

MethylFlash™ 5-Formylcytosine (5-fC) DNA Quantification Kit (Colorimetric)

Base Catalog # P-1041

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The MethylFlash™ 5-Formylcytosine (5-fC) DNA Quantification Kit (Colorimetric) is a complete set of optimized buffers and reagents to colorimetrically quantify 5-formylcytosine (5-fC) in a microplate-based format. It is suitable for use with DNA isolated from any species (mammals, plants, fungi, bacteria, viruses, etc.) in a variety of forms including, but not limited to, cultured cells, fresh and frozen tissues, paraffin-embedded tissues, and body fluid samples.

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 300 ng, as 5-fC content is generally less than 0.01% of total DNA.

Starting Material: Starting materials can include various tissue or cell samples, for example, 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 DNA controls are provided in this kit. A standard curve can be performed (range: 10 pg to 400 pg of 5-fC) or a single quantity of 5-fC can be used as a positive control. Because 5-fC 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 5-fC and determine the relative 5-fC 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-1041-48	96 Assays Cat. #P-1041-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
BS (Binding Solution)	5 ml	10 ml	RT
NC (Negative Control, 10 µg/ml)*	10 µl	20 µl	-20°C
PC (Positive Control, 5-fC 1 µg/ml)*	10 µl	20 µl	-20°C
CA (Capture Antibody, 1000X) *	4 µl	8 µl	4°C
DA (Detection Antibody, 2000X)*	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 an oligo containing no 5-fC. The **PC** Positive Control is a 5-fC oligo and is normalized to have 100% of 5-fC.

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**, 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 the 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™ 5-Formylcytosine (5-fC) 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. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigenetek.com/datasheet.

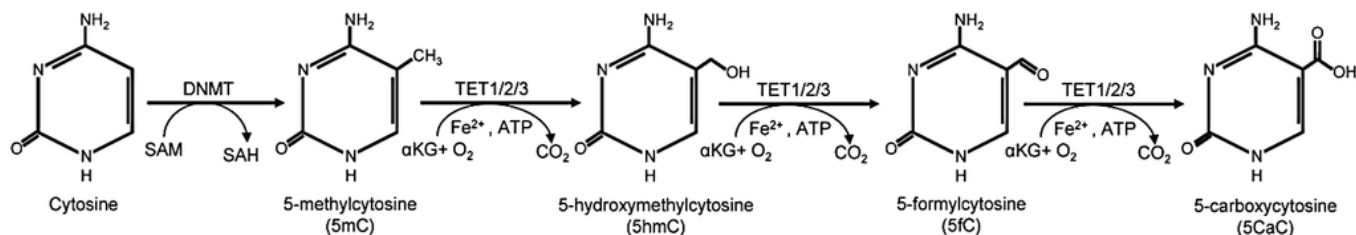
Usage Limitation: The MethylFlash™ 5-Formylcytosine (5-fC) DNA Quantification Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The MethylFlash™ 5-Formylcytosine (5-fC) 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 by DNA methyltransferases, resulting in 5-methylcytosine (5mC). In somatic cells, 5mC 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 5mC is also observed in non-CpG contexts. The biological importance of 5mC as a major epigenetic modifier of phenotype and gene expression has been recognized widely.

5-hydroxymethylcytosine (5hmC), as a sixth DNA base with functions in transcription regulation, has been detected to be abundant in the human brain, the mouse brain, and in embryonic stem (ES) cells. In mammals, it can be generated by the oxidation of 5mC, a reaction mediated by the ten-eleven translocation (TET) family of 5mC-hydroxylases. 5hmC can be further oxidized to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) through TET hydroxylases. 5-formylcytosine (5-fC) has recently been found in mammalian tissue and cells and has been classified as a seventh DNA base. The function of 5-fC in gene regulation is not yet entirely clear, but it has been shown that 5-fC exhibits replication-dependent dilution during mouse pre-implantation development and could be functional in regulating pre-implantation development as a whole. The detection of 5-fC in various tissues and cells is important because 5-fC could be a marker to indicate active DNA demethylation. 5-fC can also be directly excised by Thymine DNA glycosylase (TDG) to allow subsequent base excision repair (BER) processing, which converts modified cytosine back to its unmodified state.



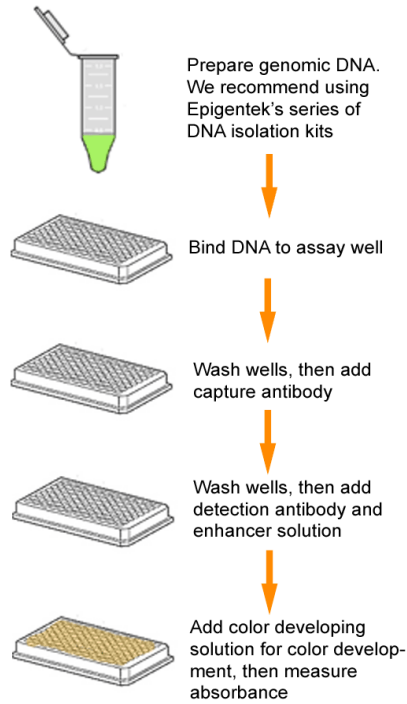
Active demethylation of 5-methylcytosine by TET-mediated oxidation to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxycytosine, followed by decarboxylation/base excision repair. Wu et al: Nat. Rev. Mol. Cell Biol. 11, 607-620, 2010.

Several chromatography-based techniques such as HPLC-MS are used for the detection of 5-fC in tissue and cells. However, these methods are time consuming, require large amounts of DNA and have low throughput with high costs. To address these problems, EpigenTek offers the MethylFlash™ 5-Formylcytosine (5-fC) DNA Quantification Kit (Colorimetric), which uses a unique microplate-based procedure to directly quantify 5-fC. The kit has the following advantages and features:

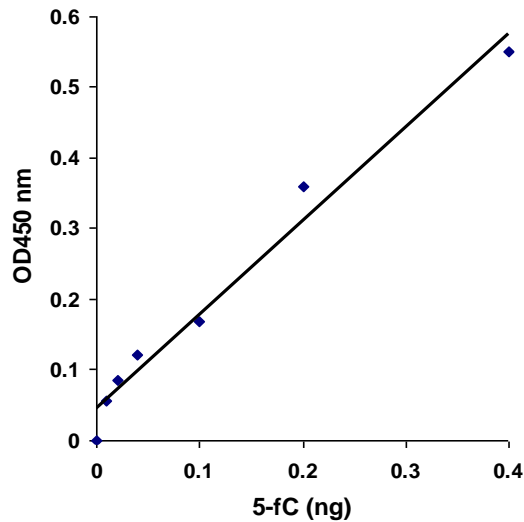
- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be completed within 3 hours and 45 minutes.
- High sensitivity, of which the detection limit can be as low as 1 pg of 5-fC.
- High specificity with detection of only 5-fC. No cross-reactivity to cytosine, 5-mC, 5-hmC and 5-caC within the indicated concentration range of the sample DNA.
- Highly convenient assay with direct use of DNA isolated from cells or tissues, no need for DNA digestion or hydrolysis.
- Universal positive and negative controls are included, which are suitable for quantifying 5-fC from any species.
- Strip-well microplate format makes the assay flexible: manual or high throughput analysis.
- Simple, reliable, and consistent assay conditions

PRINCIPLE & PROCEDURE

The MethylFlash™ 5-Formylcytosine (5-fC) DNA Quantification Kit (Colorimetric) contains all the reagents necessary for the quantification of 5-fC. In this assay, DNA is bound to strip-wells that are specifically treated to have a high DNA affinity. 5-fC 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 5-fC is proportional to the OD intensity measured.



Schematic procedure of the MethylFlash™ 5-Formylcytosine (5-fC) DNA Quantification Kit (Colorimetric)



5-fC standard control was added into the assay wells at different concentrations and then measured with the MethylFlash™ 5-Formylcytosine (5-fC) DNA Quantification Kit (Colorimetric)

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 100 ng to 300 ng per reaction. An optimal amount is 300 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. 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 (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** solution 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** solution 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** solution will be required for each assay well.

- e. Prepare **Diluted PC** Positive Control:

Single Point Control Prep: Dilute **PC** Positive Control with 1X TE to 200 pg/µl (2 µl **PC** + 8 µl TE).

Suggested Standard Curve Prep: First, dilute **PC** to 400 pg/µl (4 µl of **PC** + 6 µl of 1X TE). Then, further prepare 6 different concentrations with the 400 pg/µl diluted **PC** and 1X TE into 10, 20, 40, 100, 200, and 400 pg/µl according to the following dilution chart:

Tube	PC (400 pg/μl)	1X TE	Resulting PC Concentration
1	1.0 μl	39.0 μl	10 pg/μl
2	1.0 μl	19.0 μl	20 pg/μl
3	1.0 μl	9.0 μl	40 pg/μl
4	1.0 μl	3.0 μl	100 pg/μl
5	2.0 μl	2.0 μl	200 pg/μl
6	3.0 μl	0.0 μl	400 pg/μl

Note: Keep each of the diluted solutions (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.

2. 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 1 μl of **NC**, 1 μl of **Diluted PC** (see note below), and 300 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 **PC** at a concentration of 200 pg/μl as prepared in Step 1e; For the standard curve, add 1 μl of **Diluted PC** at concentrations of 10 to 400 pg/μl (see the chart in Step 2). The final amounts should be 10, 20, 40, 100, 200 and 400 pg per well. (2) For optimal binding, sample DNA volume added should not exceed 8 μl. (3) To ensure that the **NC**, **Diluted PC**, and sample DNA are completely added into the wells, the pipette tip should be placed in the **BS** Bind 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 the **Diluted WB** 1X Wash Buffer each time for three times. This can be done by simply pipetting **Diluted WB** in and out of the wells.

3. 5-fC DNA Capture

- a. Add 50 μl of the **Diluted CA** to each well, then cover and incubate at room temperature for 60 min.
- b. Remove the **Diluted CA** solution from each well.
- c. Wash each well with 150 μl of the **Diluted WB** each time for three times.
- d. Add 50 μl of the **Diluted DA** to each well, then cover and incubate at room temperature for 30 min.
- e. Remove the **Diluted DA** solution from each well.
- f. Wash each well with 150 μl of the **Diluted WB** each time for four times.
- g. Add 50 μl of the **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 μ l of the **Diluted WB** each time for five times.

4. Signal Detection

- a. Add 100 μ l 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 5-fC.
- b. Add 100 μ l of **SS** 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 completely stop. 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. 5-fC Calculation

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

$$5\text{-fC } \% = \frac{(\text{Sample OD} - \text{NC OD}) \div S}{(\text{PC OD} - \text{NC OD}) \div P} \times 100\%$$

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 NC is 0.120

Average OD450 of PC is 0.320

Average OD450 of Sample is 0.141

S is 300 ng

P is 0.2 ng (200 pg)

$$5\text{-fC } \% = \frac{(0.141 - 0.120) \div 300}{(0.320 - 0.120) \div 0.2} \times 100\% = 0.007\%$$

Absolute Quantification: To quantify the absolute amount of 5-fC using an accurate calculation, first generate a standard curve and plot the OD values 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) 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-fC in your total DNA using the following formulas:

$$5\text{-fC (ng)} = \frac{\text{Sample OD} - \text{NC OD}}{\text{Slope}} \quad 5\text{-fC } \% = \frac{5\text{-fC Amount (ng)}}{S} \times 100\%$$

S is the amount of input sample DNA in ng.

Example calculation:

Average OD450 of NC is 0.120
 Average OD450 of sample is 0.141
 Slope is 1 OD/ng
 S is 300 ng

$$5\text{-fC (ng)} = \frac{0.141 - 0.120}{1} = 0.021 \text{ ng}$$

$$5\text{-fC \%} = \frac{0.021}{300} \times 100\% = 0.007\%$$

SUGGESTED STRIP-WELL SETUP

Table 1. Single Point Positive Control. 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. Standard Curve Preparation. 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 10 pg/μl	PC 10 pg/μl	Sample	Sample	Sample	Sample
C	PC 20 pg/μl	PC 20 pg/μl	Sample	Sample	Sample	Sample
D	PC 40 pg/μl	PC 40 pg/μl	Sample	Sample	Sample	Sample
E	PC 100 pg/μl	PC 100 pg/μl	Sample	Sample	Sample	Sample
F	PC 200 pg/μl	PC 200 pg/μl	Sample	Sample	Sample	Sample
G	PC 400 pg/μl	PC 400 pg/μl	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
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
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 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 are followed correctly.
	Insufficient input materials.	Ensure that a sufficient amount of positive control (> 50 pg) and samples (300 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 is insufficiently added to the well in Step 2c.	Ensure a sufficient amount of positive control DNA is added.
	The PC Positive Control is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance 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 from accidentally adding sample or positive control DNA or from using contaminated tips.
	Incubation time is too long.	The incubation time at Step 2d should not exceed 2 h.
	Over development of color.	Decrease the development time in Step 4a before adding SS Stop Solution in Step 4b.
No signal or weak signal only in sample wells	DNA sample is not properly extracted or purified	Ensure the DNA sample is in good quality 260/280 ratio should be >1.7 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 2.
	Little or no 5-fC contained in the sample.	N/A
Uneven color development	Insufficient washing of the wells.	Ensure the wells are washed according to the guidance of washing and residue 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 in which you added the other reagents (e.g., from well A to well G or from well 1 to well 12).
Large variation between replicate wells	Color reaction is not evenly stopped due to an inconsistency in pipetting time.	Ensure DS Developer Solution and SS 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 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 volumes (e.g., 1 ul) are completely added into the wells.
	Solutions or antibodies were 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 after adding SS Stop Solution at Step 4b	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

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-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

Methylated and Hydroxymethylated DNA Quantification

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)

Hydroxymethylated DNA Immunoprecipitation

P-1038	MethylFlash™ Hydroxymethylated DNA Immunoprecipitation (hmeDIP) Kit
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DNA Demethylation Enzyme Activity Assay

P-3019	EpiQuik™ DNA Demethylase Activity/Inhibition Assay Kit
P-3086	Epigenase™ 5mC Hydroxylase TET Activity/Inhibition Assay Kit (Colorimetric)
P-3087	Epigenase™ 5mC Hydroxylase TET Activity/Inhibition Assay Kit (Fluorometric)