MethylFlash™ Hydroxymethylated DNA Quantification Kit (Colorimetric)

Base Catalog # P-1036

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The MethylFlash™ Hydroxymethylated DNA Quantification Kit (Colorimetric) is suitable for detecting global DNA hydroxymethylation status using DNA isolated from any species such as mammals, plants, fungi, bacteria, and viruses in a variety of forms including, but not limited to, cultured cells, fresh and frozen tissues, paraffin-embedded tissues, plasma/serum samples, and body fluid samples.

Input DNA: The amount of DNA for each assay can be 50 to 200 ng. For optimal quantification, the input DNA amount should be 200 ng, as hydroxymethylated DNA (hmDNA) is generally less than 0.6% 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, plasma/serum samples, body fluid samples, etc.

Internal Control: Both negative and positive DNA controls are provided in this kit. A standard curve can be performed (range: 0.5 to 10 ng) or a single quantity of hydroxymethylated DNA can be used as a positive control. Because global hydroxymethylation 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. This kit will allow the user to quantify an absolute amount of hydroxymethylated DNA and determine the relative hydroxymethylation 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-1036-48	96 Assays Cat. #P-1036-96	Storage Upon Receipt
HC1 (10X Wash Buffer)	14 ml	28 ml	4°C
HC2 (Binding Solution)	5 ml	10 ml	RT
HC3 (Negative Control I, 20 µg/ml)*	10 µl	20 µl	–20°C
HC4 (Negative Control II, 20 µg/ml)*	10 µl	20 µl	–20°C
HC5 (Positive Control, 20 µg/ml)*	10 µl	20 µl	–20°C
HC6 (Capture Antibody, 1000 µg/ml*	4 µl	8 µl	4°C
HC7 (Detection Antibody, 400 µg/ml)*	8 µl	16 µl	–20°C
HC8 (Enhancer Solution)*	8 µl	16 µl	–20°C
HC9 (Developer Solution)	5 ml	10 ml	4°C
HC10 (Stop Solution)	5 ml	10 ml	RT
8-Well Assay Strips (With Frame)	6	12	4°C
User Guide	1	1	RT

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

***Note**: The **HC3** Negative Control I is an unmethylated polynucleotide containing 20% of cytosine. The **HC4** Negative Control II is a methylated polynucleotide containing 20% of 5-methylcytosine. The **HC5** Positive Control is a hydroxymethylated polynucleotide containing 20% of hydroxymethylcytosine.*

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 **HC3**, **HC4**, **HC5**, **HC7**, and **HC8** at –20°C away from light; (2) Store **HC1**, **HC6**, **HC9**, and **8-Well Assay Strips** at 4°C away from light; (3) Store remaining components (**HC2** and **HC10**) at room temperature away from light.

Note: Check if wash buffer, **HC1**, 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
- ☐ Isolated DNA of interest

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the Methylflash™ Hydroxymethylated DNA Quantification 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. Thus, only use the User Guide that was supplied with the kit when using that kit.

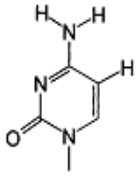
Usage Limitation: The Methylflash™ Hydroxymethylated DNA Quantification Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The Methylflash™ Hydroxymethylated DNA Quantification Kit (Colorimetric) and methods of use contain proprietary technologies by Epigentek.

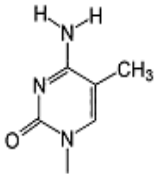
A BRIEF OVERVIEW

DNA methylation occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring, resulting in 5-methylcytosine (5-mC). In somatic cells, 5-mC is found almost exclusively in the context of paired symmetrical methylation of the dinucleotide CpG, whereas in embryonic stem (ES) cells, a substantial amount of 5-mC is also observed in non-CpG contexts. The biological importance of 5-mC as a major epigenetic modification in phenotype and gene expression has been widely recognized.

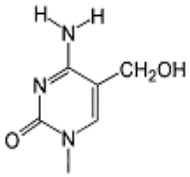
Quite recently, a novel modified nucleotide called 5-hydroxymethyl-cytosine (5-hmC) has been detected to be abundant in mouse brains and embryonic stem cells. 5-hydroxymethylcytosine was first seen in bacteriophages in 1952. In mammals, it can be generated by the oxidation of 5-methylcytosine, a reaction mediated by the Tet family of enzymes and Dnmt proteins. It is a hydroxylated and methylated form of cytosine.



C



$5^{m}C$



^{hm}C

Unmethylated DNA	**Methylated DNA**	**Hydroxymethylated DNA**
T-C-G-T-C-G-A-C-G	T-^{m}C-G-T-^{m}C-G-A-^{m}C-G	T-^{hm}C-G-T-^{hm}C-G-A-^{hm}C-G

The broader functions of 5-hmC in epigenetics are still a mystery today. However, a line of evidence does show that 5-hmC plays a role in DNA demethylation, chromatin remodeling, and gene expression regulation, specifically in brain-specific gene regulation:

1) Conversion of 5-mC to 5-hmC greatly reduced the affinity of MBD proteins to methylated DNA.
2) The observation that formation of 5-hmC by oxidative damage or by addition of aldehydes via DNMTs prevents DNMT-mediated methylation of the target cytosine.
3) 5-hmC may recruit specific binding proteins that alter chromatin structure or DNA methylation patterns.
4) 5-hmC accounts for roughly 40 percent of the methylated cytosine in Purkinje cells and 10 percent in granule neurons.

Because of the presence of 5-hmC in DNA with unclear functions in gene regulation and the discovery of the enzymes that produce 5-hmC, it is considered rather important to know the distribution of this base in different cell types and in different compartments of the genome of mammalians. It is particularly important to identify hydroxymethylation status in human cell/tissues with and without diseases. Several chromatography-based techniques such as HPLC and TLC mass spectrometry are used for detecting 5-hmC. However these methods are time consuming and have low throughput with high costs. Currently, used methylated DNA analysis methods including restriction enzyme digestion and bisulfite or MeDIP-mediated MS-PCR and sequencing are also not suitable for 5-hmC detection as 5-hmC and 5-mC are virtually indistinguishable with these methods. To address this problem, Epigentek offers the *MethylFlash*™ Hydroxymethylated DNA Quantification Kit (Colorimetric) which uses a unique procedure to quantify global DNA hydroxymethylation. The kit has the following advantages and features:

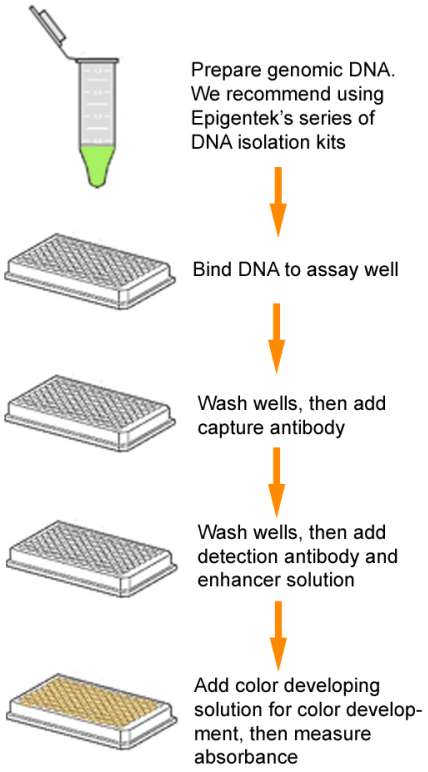
- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be completed within 3 hours and 45 minutes.
- High sensitivity, of which the detection limit can be as low as 40 pg of hydroxymethylated DNA.
- High specificity with no cross-reactivity to unmethylated cytosine and methylcytosine. Only hydroxymethylated DNA (5-hmC) is detected.
- Universal positive and negative controls are included, which are suitable for quantifying hydroxymethylated DNA from any species.
- Strip-well microplate format makes the assay flexible: manual or high throughput analysis.
- Simple, reliable, and consistent assay conditions

References

1. Robertson KD. *Nat Rev Genet*. 6:597-610, 2005.
2. Kriaucionis S et al: *Science*. 324: 929-930, 2009.
3. WYATT GR et al: *Biochem J*. 55:774-8, 1953.
4. Tahiliani M et al: *Science*. 324: 930-935, 2009.
5. Valinluck V et al: *Nucleic Acids Res*. 32: 4100-4108. 2004.
6. Valinluck V et al: *Cancer Res*. 67:946-50, 2007.
7. Jin SG et al: *Nucleic Acids Res*. 38: e125, 2010.

PRINCIPLE & PROCEDURE

The MethylFlash™ Hydroxymethylated DNA Quantification Kit (Colorimetric) contains all reagents necessary for the quantification of global DNA hydroxymethylation. In this assay, DNA is bound to strip wells that are specifically treated to have a high DNA affinity. The hydroxymethylated fraction of DNA is detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of hydroxymethylated DNA is proportional to the OD intensity measured.



Schematic procedure of the MethylFlash™ Hydroxymethylated DNA Quantification Kit (Colorimetric).

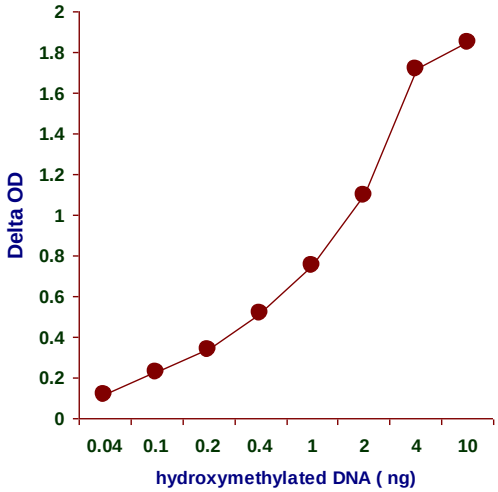


Fig. 1. Demonstration of high sensitivity of 5-hydroxymethylcytosine detection achieved by the MethylFlash™ kit. Synthetic hydroxymethylated DNA was added into the assay wells at different concentrations and then measured with the MethylFlash™ Hydroxymethylated DNA Quantification Kit

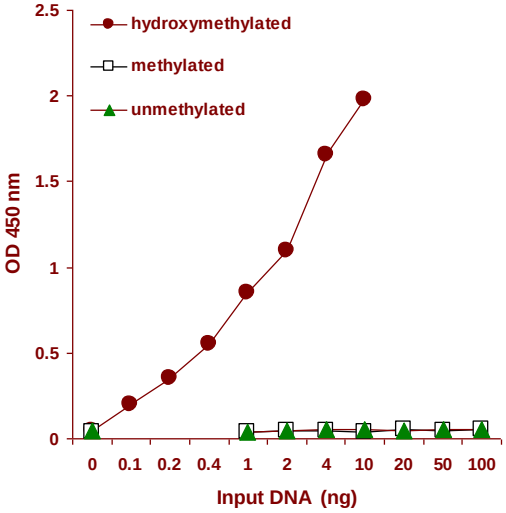


Fig. 2. Demonstration of high specificity of 5-hydroxymethylcytosine detection achieved by the MethylFlash™ kit. Synthetic unmethylated DNA (contains only cytosine), methylated DNA (contains only 5-methylcytosine), and hydroxymethylated DNA standard (contains only 5-hydroxymethylcytosine) were added into the assay wells at different concentrations and then measured with the MethylFlash™ Hydroxymethylated DNA Quantification Kit (Colorimetric).

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ASSAY PROTOCOL

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

Starting Materials

Input DNA Amount: DNA amount can range from 50 ng to 200 ng per reaction. An optimal amount is 200 ng per reaction. Starting DNA may be in water or in a buffer such as TE.

DNA Isolation: You can use your method of choice for DNA isolation. Epigentek offers a series of genomic DNA isolation kits for your convenience (see "Ordering Information").

DNA Storage: Isolated genomic DNA can be stored at 4°C (short term) or –20°C (long term) until use.

1. Preparation of 1X Wash Buffer (HC1)

48-Assay Kit: Add 13 ml of **HC1** 10X Wash Buffer to 117 ml of distilled water (pH 7.2-7.5).

96-Assay Kit: Add 26 ml of **HC1** 10X Wash Buffer to 234 ml of distilled water (pH 7.2-7.5).

*Note: This **Diluted HC1** 1X Wash Buffer can now be stored at 4°C for up to six months. All other diluted solutions should be kept on ice at all times and should be discarded if not used within the same day.*

2. Preparation of Diluted Positive Control (HC5)

Single Point Control Prep: Dilute **HC5** Positive Control with 1X TE to 5 ng/µl (1 µl **HC5** + 3 µl TE).

Suggested Standard Curve Prep: First, dilute **HC5** to 10 ng/µl (5 µl of **HC5** + 5 µl of 1X TE). Then, further prepare five different concentrations with the 10 ng/µl diluted **HC5** and 1X TE into 0.5, 1.0, 2.0, 5.0, and 10.0 ng/µl according to the following dilution chart:

Tube	HC5 (10 ng/µl)	1X TE	Resulting HC5 Concentration
1	1.0 µl	19.0 µl	0.5 ng/µl
2	1.0 µl	9.0 µl	1.0 ng/µl
3	1.0 µl	4.0 µl	2.0 ng/µl
4	2.5 µl	2.5 µl	5.0 ng/µl
5	4.5 µl	0.0 µl	10.0 ng/µl

The standard curve can also be generated with a lower concentration range if needed (ex: 0.05, 0.1, 0.2, 0.5, 1, and 2 ng/µl).

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 **HC2** Binding Solution to each well.

c. Add 1 µl of **HC3**, 1 µl of **HC4**, 1 µl of **Diluted HC5** (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 1 µl of **HC5** at a concentration of 5 ng/µl as prepared in Step 2; For the standard curve, add 1 µl of **Diluted HC5** at concentrations of 0.5 to 10 ng/µl (see the chart in Step 2). The final amounts should be 0.5, 1, 2, 5, and 10 ng per well. (2) For optimal binding, sample DNA volume added should not exceed 8 µl. (3) To ensure that **HC3**, **HC4**, **Diluted HC5**, and sample DNA are completely added into the wells, the pipette tip should be placed into the **HC2** 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 **HC2** Binding Solution from each well. Wash each well with 150 µl of the **Diluted HC1** 1X Wash Buffer each time for three times. This can be done by simply pipetting **Diluted HC1** in and out of the wells.

4. Hydroxymethylated DNA Capture

a. Dilute **HC6** (at 1:1000 ratio) with the **Diluted HC1**.

b. Add 50 µl of the **Diluted HC6** to each well, then cover and incubate at room temperature for 60 min.

c. Remove the **Diluted HC6** solution from each well.

d. Wash each well with 150 µl of the **Diluted HC1** each time for three times.

e. Dilute **HC7** (at 1:1000 ratio) with the **Diluted HC1**.

f. Add 50 µl of the **Diluted HC7** to each well, then cover and incubate at room temperature for 30 min.

g. Remove the **Diluted HC7** solution from each well.

h. Wash each well with 150 µl of the **Diluted HC1** each time for four times.

i. Dilute **HC8** (at 1:5000 ratio) with the **Diluted HC1**.

j. Add 50 µl of the **Diluted HC8** to each well, then cover and incubate at room temperature for 30 min.

k. Remove the **Diluted HC8** solution from each well.

l. Wash each well with 150 µl of the **Diluted HC1** each time for five times.

5. Signal Detection

a. Add 100 µl of **HC9** 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 **HC9** solution will turn blue in the presence of sufficient hydroxymethylated DNA.

b. Add 100 µl of **HC10** to each well to stop enzyme reaction when color in the positive control wells turns 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 **HC10** 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. 5-hmC Calculation

Relative Quantification: To determine the relative hydroxymethylation status of two different DNA samples, simple calculation for the percentage of 5-hmC in your total DNA can be carried out using the following formula:

$$5\text{-}hmC\ \% = \frac{(Sample\ OD - HC4\ OD) \div S}{(HC5\ OD - HC4\ OD) \times 5^* \div P} \times 100\%$$

S is the amount of input sample DNA in ng.
P is the amount of input positive control (**HC5**) in ng.

***** *5 is a factor to normalize 5-hmC in the positive control to 100%, as the positive control contains only 20% of 5-hmC.*

Example calculation:

Average OD450 of HC4 is 0.085
Average OD450 of HC5 is 0.780
Average OD450 of Sample is 0.158
S is 200 ng
P is 5 ng

$$5\text{-}hmC\ \% = \frac{(0.158 - 0.085) \div 200}{(0.780 - 0.085) \times 5 \div 5} \times 100\% = \mathbf{0.0525\%}$$

Absolute Quantification: To quantify the absolute amount of hydroxymethylated DNA using an accurate calculation, first generate a standard curve and plot the OD values versus the amount of **HC5** 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 (at least 4 concentration points including 0 point) of the standard curve for optimal slope calculation. Now calculate the amount and percentage of 5-hmC in your total DNA using the following formulas:

$$5\text{-}hmC\ (ng) = \frac{Sample\ OD - HC4\ OD}{Slope \times 5^*}$$

$$5\text{-}hmC\ \% = \frac{5\text{-}hmC\ Amount\ (ng)}{S} \times 100\%$$

S is the amount of input sample DNA in ng.

***** *5 is a factor to normalize 5-hmC in the positive control to 100%, as the positive control contains only 20% of 5-hmC.*

Example calculation:

Average OD450 of HC4 is 0.085
Average OD450 of sample is 0.158

Slope is 0.14 OD/ng
S is 200 ng

$$5\text{-hmC (ng)} = \frac{0.158 - 0.085}{0.14 \times 5} = \mathbf{0.104\ ng}$$

$$5\text{-hmC \%} = \frac{0.104}{200} \times 100\% = \mathbf{0.052\%}$$

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	HC3	HC3	Sample	Sample	Sample	Sample
B	HC4	HC4	Sample	Sample	Sample	Sample
C	HC5	HC5	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	HC3	HC3	Sample	Sample	Sample	Sample
B	HC4	HC4	Sample	Sample	Sample	Sample
C	HC5 0.5 ng/µl	HC5 0.5 ng/µl	Sample	Sample	Sample	Sample
D	HC5 1 ng/µl	HC5 1 ng/µl	Sample	Sample	Sample	Sample
E	HC5 2 ng/µl	HC5 2 ng/µl	Sample	Sample	Sample	Sample
F	HC5 5 ng/µl	HC5 5 ng/µl	Sample	Sample	Sample	Sample
G	HC5 10 ng/µl	HC5 10 ng/µl	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

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 **HC2** 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 are followed correctly.
	Insufficient input materials.	Ensure that a sufficient amount of positive control (> 1 ng) and samples (>100 ng) 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 capped after each opening or use.
No signal or weak signal in only the positive control wells	The positive control DNA is insufficiently added to the well in Step 3c.	Ensure a sufficient amount of positive control DNA is added.
	The **HC5** Positive Control is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance in the User Guide for storage of **HC5** Positive Control.
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 sample or positive control DNA.	Ensure the well is not contaminated from adding sample or positive control DNA accidentally or from using 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 **HC10** Stop Solution in Step 5b.
Large variation between replicate wells	Color reaction is not evenly stopped due to inconsistency in pipetting time.	Ensure **HC9** Developer Solution and **HC10** Stop Solution are added at the same time between replicates or otherwise maintain a 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 **HC9** Developer Solution and **HC10** Stop Solution, are added in the same order each time as all other solutions.
	Color reaction is not evenly stopped 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. Do not expose the pipette tip to any solutions already in the wells

	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 **HC10** Stop Solution in Step 5b.	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.	Use the same multi-channel pipette throughout the entire experiment, as different pipettes 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

DNA Sample Preparation

P-1003 FitAmp™ General Tissue Section DNA Isolation Kit
P-1004 FitAmp™ Plasma/Serum DNA Isolation Kit
P-1006 DNA Concentrator Kit
P-1007 FitAmp™ Gel DNA Isolation Kit
P-1009 FitAmp™ Paraffin Tissue Section DNA Isolation Kit
P-1017 FitAmp™ Urine DNA Isolation Kit
P-1018 FitAmp™ Blood and Cultured Cell DNA Extraction Kit

DNA Methylation Quantification

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

Hydroxymethylated DNA Immunoprecipitation

P-1038 MethylFlash™ Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit