5-mC RNA Methylation ELISA Easy Kit

—— GK- 4046

Product Manual

(Fluorometric)

*PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures

Uses: The Genelily™ 5-mC RNA Methylation ELISA Easy Kit (Fluorometric) is suitable for detecting global 5-mC RNA methylation levels using total RNA 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, plasma/serum samples and body fluid samples, etc.

Input RNA: The amount of RNA for each assay can be 50 to 300 ng. For the most ideal quantification, the input RNA amount should be 200 ng, as 5-mC in RNA varies from tissue to tissue and can be less than 0.1% of total RNA 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, plasma/serum samples, body fluid samples, etc.

Internal Control: Both negative and positive RNA controls are provided in this kit. A standard curve can be performed (range: 0.05% to 2%). Because global 5-mC in RNA 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 5-mC in RNA and determine the relative 5-mC states of two different RNA samples.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the stripwells. 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.

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the Genelily™ 5-mC RNA Methylation ELISA Easy Kit (Fluorometric)is tested against predetermined specifications to ensure consistent product quality. EpiGentekguarantees 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: We 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 Genelily™ 5-mC RNA Methylation ELISA Easy Kit (Fluorometric) is for research use only and is not intended for diagnostic or therapeutic applications.

Intellectual Property: The Genelily™ 5-mC RNA Methylation ELISA Easy Kit (Fluorometric) and methods of use contain proprietary technologies by EpiGentek.

KIT CONTENTS

Component	48 Reactions	96 Reactions	Storage Upon Receipt
WB (10XWash Buffer)	14ml	28 ml	4℃
BS (Binding Solution)	5 ml	10 ml	RT
NC (Negative Control 0% 5-mC,100μg/ml) *	50 μl	100 μl	-20℃
PC(Positive Control, 2% 5-mC,100μg/ml *	10 μl	20 μl	-20℃
mcAb (5-mC Antibody, 1000X)*	5 μl	10 μl	4℃
SI (Signal Indicator, 1000X)*	5 μl	10 μl	-20℃
ES (Enhancer Solution, 1000X)*	5 μl	10 μl	-20℃
FD (Fluoro Developer)	12 μl	24μl	-20℃
FE (Fluoro Enhancer)	12 μl	24μl	-20℃
DB (Dilution buffer)	5 ml	10 ml	RT
8-Well Assay Strips (With Frame)	6	12	4℃
User Guide	1	1	RT

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

__Take Note!__ The __NC__ is unmethylated RNA containing 0% of 5-methylcytosine. The PC is methylated RNA containing 2% of 5-methylcytosine.

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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, ES, FD and FE at －20°C away from light;
(2) Store mCAband 8 -Well Assay Strips at 4°C away from light;
(3) Store BS and DB at room temperature away from light.

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.

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

MATERIALS REQUIRED
(BUT NOT SUPPLIED)

- Adjustable 8-channel pipette
- Aerosol resistant pipette tips
- Distilled water
- 1X TE buffer pH 7.5 to 8.0
- 1.5 ml microcentrifuge tubes
- Plate seal or Parafilm M
- Isolated RNA of interest
- Incubator for 37°C incubation
- Microplate reader capable of reading absorbance at $530_{ex}/590_{em}$ nm

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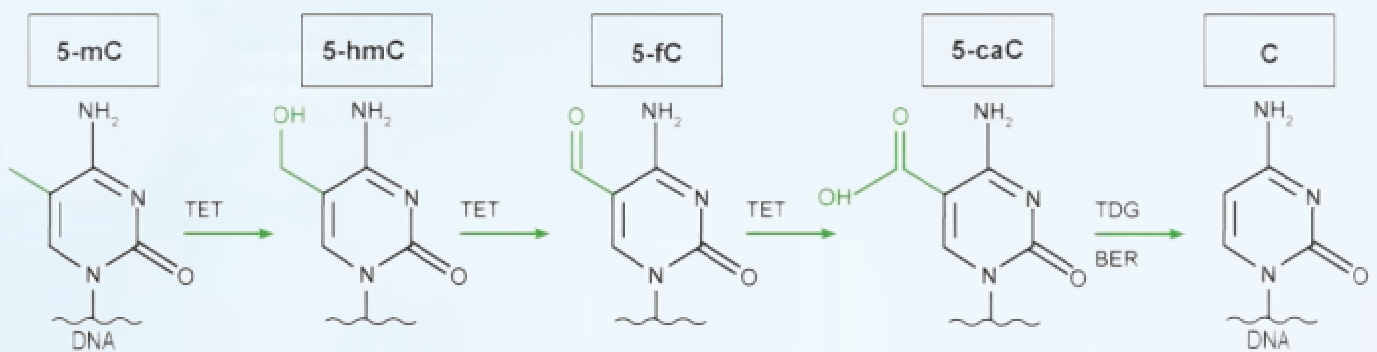
A BRIEF OVERVIEW

5-methylcytosine (5-mC) in DNA occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNA methyltransferases. This process has been well studied and is generally associated with repression of gene expression. It was also observed that in humans, 5-mC occurs in various RNA molecules including tRNAs, rRNAs, mRNAs and non-coding RNAs (ncRNAs). 5-mC seems to be enriched in some classes of ncRNA, but relatively depleted in mRNAs. Levels of 5-mC are variable in animal genomes, ranging from undetectable amounts in some insects to about 0.1-0.45% of total RNA in human cells.

The majority (83%) of 5-mC sites were found in mRNAs. Within these transcripts 5-mC appears to be depleted within protein coding sequences but enriched in 5 ’ and 3 ’ UTRs. Two different methyltransferases, NSUN2 and Dnmt2 are known to catalyze 5-mCmodification in eukaryotic RNA.There has been strong evidence that RNA cytosine methylation affects the regulation of various biological processes such as RNA stability and mRNA translation.

Furthermore, loss of 5-mC in vault RNAs causes aberrant processing into Argonaute-associated smallRNA fragments that can function as microRNAs. Thus, impaired processing of vault ncRNA may contribute to the etiology of human disorders related to NSUN2-deficiency.Very recently, a few novel modified nucleotides previously found in the DNA such as 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC) have also been detected in RNA of human and mouse tissues. These modified nucleotides are also generated by iterative oxidation of 5-methylcytosine, a reaction mediated by the TET family of enzymes.



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Accurate and convenient detection of 5-mC would be extremely useful for identifying and understanding the 5-mC methylation changes that occur in RNA during various physiological and pathological processes. Currently only chromatography-based techniques such as MS-LC are used for detecting total 5-mC in RNA. However, these methods are time consuming and have low throughput with high costs. To address this problem, Genelily developed 5-mC RNA Methylation ELISA Easy Kit. This kit has the following advantages and features:

- Fast - The entire procedure only needs 2 hours and 40 minutes*
- Robust - The kit composition allows the assay to have a large “ signal window ” with less variation between replicates
- Convenient - Inherently low background noise, thereby eliminating the need for plate blocking steps
- Sensitive - Detection limit can be as low as 0.02% of 5-mC RNA from 200 ng of input RNA
- Specific - High specificity to 5-mC, with no cross-reactivity to unmethylated cytosine orhydroxymethylated cytosine within the indicated concentration range of the sample RNA
- Universal － Positive and negative controls allow detection of 5-mC RNA methylation in any species
- 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*

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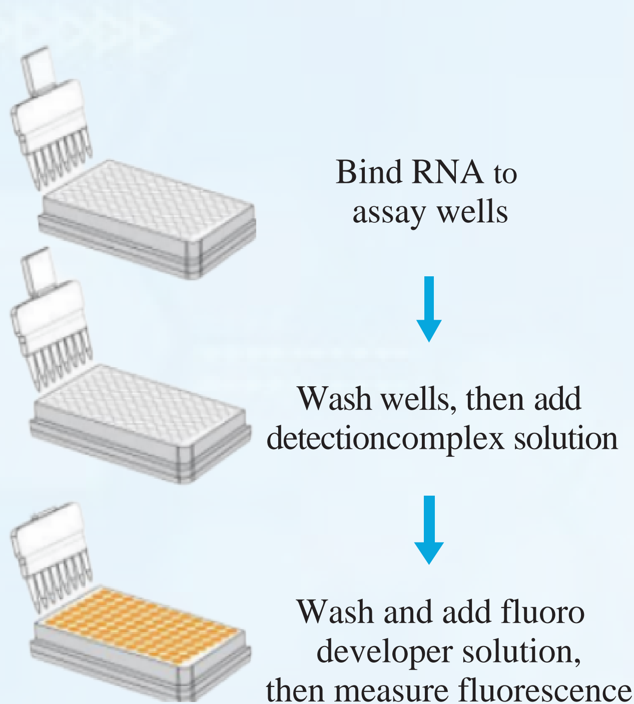
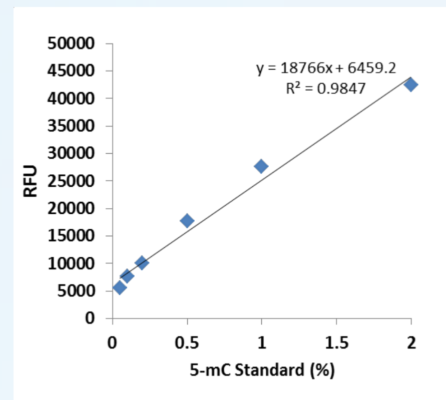
PRINCIPLE & PROCEDURE

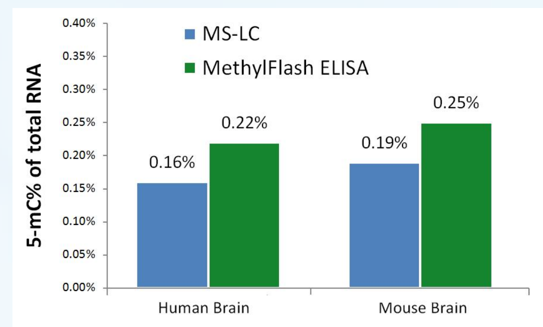
The Genelily™ 5-mC RNA Methylation ELISA Easy Kit (Fluorometric) contains all reagents necessary for the quantification of global 5-methylcytosine (5-mC) RNA methylation. In this assay,RNA is bound to strip-wells that are specifically treated to have a high nucleic acid affinity. 5-mC inRNA is detected using capture and detection antibodies and then quantified fluorometrically by reading the fluorescence in a microplate spectrophotometer. The percentage of 5 -mC RNA is proportional to the fluorescence intensity measured.



Schematic procedure for the Genelily™ 5-mC RNA Methylation ELISA Easy Kit (Fluorometric).



An example of an optimal standard curvegenerated with 5-mC standard control.



Accurate quantification of 5-mC content of various RNA samples from different species using the Genelily™ 5-mC RNA Methylation ELISA Easy Kit (Fluorometric). The results are closely correlated with those obtained by MS-LC.

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ASSAY PROTOCOL

Starting Materials

Input RNA Quality and Amount: Input RNA should be highly pure with 260/280 ratio >2.0 and relatively free of DNA. DNase I can be used to remove DNA. RNA should be eluted in RNase-free water.The RNA amount can range from 50 ng to 300 ng per reaction. However, we recommend using 200 ng of RNA, which is theoptimized input amount for the best results.

RNA Storage: Isolated RNA can be stored at －20°C until use.

1. Preparation of Working Buffer

Diluted Wash Buffer: For a 48-reaction size kit, prepare **Diluted WB** (1X WashBuffer) 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** (10XWash 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

Fluorescence Development Solution: Add 1 µl of **FD** (Fluoro Developer) and 1 µl of **FE** (Fluoro Enhancer) to every 500 µl of **DB** (Dilution Buffer). About 50 µl of this **Fluorescence Development Solution** will be required for each assay well.

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

Take 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.

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	100µl	800 µl	1600 µl	4800 µl	9600 µl
5-mC Detection Complex	50 µl	400 µl	800 µl	2400 µl	4800 µl
Fluorescence Development Solution	50 µl	400 µl	800 µl	2400 µl	4800 µl
NC	N/A	2 µl	2 µl	4 µl	8 µl
PC	N/A	N/A	Optional	4 µl	8 µl

2. Preparation of Standard Curve

Dilute 1 µl of PC with 7 µl of NC to make Diluted PC. Mix well. Then, prepare 6 concentration pointsfor the control by combining PC, Diluted PC, and NC according to the following chart. Mix well to ensure the accuracy of the concentration.

Control		PC (2.0%)		Diluted PC (0.25%)		NC
0.05% PC/well	=	0.0 µl	+	1.0 µl	+	9.0 µl
0.1% PC/well	=	0.0 µl	+	1.0 µl	+	4.0 µl
0.5% PC/well	=	0.0 µl	+	2.0 µl	+	3.0 µl
0.2% PC/well	=	1.0 µl	+	0.0 µl	+	7.0 µl
1.0% PC/well	=	2.0 µl	+	0.0 µl	+	6.0 µl
2.0% 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).*

3.RNA Binding

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	Stirp 2	Stirp 3	Stirp 4	Stirp 5	Stirp 6
A	NC	0.05%PC	Sample2	Sample6	Sample10	Sample14
B	PC	0.05%PC	Sample2	Sample6	Sample10	Sample14
C	0.05%PC	0.1%PC	Sample3	Sample7	Sample11	Sample15
D	0.05%PC	0.1%PC	Sample3	Sample7	Sample11	Sample15
E	0.1%PC	0.2%PC	Sample4	Sample8	Sample12	Sample16
F	0.1%PC	0.2%PC	Sample4	Sample8	Sample12	Sample16
G	0.2%PC	Sample1	Sample5	Sample9	Sample13	Sample17
H	0.2%PC	Sample1	Sample5	Sample9	Sample13	Sample17

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 (0.05%-2%) to generate a standard curve (see note below).

d. For sample wells: Add 100 µl of BS and 200 ng of your sample RNA (2-8 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.

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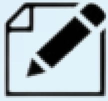

(2) For the positive controls, total RNA is 200 ng per well with different methylation percentages (0.05%, 0.1%, 0.2%, 0.5%, 1%, and 2%). The positive controls should be assayed in parallel with the samples in the same plate and a new positive control standard curve should be generated for each assay.
(3) For optimal binding and to reduce pipetting error, sample RNA volume added should be 2 µl or more, but should not exceed 5 µl. If the sample RNA is not 200 ng per well, the amount of positive control RNA should be adjusted accordingly to be equal to the amount of the sample RNA that is used to ensure the accuracy of 5-mC quantification.
(4) To ensure that **NC, PC,** and sample RNA are completely added into the wells, the input RNA 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 90 minutes.
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 1 µl of ES. Mix well.
g. Remove the BS from each well after 90 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. 5-mC RNA 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.

5. Signal Detection

a. Add 50 µl of **Fluorescence Development Solution** to each well and incubate at room temperature for 2 to 4 minutes away from direct light. The **Fluorescence Development Solution** will turn pink in the presence of sufficient 5-mC products.
b. Read the fluorescence on a fluorescence microplate reader within 2 to 10 minutes at $530_{ex}/590_{em}$ nm.

Take Note!
(1) The fluorescence development time may vary from 1-10 minutes based on the speed of color change, but is typically 3-4 minutes.
(2) If the strip-well plate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.

6. 5-mC% Calculation

To calculate percentage of 5-mC RNA, first generate a standard curve and plot the RFU values versus the **PC** at each percentage point. Next, determine the slope (RFU/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 RNA (5-mC) in total RNA using the following formula:

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

S is the amount of input sample RNA in ng.

Example Calculation:
Average RFU of NC is 1250
Average RFU of sample is 11250
Slope is 25000/1%
S is 200 ng

$$5\text{-mC}\% = \frac{11250-1250}{25000 \times 200} \times 100\% = 0.2\%$$

Take Note!
(1) The calculated 5-mC% is 5-mC/total RNA (A+G+C+U). 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.
(2) In the event that the standard curve is flat due to high RFUs starting from the lowest %PC or is flat at high %PCs because of a saturated signal intensity due to extended fluoro development time, the 5-mC% can be calculated with logarithmic or polynomial second order regression, respectively (see the Appendix).

APPENDIX

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

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

a. Plot the average RFU 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 RFU>; **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 RNA in ng.

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

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

a. Plot the average RFU 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:

$$Y = aX2 + bX$$

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

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

$$5\text{-mC}\% = \frac{(b2 + 4aY)\,0.5 - b}{2a} \div S \times 100\%$$

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

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 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 (530_{ex}/590_{em} 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.
Large variation between sample replicate wells only	Sample RNA is sedimented or uneven prior to loading to wells.	Mix your sample RNA sufficiently and evenly prior to loading it into wells.
mcAb (5-mC An tibody) vial appe ars 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.

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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 positive control RNA 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.	Ensure the well is not contaminated from adding sample or positive control accidentally or from using contamin ated tips.
	Incubation time is too long.	The incubation time at **Step 4a** should not exceed 2 hours.
	Over development of fluorescence.	Decrease the development time at **Step 5a.**
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 3**. Ensure loading of reagents is also in vertical order with a multi-channel pipette, especially when adding F**luorescence Development Solution at Step 5a.**
	Residue wash buffer is present in some of the wells.	Ensure the wash buffer is complet ely 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 preve nt solutions from sticking to the surface.
	Did not use the same pipette device throughout the experiment.	Use the same multi-channel pipette device throughout the entire experiment, as diffe rent 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 temper ature environment, away from drafts.

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