WB**, **CA**, **FE**, and **8-Well Assay Strips** at 4°C away from light; (3) Store remaining components (**BS** and **DB**) 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
- ☐ Fluorescence microplate reader capable of reading fluorescence at excitation 530 and emission 590
- ☐ 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 RNA of interest

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Fluorometric) 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.

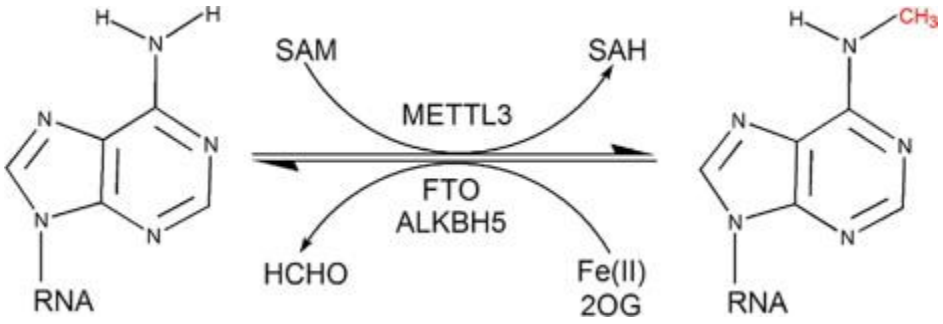
Product Updates: EpigenTek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigentek.com/datasheet.

Usage Limitation: The EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Fluorometric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Fluorometric) 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. The m^{6}A modification is catalyzed by a methyltransferase complex METTL3 and removed by the recently discovered m^{6}A RNA demethylases FTO and ALKBH5, which catalyze m^{6}A demethylation in an α-ketoglutarate (α-KG)- and Fe^{2+}-dependent manner. It was shown that METTL3, FTO and ALKBH5 play important roles in many biological processes, ranging from development and metabolism to fertility. m^{6}A accounts for more than 80% of all RNA base methylations and exists in various species. m^{6}A is mainly distributed in mRNA and also occurs in non-coding RNA such as tRNA, rRNA and snRNA. The relative abundance of m^{6}A in mRNA transcripts has been shown to affect RNA metabolism processes such as splicing, nuclear export, translation ability and stability and RNA transcription. Abnormal m^{6}A methylation levels induced by defects in m^{6}A RNA methylase and demethylase could lead to dysfunction of RNA and cause disease. For example, abnormally low levels of m^{6}A in target mRNAs due to increased FTO activity in patients with *FTO* mutations, through an as-yet undefined pathway, contributes to the onset of obesity and related diseases. The dynamic and reversible chemical m^{6}A modification on RNA may also serve as a novel epigenetic marker of profound biological significance. Therefore, more useful information for better understanding of m^{6}A RNA methylation levels and distribution on RNA transcripts could benefit diagnostics and therapeutics of disease.



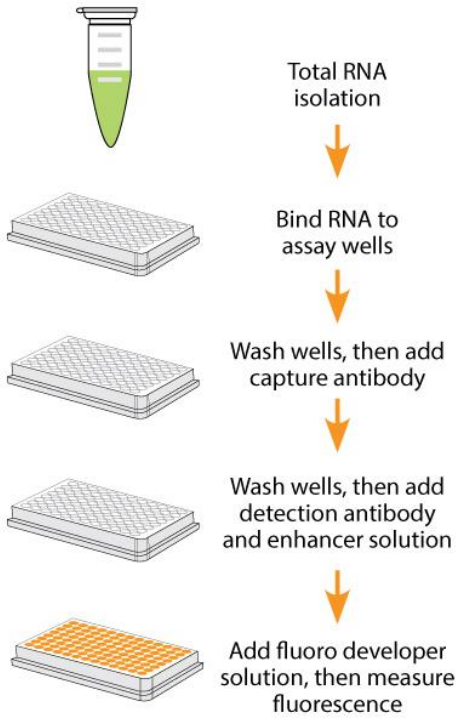
Reversible m^{6}A methylation in mRNA (Niu Y et al: Genomics, Proteomics & Bioinformatics, 11: 2013)

Several chromatography-based techniques such as HPLC-ECD and LC-MS are used for detecting 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 EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Fluorometric) which uses a unique procedure to directly quantify m^{6}A RNA methylation status using total RNA isolated from cells/tissues. The kit has the following advantages and features:

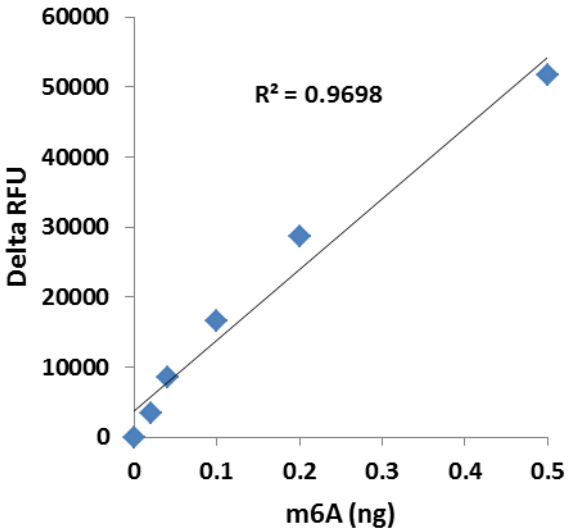
- Fluorometric assay with easy-to-follow steps for convenience and speed. The entire procedure can be completed within 3 hours and 45 minutes.
- High sensitivity, of which the detection limit can be as low as 5 pg of m^{6}A.
- Unique binding solution allows that RNA >70 nts can be tightly bound to the wells, which enables quantification of m^{6}A from both mRNA and nc-RNA such as tRNA, rRNA and snRNA.
- 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 RNA.
- 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 EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Fluorometric) contains all reagents necessary for the quantification of m^{6}A in RNA. In this assay, total RNA is bound to strip wells using RNA high binding solution. m^{6}A is detected using capture and detection antibodies. The detected signal is enhanced and then quantified fluorometrically by reading the RFU (relative fluorescence units) with a fluorescence spectrophotometer. The amount of m^{6}A is proportional to the fluorescence intensity measured.



Schematic procedure of the EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Fluorometric).



m^{6}A standard control was added into the assay wells at different concentrations and then measured with the EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Fluorometric).

ASSAY PROTOCOL

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

1. Starting Materials

Input RNA Amount: Total RNA amount can range from 100 ng to 300 ng per reaction. An optimal amount is 200 ng per reaction. Starting RNA may be in water or in a buffer such as TE. You can use your method of choice for RNA isolation.

RNA Storage: Isolated total RNA 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 to 0.5 ng/µl (1 µl **PC** + 3 µl TE).

Suggested Standard Curve Prep: First, dilute **PC** to 0.5 ng/µl (ex: 3 µl of **PC** + 9 µl of 1X TE). Then, further prepare 6 different concentrations with the 0.5 ng/µl **PC** and 1X TE into 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 ng/µl according to the following dilution chart:

Tube	PC (0.5 ng/µl)	1X TE	Resulting PC Concentration
1	1.0 µl	49.0 µl	0.01 ng/µl
2	1.0 µl	24.0 µl	0. 02 ng/µl
3	1.0 µl	9.0 µl	0.05 ng/µl
4	1.0 µl	4.0 µl	0.1 ng/µl
5	2.0 µl	3.0 µl	0.2 ng/µl
6	4.5 µl	0.0 µl	0.5 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. RNA 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 **NC**, 2 µl of **Diluted PC** (see note below), and 200 ng of your sample RNA (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 0.01 to 0.5 ng/µl (see the chart in Step 2e). The final amounts should be 0.02, 0.04, 0.1, 0.2, 0.4 and 1 ng per well. (2) For optimal binding, sample RNA volume added should not exceed 8 µl. (3) To ensure that **NC**, **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 RNA 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. Prepare **Fluoro-Development Solution** by adding 1 µl of **FD** and 1 µl of **FE** into each 500 µl of **DB**.

b. Add 50 µl of **Fluoro-Development Solution** into the wells and incubate at room temperature for 1 to 4 minutes away from light. The color in the standard wells containing the higher concentrations may turn pink during this period. Measure and read RFU (relative fluorescence units) on a fluorescence microplate reader at $530_{EX}/590_{EM}$ nm.

***Note:** If the strip well frame does not fit the microplate reader, transfer the solution to a standard 96-well microplate and read the RFU on a fluorescence microplate reader at $530_{EX}/590_{EM}$ nm.*

6. m^{6}A Calculation

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

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

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

Example calculation:

Average RFU of NC is 900
Average RFU of PC is 10900
Average RFU of Sample is 1900
S is 200 ng
P is 1 ng

$$m^6A\ \% = \frac{(1900 - 900) \div 200}{(10900 - 900) \div 1} \times 100\% = \mathbf{0.05\%}$$

Absolute Quantification: To quantify the absolute amount of m^6A using an accurate calculation, first generate a standard curve and plot the RFU values (background (NC)-subtracted) versus the amount of **PC** at each concentration point. Next, determine the slope (RFU/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^6A in your total RNA using the following formulas:

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

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

S is the amount of input sample RNA in ng.

Example calculation:

Average RFU of NC is 900
Average RFU of sample is 1900
Slope is 10000 RFU/ng
S is 200 ng

$$m^6A\ (ng) = \frac{1900 - 900}{10000} = \mathbf{0.1\ ng}$$

$$m^6A\ \% = \frac{0.1}{200} \times 100\% = \mathbf{0.05\%}$$

SUGGESTED STRIP WELL SETUP

Table 1. The suggested strip-well plate setup using a single point positive control in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can be measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	NC	Sample	Sample	Sample	Sample
B	PC	PC	Sample	Sample	Sample	Sample
C	Sample	Sample	Sample	Sample	Sample	Sample
D	Sample	Sample	Sample	Sample	Sample	Sample
E	Sample	Sample	Sample	Sample	Sample	Sample
F	Sample	Sample	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

Table 2. 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 be measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	NC	Sample	Sample	Sample	Sample
B	PC 0.02 ng/well	PC 0.02 ng/well	Sample	Sample	Sample	Sample
C	PC 0.04 ng/well	PC 0.04 ng/well	Sample	Sample	Sample	Sample
D	PC 0.1 ng/well	PC 0.1 ng/well	Sample	Sample	Sample	Sample
E	PC 0.2 ng/well	PC 0.2 ng/well	Sample	Sample	Sample	Sample
F	PC 0.4 ng/well	PC 0.4 ng/well	Sample	Sample	Sample	Sample
G	PC 1 ng/well	PC 1 ng/well	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

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
Fluoro-Developer Solution	50 µl	400 µl	800 µl	2400 µl	4800 µl
NC	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 RNA 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 (> 0.02 ng) and sample (200 ng) is added into the wells.
	Incorrect fluorescence reading.	Check if appropriate fluorescence wavelength (530EX/590EM 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 fluorescence.	Decrease the development time in Step 5b.
No signal or weak signal only in sample wells	RNA sample is not properly extracted or purified.	Ensure the RNA sample is good quality. The 260/280 ratio should be >1.9 with no or minimal DNA contamination.
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of RNA is used as indicated in Step 3c.

	Little or no m^{6}A contained in the sample.	N/A
Large variation between replicate wells	Fluorescent reaction is not evenly occurring due to an inconsistency in pipetting time.	Ensure **Fluoro-Development Solution** is added at the same time between replicates or otherwise maintain a consistent timing in between each addition of solutions.
	Fluorescent reaction is not occurring evenly due to an inconsistent order of adding solutions.	Ensure all solutions, particularly **Fluoro-Development Solution**, are added in the same order each time as all other solutions.
	The solutions are not evenly added due to an 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 volumes (e.g., 1 ul) are completely added into the wells.
	Solutions or antibodies were not actually added into the wells.	Do not allow the pipette tip to touch the outer edges or inner sides of the wells in order to prevent solutions from sticking to the surface.
	Did not sufficiently shake the solutions in the wells after adding sample or positive control at Step 3c	Gently and evenly 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

Methylated and Hydroxymethylated DNA Quantification

P-1034 MethylFlash™ Methylated DNA 5-mC Quantification Kit (Colorimetric)
P-1035 MethylFlash™ Methylated DNA Quantification Kit (Fluorometric)
P-1036 MethylFlash™ Hydroxymethylated DNA 5-hmC Quantification Kit (Colorimetric)
P-1037 MethylFlash™ Hydroxymethylated DNA Quantification Kit (Fluorometric)

RNA Methylation Detection

P-9003 Methylamp™ RNA Bisulfite Kit
P-9005 EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Colorimetric)
P-9010 MethylFlash™ m6A DNA Methylation ELISA Kit (Colorimetric)
P-9015 MethylFlash™ Urine N6-methyladenosine (m6A) Quantification Kit (Colorimetric)