EPIGENTEK
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MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric)

Base Catalog # P-1030

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) is suitable for detecting global DNA methylation levels using DNA isolated from any species including 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. Both single stranded DNA and double stranded DNA with a size of 200 bps to full length are suitable for use.

Input DNA: The amount of DNA for each assay can be 20 to 200 ng. For the most ideal quantification, the input DNA amount should be 100 ng, as methylated DNA varies from tissue to tissue and can be less than 1% of total DNA in some species.

Starting Materials: Starting materials can include various tissue or cell samples such as cells from a 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: 0.1% to 5%). Because global methylation 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 methylated DNA and determine the relative methylation states of two different DNA samples.

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

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KIT CONTENTS

Component	48 Reactions Cat. #P-1030-48	96 Reactions Cat. #P-1030-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
BS (Binding Solution)	5 ml	10 ml	RT
NC (Negative Control containing 0% 5-mC, 50 µg/ml)*	50 µl	100 µl	–20°C
PC (Positive Control containing 5% 5-mC, 50 µg/ml)*	10 µl	20 µl	–20°C
mcAb (5-mC Antibody, 1000X)*	5 µl	10 µl	4°C
SI (Signal Indicator, 1000X)*	5 µl	10 µl	–20°C
ES (Enhancer Solution, 1000X)*	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
User Guide	1	1	RT

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

Take Note! *The **NC** is unmethylated DNA containing 0% of 5-methylcytosine. The **PC** is methylated DNA containing 5% of 5-methylcytosine.*

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**, **SI** and **ES** at –20°C away from light; (2) store **WB**, **mcAb**, **DS**, and **8-Well Assay Strips** at 4°C away from light; (3) Store **BS** and **SS** at room temperature away from light.

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

Take Note! *Check if **WB** 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 8-channel 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™ Global DNA Methylation (5-mC) ELISA Easy 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. Thus, use only the User Guide that was supplied with the kit when using that kit.

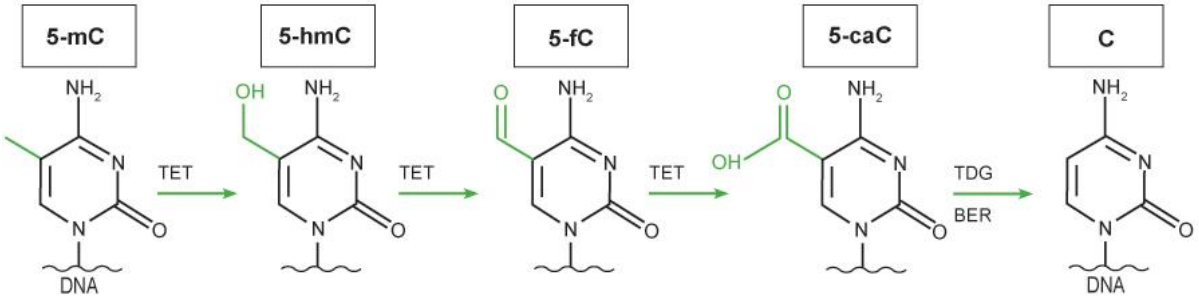
Usage Limitation: The MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit is for research use only and is not intended for diagnostic or therapeutic applications.

Intellectual Property: The MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit 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 (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. Levels of 5-mC are variable in animal genomes, ranging from undetectable amounts in some insects to about 2% of total DNA in vertebrates. The level of 5-mC in plants generally accounts for 0.5-2% and can be as high as 8% of total DNA in some other species. The biological importance of 5-mC as a major epigenetic modification in phenotype and gene expression has been recognized widely. For example, global decrease in 5-mC content (DNA hypomethylation) is likely caused by methyl-deficiency due to a variety of environmental influences, and has been proposed as a molecular marker in multiple biological processes such as cancer. It has been well demonstrated that the decrease in global DNA methylation is one of the most important characteristics of cancer.

A few novel modified nucleotides, 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC), have been detected in human and mouse tissues as well as embryonic stem cells. In mammals, these modified nucleotides can be generated by iterative oxidation of 5-methylcytosine, a reaction mediated by the TET family of enzymes.



A line of evidence shows that these modified cytosines play a critical role in regulating gene function by impacting DNA methylation structures and patterns.

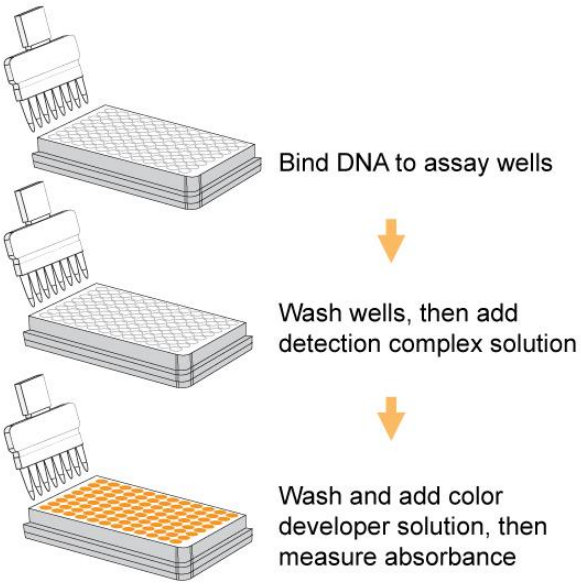
Accurate and convenient detection of 5-mC would be extremely useful for identifying and understanding the global methylation changes that occur in DNA during various physiological and pathological processes such as in cancer. Several chromatography-based techniques such as HPLC and TLC mass spectrometry are used for detecting 5-mC. However, these methods are time consuming and have low throughput with high costs. To address this problem, EpiGentek offers a series of Methylated DNA Quantification Kits to quantify 5-mC or methylated DNA and further refines its methylated DNA assay expertise with the development of the MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit. This kit has the following advantages and features:

- **Fast** - Reduced steps so that the entire procedure only needs 2 hours*
- **Robust** - Improved kit composition allows the assay to have a greater "signal window" with reduced variation between replicates
- **Convenient** - Inherently low background noise, thereby eliminating the need for DNA denaturation and plate blocking steps
- **Sensitive** - Detection limit can be as low as 0.05% methylated DNA from 100 ng of input DNA
- **Specific -** High specificity to 5-mC, with no cross-reactivity to unmethylated cytosine or hydroxymethylated cytosine within the indicated concentration range of the sample DNA
- **Universal** – Positive and negative controls and allow detection of DNA methylation in any species from either single-stranded or double-stranded input DNA
- **Accurate -** Optimized positive controls that can be fractionalized in percentage scale, allowing the assay to be more accurate and highly comparable with HPLC-MS analysis
- **Flexible** - Strip-well microplate format makes the assay available for manual or high throughput analysis

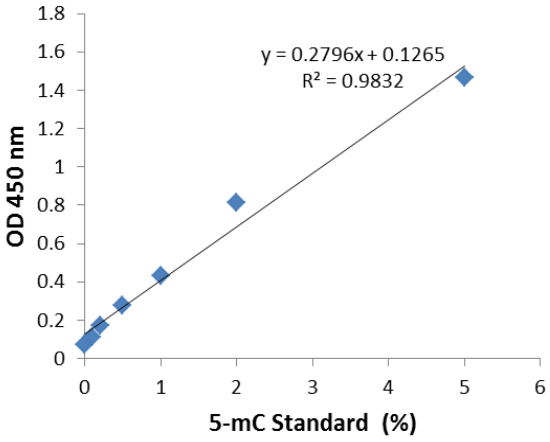
** Based on a single sample assay in duplicate*

PRINCIPLE & PROCEDURE

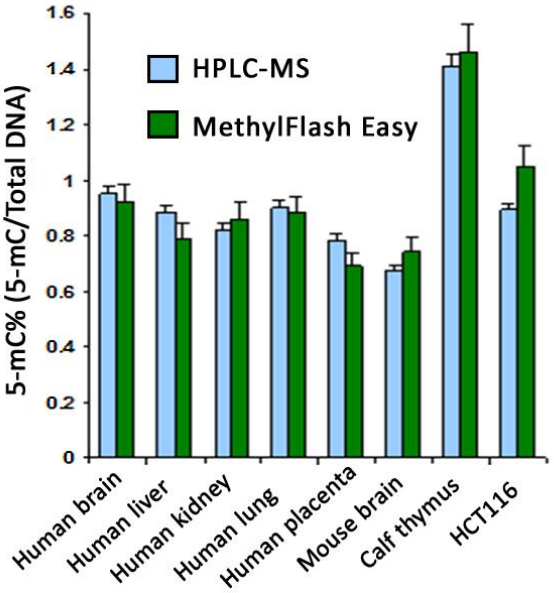
The MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) contains all reagents necessary for the quantification of global DNA methylation. In this assay, DNA is bound to strip-wells that are specifically treated to have a high DNA affinity. The methylated fraction of DNA is detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The percentage of methylated DNA is proportional to the OD intensity measured.



▲ Schematic procedure for the MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric).



▲ An example of an optimal standard curve generated with 5-mC standard control.



▲ Accurate quantification of 5-mC content of various DNA samples from different species using the MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric). The results are closely correlated with those obtained by HPLC-MS.

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ASSAY PROTOCOL

Starting Materials

Input DNA Quality and Amount: Input DNA should be relatively pure with 260/280 ratio >1.6 and can be diluted with water or TE buffer. The DNA amount can range from 20 ng to 200 ng per reaction. However, we recommend using 100 ng of DNA, which is the optimized input amount for the best results.

DNA Isolation: You can use your method of choice for DNA isolation. EpiGentek offers a series of genomic DNA isolation kits for your convenience.

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

1. Working Buffer and Solution Preparation

For 3 columns, I made 40mL of WB:
4mL 10x WB
36mL Nanopure H2O

For a 48-reaction size kit, prepare **Diluted WB** (1X Wash Buffer) by adding 13 ml of **WB** (10X Wash Buffer) to 117 ml of distilled water and adjust pH to 7.2-7.5. For the 96-reaction size kit, add 26 ml of **WB** (10X Wash Buffer) to 234 ml of distilled water and adjust pH to 7.2-7.5.

Take Note! *This **Diluted WB** can now be stored at 4°C for up to six months.*

The anticipated approximate volumes of reagents needed are reflected below for this assay.

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	100 µl	800 µl	1600 µl	4800 µl	9600 µl
5-mC Detection Complex	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	2 µl	2 µl	45 µl	45-90 µl
PC	N/A	N/A	Optional	6 µl	6-12 µl

2. Preparation of Standard Curve

Dilute 1 µl of **PC** with 9 µl of **NC** to make **Diluted PC**. Mix well. Then, prepare 6 concentration points for the control by combining **PC**, **Diluted PC**, and **NC** according to the following chart. Mix well to ensure the accuracy of the concentration.

I did not make the 2% standard.
All leftover standard dilutions are in my box (labeled 'Shelly 10-17-18') in the -20C in 213.
I also used non-diluted 5% PC as a control

Control		PC (5.0%)		Diluted PC (0.5%)		NC
0.1% PC/well	=	0.0 µl	+	1.0 µl	+	9.0 µl
0.2% PC/well	=	0.0 µl	+	1.0 µl	+	4.0 µl
0.5% PC/well	=	0.0 µl	+	3.0 µl	+	3.0 µl
1.0% PC/well	=	1.0 µl	+	0.0 µl	+	9.0 µl
2.0% PC/well	=	1.0 µl	+	0.0 µl	+	4.0 µl
5.0% PC/well	=	3.0 µl	+	0.0 µl	+	3.0 µl

Take Note! *The above volumes will be sufficient for one standard curve in duplicate (12 wells total). The PC concentrations are based on per assay well, not per microliter.*

3. DNA Binding

I removed the 9 columns I didn't need to use and placed them back in the bag at 4C
I spread the 3 columns out, so controls were in lane 1, samples were in lane 6 and lane 12.
I did this to prevent any well-to-well color intensity contamination during plate reading
Then I added 100uL BS to each column.

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

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	1%PC	Sample 2	Sample 6	Sample 10	Sample 14
B	NC	1%PC	Sample 2	Sample 6	Sample 10	Sample 14
C	0.1%PC	2%PC	Sample 3	Sample 7	Sample 11	Sample 15
D	0.1%PC	2%PC	Sample 3	Sample 7	Sample 11	Sample 15
E	0.2%PC	5%PC	Sample 4	Sample 8	Sample 12	Sample 16
F	0.2%PC	5%PC	Sample 4	Sample 8	Sample 12	Sample 16
G	0.5%PC	Sample 1	Sample 5	Sample 9	Sample 13	Sample 17
H	0.5%PC	Sample 1	Sample 5	Sample 9	Sample 13	Sample 17

I did not run duplicate samples or controls.
I ran 2 uL NC in A1
2 uL 0.1% PC in B1
2 uL 0.2% in C1
2 uL 0.5% in D1
2 uL 1% in E1
2 uL 5% in F1
2 uL nondiluted 5% in G1
I ran a blank in H1
For sample volumes refer to the suggested set

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. For negative control wells: Add 100 µl of **BS** and 2 µl of **NC**.

c. For positive control wells: Add 100 µl of **BS** and 2 µl of **PC** at different concentrations (0.1%-5%) to generate a standard curve (see note below).

d. For sample wells: Add 100 µl of **BS** and 100 ng of your sample DNA (2-4 µl).

Take Note! *(1) To reduce cross variation between replicates, it is important to load the wells in vertical formation according to the plate layout depicted above. (2) For the positive controls, total DNA is 100 ng per well with different methylation percentages (0.1%, 0.2%, 0.5%, 1%, 2%, and 5%). The positive controls should be assayed in parallel with the samples in the same plate and a new positive control standard curve should be generated for each assay. (3) For optimal binding and to reduce pipetting error, sample DNA volume added should be 2 µl or more, but should not exceed 5 µl. If the sample DNA is not 100 ng per well, the amount of positive control DNA should be adjusted accordingly to be equal to the amount of the sample DNA that is used to ensure the accuracy of 5-mC quantification. (4) To ensure that **NC**, **PC**, and sample DNA are completely added into the wells, the DNA should be mixed well before use and the pipette tip should be placed into the **BS** solution in the well and aspirated in/out 1-2 times. Changing the tips each time when adding the sample will increase sample volume accuracy added into each well.*

e. Mix solution by gently tilting from side to side or by gently shaking the plate several times to ensure the solution coats the bottom of the well evenly. Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 60 minutes.

I used the incubator in 228 set at 42C because that is actually 37C.

f. During the last 10 minutes of sample incubation, prepare the **5-mC Detection Complex Solution**: In each 1 ml of **Diluted WB** add 1 µl of **mcAb**, mix and then add 1 µl of **SI** and 0.5 µl of **ES**. Mix well.

I made 1.5mL of DCS

g. Remove the **BS** from each well after 60 minute incubation. Wash each well with 150 µl of the **Diluted WB** each time for three times. This can be done by simply pipetting **Diluted WB** in and out of the wells.

4. Methylated DNA Detection and Signal Measurement

a. Add 50 µl of the **5-mC Detection Complex Solution** to each well, then cover and incubate at room temperature for 50 minutes.

b. Remove the **5-mC Detection Complex Solution** from each well.

c. Wash each well with 150 µl of the **Diluted WB** each time for five times.
I did this 6 times because I had extra WB

d. Add 100 µl of **DS** to each well in a column, not row, simultaneously in a vertical fashion with a multi-channel pipette so that replicates are developed at the same time. Gently shake the plate against a flat surface for 5-10 seconds and incubate at room temperature for 3-4 minutes. Monitor color development in the sample wells and control wells. After a few minutes, the **DS** will turn blue in the presence of sufficient methylated DNA. The color in the **NC** wells will remain generally unchanged.
I developed for 4 minutes. See picture for what the plate looked like at 3 minutes

e. When the color in the 5% **PC** wells turns deep blue, stop the enzyme reaction by adding 100 µl of **SS** to each well in a column, not row, simultaneously in a vertical fashion with a multi-channel pipette so that replicates are stopped at the same time. Mix the solution by gently shaking the plate against a flat surface and wait 1-2 minutes 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 2-15 minutes.
I ended up 130uL instead of 100uL of SS to each well by accident.

Take Note! *(1) The color development time may vary from 1-10 minutes based on the speed of color change, but is typically 4-5 minutes. (2) If the strip-well plate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

5. 5-mC% Calculation

To calculate percentage of methylated DNA, first generate a standard curve and plot the OD values versus the **PC** at each percentage point. Next, determine the slope (OD/1%) of the standard curve using linear regression (*Microsoft Excel* can be used) and the most linear part (at least 4 concentration points including 0 point) of the standard curve for optimal slope calculation. Now, calculate the percentage of methylated DNA (5-mC) in total DNA using the following formula:

$$5\text{-}mC\% = \frac{Sample\ OD - NC\ OD}{Slope \times S} \times 100\%$$

S is the amount of input sample DNA in ng.

Example Calculation:

```
Average OD450 of NC is 0.065
Average OD450 of sample is 0.305
Slope is 0.15 OD/1%
S is 100 ng
```

$$5\text{-}mC\% = \frac{0.305 - 0.065}{0.15 \times 100} \times 100\% = \mathbf{1.60\%}$$

Take Note! *(1) The calculated 5-mC% is 5-mC/total DNA (A+G+C+T). If the 5-mC% would be presented as 5-mC/(5-mC+C), simply divide the calculated 5-mC% by cytosine content of the species if it is available. For example, cytosine content is 21% in human DNA, thus 5-mC/(5-mC+C) is 1.6% ÷ 0.21 = 7.62%. (2) In the event that the standard curve is flat due to high ODs starting from the lowest %PC or is flat at high %PCs because of a saturated signal intensity due to extended color development time, the 5-mC% can be calculated with logarithmic or polynomial second order regression, respectively (see the Appendix).*

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 gently 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 and samples are 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 caps are tightly capped after each opening or use.
No signal or weak signal in only the positive control wells	The **PC** (Positive Control) DNA is insufficiently added to the well in **Step 3c**.	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 in 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 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 4a** should not exceed 2 hours.
	Over development of color.	Decrease the development time in **Step 4d** before adding **SS** (Stop Solution) in **Step 4e**.
Large variation between replicate wells	Horizontal positioning of well replicates causes inconsistent delays in pipetting and loading of reagents.	Follow the vertical layout example provided in **Step 2**. Ensure loading of reagents is also in vertical order with a multi-channel pipette, especially when adding **DS** (Developer Solution) and **DS** (Stop Solution) in **Step 4**.
	Color reaction is not evenly stopped due to an inconsistency in pipetting time or in pipetting volume.	Ensure **DS** (Developer Solution) and **SS** (Stop Solution) is added at the same time between replicates or otherwise maintains a

		consistent timing in between each addition of solutions. 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 µl) are completely added into the wells. Pay special attention at the steps of adding DNA samples and preparing **5-mC Detection Complex Solution.**
	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.
	Residue wash buffer is present in some of the wells.	Ensure the wash buffer is completely removed at each wash step.
	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 at **Step 4d** and **Step 4e**.	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.
	Splashing of reagents between wells.	Pipette carefully against the sides of the wells to avoid splashing.
	Temperature variations across the plate.	Ensure plates are evenly and fully covered during incubation steps in a stable temperature environment, away from drafts.
Large variation between sample replicate wells only	Sample DNA is sedimented or uneven prior to loading to wells.	Mix your sample DNA sufficiently and evenly prior to loading it into wells.
mcAb (5-mC 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 **mcAb** (5-mC 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.

APPENDIX

Method 1: 5-mC% Calculation Using Logarithmic Second Order Regression

Use this method when the standard curve is flat due to high ODs starting from the lowest %PC.

a. Plot the average OD value on the Y-axis versus the known 5-mC percentage of each **PC** point on the X-axis.

b. Graph the second order logarithmic curve* (also see "Example Calculation" below) and obtain second order logarithmic regression equation:

$$Y = a\ \ln(X) + b$$

Here, X = 5-mC%; Y = <Sample OD>; a is Slope and b is Y-intercept, respectively.

** Microsoft Excel's logarithmic regression function can be used for easy and convenient calculation of 5-mC%.*

c. Calculate 5-mC% of the samples based on the following equation, derived from the above equation

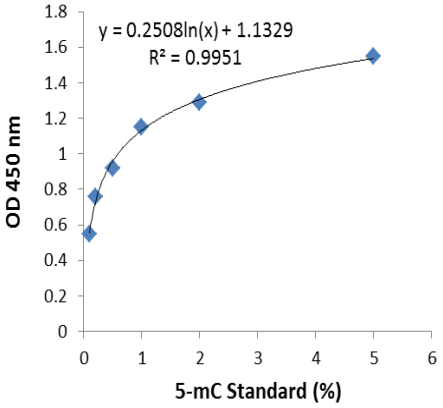
$$5\text{-}mC\% = e^{[(Y-b)/a]} \div S \times 100\%$$

Here, **S** is the amount of input sample DNA in ng.

Example Calculation

The example positive control ODs are as follows:

PC%	Mean OD
0.1	0.55
0.2	0.76
0.5	0.92
1.0	1.15
2.0	1.29
5.0	1.55



▲ The average **NC** OD is 0.06 and the sample OD is 1.274.

▲ Standard curve generated with use of the positive controls.

Data was graphed using a Standard Scatter (XY) Chart in *Microsoft Excel*. In the figure above, a = 0.2508, b = 1.1329, Y = 1.274. Input DNA amount was 100 ng.

5-mC% of the sample was calculated accordingly:

$$5\text{-}mC\% = e^{[(1.274-1.1329)\ /\ 0.2508]} \div 100 \times 100\% = 1.75\%$$

Method 2: 5-mC% Calculation Using Polynomial Second Order Regression

Use this method when the standard curve is flat due to a saturated signal intensity at high %PCs.

a. Plot the average delta OD values on the Y-axis versus the known 5-mC percentage of each **PC** point on the X-axis.

b. Graph the second order polynomial curve* (also see "Example Calculation" below) and obtain second order polynomial regression equation:

$$Y = aX^2 + bX$$

Here, X = 5-mC%; Y = <Sample OD> – <NC OD>; a and b is known Slope 1 and Slope 2, respectively.

** Microsoft Excel's polynomial regression function could be used for easy and convenient calculation of 5-mC%.*

c. Calculate 5-mC% of the samples based on the following equation, derived from the above equation.

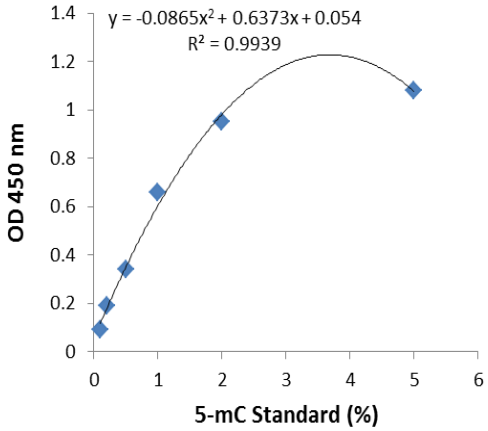
$$5\text{-}mC\% = \frac{(b^2 + 4aY)^{0.5} - b}{2a} \div S \times 100\%$$

Here, S is the amount of input sample DNA in ng.

Example Calculation

The example positive control ODs are as follows:

PC%	Mean OD	Delta OD
0.1	0.15	0.09
0.2	0.25	0.19
0.5	0.4	0.34
1.0	0.72	0.66
2.0	1.01	0.95
5.0	1.14	1.08



▲ The average Negative Control OD is 0.06 and the sample OD is 0.621.

▲ Standard curve generated with use of the positive controls.

Data was graphed using a Standard Scatter (XY) Chart in *Microsoft Excel*. In the figure above, a = −0.0865, b = 0.6373, Y = (0.621−0.060) = 0.561. Input DNA amount was 100 ng.

5-mC% of the sample was calculated accordingly:

$$5\text{-}mC\% = \frac{[(0.6373)^2 + (4 \times -0.0865 \times 0.561)]^{0.5} - 0.6373}{2 \times -0.0865} \div 100 \times 100\% = 1.02\%$$

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