EpiQuik™ Circulating Modified Histone H3 Multiplex Assay Kit (Colorimetric)

Base Catalog # P-3106

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The EpiQuik™ Circulating Modified Histone H3 Multiplex Assay Kit (Colorimetric) is suitable for specifically measuring up to 22 histone H3 modifications from biological fluid samples such as plasma and serum from human, mouse or rat. Each kit can be used for two different samples or a pair of samples: control and treated, normal and diseased, and other paired comparisons.

Input Material: Input materials should be plasma or serum. The amount of plasma or serum for each assay can be 10 to 40 µl with an optimal amount of 30 µl.

Internal Control: An assay standard control is provided in this kit. It can be used for signal intensity comparison between the assay control and the samples to indicate the amount of each histone H3 modification captured from the samples. Because the content of each histone H3 modification can vary from tissue to tissue, and from normal and diseased states, or from different treatments, it is advised to run duplicate for each sample to ensure that the signal generated is validated.

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.

110 Bi County Blvd. Ste. 122, Farmingdale, NY 11735
Tel: 1-877-374-4368 ■ Fax: 1-718-484-3956 ■ E-mail: info@epigentek.com ■ Web: www.epigentek.com

KIT CONTENTS

Part 1 of 2

Component	96 Assays Cat. #P-3106-96	Shipping Temperature	Storage Upon Receipt	Storage Checklist
WB (10X Wash Buffer)	28 ml	Ambient	4°C	
HAB (Histone Assay Buffer)	8 ml	Ambient	4°C	
DS (Developer Solution)	12 ml	Ambient	4°C	
SS (Stop Solution)	12 ml	Ambient	RT	
96-Well Strip Plate (With Frame)	1	Ambient	4°C	
Extra 8-Well Strips	2	Ambient	4°C	
Adhesive Covering Film	1	Ambient	RT	

Part 2 of 2

Component	96 Assays Cat. #P-3106-96	Shipping Temperature	Storage Upon Receipt	Storage Checklist
DAb (Detection Antibody, 1000X)*	12 µl	Ice Pack	–20°C	
SC (Standard Control, 100 µg/ml)	20 µl	Ice Pack	–20°C	

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

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 **DAb** and **SC** at –20°C away from light; (2) Store **WB**, **HAB**, **DS**, **96-Well Strip Plate**, and **Extra 8-Well Strips** at 4°C away from light; and (3) Store remaining components (**SS** and **Adhesive Covering Film**) at room temperature away from light. All components of the kit are stable for 6 months from the date of shipment, when stored properly.

Note: *(1) Check if **WB** (10X Wash Buffer) contains salt precipitates before use. If so, warm (at room temperature or 37°C) and shake the buffer until the salts are re-dissolved; and (2) Check if a blue color is present in **DS** (Developer Solution), which would indicate contamination of the solution and should not be used. To avoid contamination, transfer the amount of **DS** required into a secondary container (tube or vial) before adding **DS** into the assay wells.*

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Adjustable pipette or multiple-channel pipette
- ☐ Multiple-channel pipette reservoirs
- ☐ Aerosol resistant pipette tips
- ☐ Microplate reader capable of reading absorbance at 450 nm

- ☐ 1.5 ml microcentrifuge tubes
- ☐ Incubator for 37°C incubation
- ☐ Distilled water
- ☐ Plasma or serum
- ☐ Parafilm M or aluminum foil

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the EpiQuik™ Circulating Modified Histone H3 Multiplex Assay 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.epigentek.com/datasheet.

Usage Limitation: The EpiQuik™ Circulating Modified Histone H3 Multiplex Assay Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application.

A BRIEF OVERVIEW

Histone modifications have been defined as epigenetic modifiers. Post-translational modifications of histones include the acetylation of specific lysine residues by histone acetyltransferases (HATs), deacetylation by histone deacetylases (HDACs), methylation of lysine and arginine residues by histone methytransferases (HMTs), the demethylation of lysine residues by histone demethylases (HDMTs), and the phosphorylation of specific serine groups by histone kinases (HKs). Additional histone modifications include the attachment of ubiquitin (Ub), small ubiquitin-like modifiers (SUMOs), and poly ADP-ribose (PAR) units. Next to DNA methylation, histone acetylation and histone methylation are the most well characterized epigenetic marks. Generally, tri-methylation at H3-K4, H3-K36, or H3-K79 results in an open chromatin configuration and is therefore characteristic of euchromatin. Euchromatin is also characterized by a high level of histone acetylation, which is mediated by histone acetyltransferases. Conversely, histone deacetylases have the ability to remove this epigenetic mark, which leads to transcriptional repression. Condensed heterochromatin is enriched in tri-methylation of H3-K9 and H3-K27, and silencing of euchromatin loci caused by histone deacetylation involves the recruitment of specific K9 histone methyltransferases. Methylated H3-K9 provides a binding site for the chromodomain-containing heterochromatin protein 1 (HP1), which induces transcriptional repression

and heterochromatinization. At euchromatic loci, this process is mediated by co-repressors, such as retinoblastoma protein pRb or KAP1. Histone demethylases have the opposite effect on transcription. For example, the histone demethylase LSD1 is responsible for H3K4 demethylation, which leads to transcriptional inactivation. Other histone demethylases, such as jumonji (JHDM2A), are responsible for H3K9 demethylation, whereas JHDM1 has the ability to convert active chromatin marks such as H3-K36me2, to an unmodified state. Lysine residues can be mono-, di-, or trimethylated, each of which can differentially regulate chromatin structure and transcription. Along with other histone modifications such as phosphorylation, this enormous variation leads to a multiplicity of possible combinations of different modifications. This may constitute a “histone code”, which can be read and interpreted by different cellular factors.

Abnormal histone modification patterns have been associated with many different diseases such as cancer, autoimmune disorders, and inflammatory and neurological diseases. Circulating modified histones in the plasma or serum are demonstrated to be and considered as the markers for many different diseases or pathological change. Therefore, detection of circulating histone H3 modifications would provide useful information for a better understanding of epigenetic regulation of gene activation and silencing, histone modification-associated pathological disease process, screening of disease-related biomarkers, as well as for developing histone modification-targeted drugs. As the leading provider of histone modification assay products, we have further refined our assay expertise by developing the EpiQuik™ Circulating Modified Histone H3 Multiplex Assay Kit. As the first circulating modified histone H3 multiplex assay for detecting up to 22 modified histone H3 patterns simultaneously, this kit has the following advantages:

- Simultaneously measure 22 different histone H3 modifications, which include all of the most important and well-characterized patterns.

H3K4me1	H3K4me2	H3K4me3	H3K9me1	H3K9me2	H3K9me3
H3K27me1	H3K27me2	H3K27me3	H3K36me1	H3K36me2	H3K36me3
H3K79me1	H3K79me2	H3K79me3	H3K9ac	H3K14ac	H3K18ac
H3K27ac	H3K56ac	H3ser10P	H3cit	Total H3	

- Quick and efficient procedure, which can be finished within 2.5 hours.
- Innovative colorimetric assay without the need for radioactivity, electrophoresis, chromatography, or expensive equipment.
- High sensitivity with a detection limit as low as 0.5 ng/well for each modification pattern with dynamic range of 1-20 ng/well within the indicated amount range of the plasma/serum.
- Total histone H3 sets are included, which can be used for normalizing total histone H3 levels for relative comparison of histone H3 content between different samples or different treatment conditions.
- Strip microplate format makes the assay flexible: manual or high throughput, which enables analysis of a single modification or total 22 modification patterns within the same samples.
- An assay standard control and two extra 8-well strips are conveniently included in the kit which can be used for signal intensity comparison between the assay control and the samples to indicate the amount of each histone H3 modification captured from the samples.
- Simple, reliable, and consistent assay conditions.

PRINCIPLE & PROCEDURE

The EpiQuik™ Circulating Modified Histone H3 Multiplex Assay Kit (Colorimetric) is designed for measuring multiple histone H3 modifications simultaneously from plasma and serum. In an assay with this kit, each histone H3 modified at specific sites will be captured by an antibody that is coated on the strip wells and specifically targets the appropriate histone modification pattern. The captured histone modified at specific sites will be detected with a detection antibody, followed by a color development reagent. The ratio of modified histone is proportional to the intensity of absorbance measured by a microplate reader at a wavelength of 450 nm.

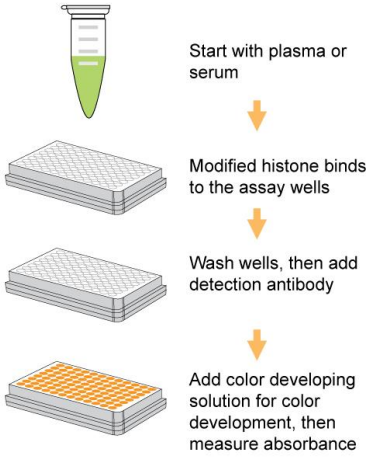

Fig. 1. Schematic procedure of the EpiQuik™ Circulating Modified Histone H3 Multiplex Assay Kit (Colorimetric).

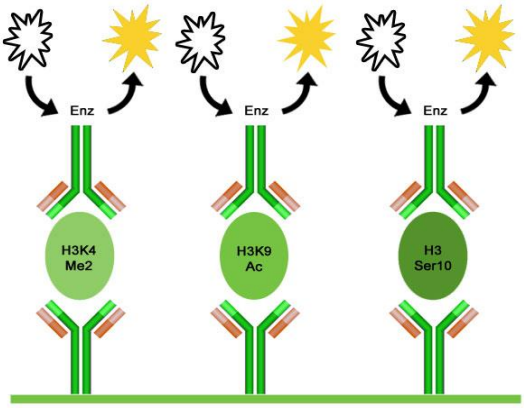

Fig. 2. Working principle of EpiQuik™ Circulating Modified Histone H3 Multiplex Assay Kit (Colorimetric).

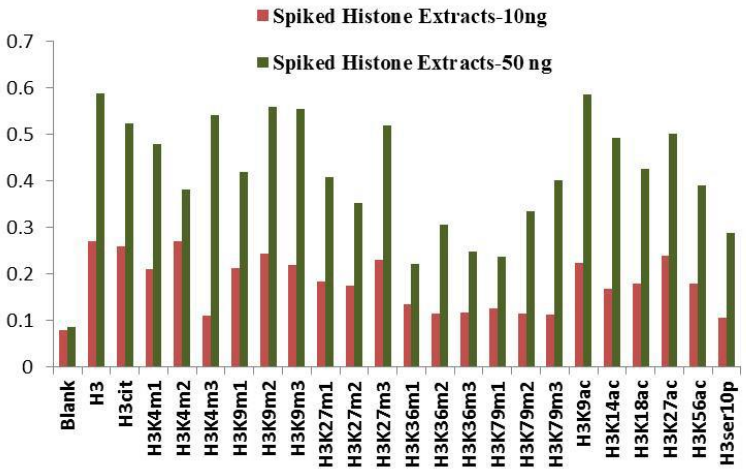

Fig. 3. Histone extracts were prepared from HL-60 cells using the EpiQuik™ Total Histone Extraction Kit and spiked into bovine plasma at different concentrations. The signal intensity of each H3 modification was measured using the EpiQuik™ Circulating Modified Histone H3 Multiplex Assay Kit (Colorimetric).

PROTOCOL

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

Starting Materials

Input Amount: The amount of plasma or serum for each assay can be 10 to 40 µl with an optimal amount of 30 µl.

Use of Extra Strips

The assay **SC** (Standard Control) and two **Extra 8-Well Strips** are conveniently included in the kit which can be used for quantification of each histone H3 modification. If necessary, the extra strips included in the kit can also be used as controls if only a few histone H3 modifications are selected for detection. The strips can be set up as indicated in Table 3 under the "Extra Strip Well Setup" section and carried out by using the same assay protocol described below.

1. Working Buffer and Solution Preparation

a. Prepare **Diluted WB** 1X Wash Buffer:

96-Assays: Add 26 ml of **WB** 10X Wash Buffer to 234 ml of distilled water and adjust pH to 7.2-7.5.

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

b. Prepare **Diluted Dab** Detection Antibody solution:

Dilute **DAb** Detection Antibody with **Diluted WB** at a ratio of 1:1000 (i.e., add 1 µl of **DAb** to 1000 µl of **Diluted WB**). 50 µl of **Diluted DAb** will be required for each assay well.

c. Prepare **Diluted SC**

Suggested Assay Standard Curve Preparation: First, dilute **SC** to 50 ng/µl by adding 5 µl of **SC** to 5 µl of **HAB** (Histone Assay Buffer) and to 5 ng/µl by adding 1 µl of **SC** to 19 µl of **HAB**. Then, further prepare seven concentrations by using the 5 ng/µl and 50 ng/µl of **Diluted SC** with **HAB** into final concentrations of 0.5, 1, 2, 5, 10, 20, and 50 ng according to the following dilution chart:

Tube	SC (5 ng/µl)	SC (50 ng/µl)	HAB	Resulting H3 Concentration
1	1.0 µl		9.0 µl	0.5 ng/µl
2	1.0 µl		4.0 µl	1 ng/µl
3	2.0 µl		3.0 µl	2 ng/µl
4	4.0 µl		0.0 µl	5 ng/µl
5		1.0 µl	4.0 µl	10 ng/µl
6		2.0 µl	3.0 µl	20 ng/µl
7		4.0 µl	0.0 µl	50 ng/µl

Note: *Keep each of the diluted solutions except* ***Diluted WB*** *on ice until use. Any remaining diluted solutions other than* ***Diluted WB*** *should be discarded if not used within the same day.*

2. Histone Binding

a. Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (include blank and positive controls) to ensure that the signal generated is validated. 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).

Note: *If removing strip wells, you must absolutely keep track of which wells have been removed, as each well represents a specific histone modification pattern according to Table 2 of the "Strip Well Setup" section.*

b. Blank Wells: Add 50 µl of **HAB** to each blank well.

c. Standard Wells: Add 50 µl of **HAB** and 1 µl of **Diluted SC (25 ng/ul, single point control)** into the standard well of the plate, or add 1 µl of **Diluted SC** into extra strip wells, each at a different concentration between 0.5 and 50 ng/µl (**Standard Curve**, based on the dilution chart in Step 1c; see Table 3 under the "Extra Strip Well Setup" section as an example).

d. Sample Wells: Add 50 µl of **HAB** and 30 µl of your plasma or serum sample.

Note: *Follow the suggested well setup diagrams.*

e. Tightly cover strip-well microplate with **Adhesive Covering Film** to avoid evaporation and incubate at 37°C for 60 min.

Note: *The **Adhesive Covering Film** can be cut to the required size to cover the strips based on the number of strips to be used.*

f. Remove the reaction solution from each well. Wash each well three times with 150 µl of the **Diluted WB** (1X Wash Buffer) each time.

3. Detection Antibody Binding

a. Prepare Dilute **DAb** with **Diluted WB** (1X Wash Buffer) at a ratio of 1:1000 (i.e., add 1 µl of **DAb** to 1000 µl of **Diluted WB**). Add 50 µl of the **Diluted DAb** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.

b. Remove the **Diluted DAb** solution from each well.

c. Wash each well four times with 150 µl of the **Diluted WB** each time.

Note: *Ensure any residual wash buffer in the wells is removed as much as possible at each wash step.*

4. Signal Detection

a. Add 100 µl 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 modified histone H3 products.

b. Add 100 µl of **SS** to each well to stop enzyme reaction when color in the positive control wells turns medium blue. The color will change to yellow after adding **SS** and the absorbance should be read on a microplate reader within 2 to 10 min at 450 nm with an optional reference wavelength of 655 nm.

Note: *(1) Most microplate readers have the capability to carry out dual wavelength analysis and will automatically subtract reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, the plate can be read twice, once at 450 nm and once at 655 nm. Then, manually subtract the 655 nm ODs from 450 nm ODs; (2) If the strip-well microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

5. Histone H3 Modification Calculation

a. Calculate the average duplicate readings for the sample wells, assay control wells and blank wells.

b. Simple amount quantification of each H3 modification or total H3 in the samples can be carried out using Formula 1 shown below:

Formula 1:

$$\text{H3 Modification or total H3 (ng/ml)} = \frac{\text{(Sample OD – Blank OD)} \div S}{\text{(Standard Control OD – Blank OD)} \div P} \times 1000$$

S is the volume of input sample.
P is the amount of input standard control in ng (use 25 ng).

Example calculation:

Average OD450 of blank is 0.085
Average OD450 of standard control is 0.885
Average OD450 of Sample (H3 modification or total H3) is 0.285
S is 25 ul
P is 25 ng

$$\text{H3 Modification or total H3 (ng/ml)} = \frac{(0.285\text{-}0.085) \div 25}{(0.885\text{-}0.085) \div 25} \times 1000 = 250 \text{ ng/ml}$$

c. Accurate amount quantification of each H3 modification or total H3 in the samples can be carried out using Formula 2 shown below:

Formula 2:

1. Generate a standard curve and plot OD value versus amount of **SC** at each concentration point.

2. Determine the slope as OD/ng (you can use Microsoft Excel statistical functions for slope calculation), then calculate the amount of H3 modification or total H3 using the following formulas:

$$\text{H3 Modification or total H3 (ng/ml)} = \frac{\text{(Sample OD – Blank OD)}}{\text{Slope x sample amount (ul)}} \times 1000$$

d. Calculate the percentage of histone H3 modification in total H3 using Formula 3 shown below:

Formula 3:

$$H3\ Modification\ \% = \frac{Amount\ of\ H3\ Modification\ (ng/ml)}{Amount\ of\ total\ H3\ (ng/ml)} \times 100\%$$

The amount of H3 modification or total H3 is calculated from Formula 1 or Formula 2.

e. Calculate the relative change of each histone H3 modification between different samples using Formula 4 shown below:

Formula 4:

$$Relative\ Change\ \% = \frac{H3\ Modification\ \%\ in\ sample\ 1\ or\ treated\ sample}{H3\ Modification\ \%\ in\ sample\ 2\ or\ control\ sample} \times 100\%$$

SUGGESTED BUFFER AND SOLUTION SETUP

Table 1. Approximate amount of required buffers and solutions for defined assay wells based on the protocol.

Reagents	1 well	1 strip (8 wells)	2 strips (16 wells)	6 strips (48 wells)	12 strips (96 wells)
Diluted WB	1.2 ml	10 ml	20 ml	60 ml	120 ml
Diluted DAb	50 µl	400 µl	800 µl	2400 µl	4800 µl
Standard Control	N/A	N/A	2 µl	2 µl	2 µl
DA (Developer Solution)	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml
SS (Stop Solution)	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml

STRIP WELL SETUP

Table 2. An antibody for each H3 modification is coated onto the indicated wells accordingly.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	H3cit	H3K4me2	H3K9me1	H3K9me3	H3K27me2	H3K36me1	H3K36me3	H3K79me2	H3K9ac	H3K18ac	H3K56ac
B	Blank	H3cit	H3K4me2	H3K9me1	H3K9me3	H3K27me2	H3K36me1	H3K36me3	H3K79me2	H3K9ac	H3K18ac	H3K56ac
C	SC 25 ng	H3cit	H3K4me2	H3K9me1	H3K9me3	H3K27me2	H3K36me1	H3K36me3	H3K79me2	H3K9ac	H3K18ac	H3K56ac
D	SC25 ng	H3cit	H3K4me2	H3K9me1	H3K9me3	H3K27me2	H3K36me1	H3K36me3	H3K79me2	H3K9ac	H3K18ac	H3K56ac
E	Total H3	H3K4me1	H3K4me3	H3K9me2	H3K27me1	H3K27me3	H3K36me2	H3K79me1	H3K79me3	H3K14ac	H3K27ac	H3ser10P
F	Total H3	H3K4me1	H3K4me3	H3K9me2	H3K27me1	H3K27me3	H3K36me2	H3K79me1	H3K79me3	H3K14ac	H3K27ac	H3ser10P
G	Total H3	H3K4me1	H3K4me3	H3K9me2	H3K27me1	H3K27me3	H3K36me2	H3K79me1	H3K79me3	H3K14ac	H3K27ac	H3ser10P
H	Total H3	H3K4me1	H3K4me3	H3K9me2	H3K27me1	H3K27me3	H3K36me2	H3K79me1	H3K79me3	H3K14ac	H3K27ac	H3ser10P

EXTRA STRIP WELL SETUP

	Strip 1	Strip 2
A	Blank	Blank
B	0.5 ng	0.5 ng
C	1 ng	1 ng
D	2 ng	2 ng
E	5 ng	5 ng
F	10 ng	10 ng
G	20 ng	20 ng
H	50 ng	50 ng

Table 3. Two extra strip wells can be set up for generating standard curve.

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal in both the assay control and sample wells	Reagents are added incorrectly.	Check if reagents are added in the proper order with the right amount, and if any steps in the protocol may have been omitted by mistake.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	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 closed after each opening or use.
No signal or weak signal in only the assay control wells	The assay **SC** amount is insufficiently added to the well in Step 2c.	Ensure a sufficient amount of **SC** is added.
	The **SC** is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance in this User Guide for storage of **SC.**
High background present in the blank wells.	Insufficient washing of wells.	Check if washing recommendations at each step are performed according to the protocol.
	Contaminated by sample or **SC**.	Ensure the well is not contaminated from adding sample or **SC** accidentally or from using contaminated tips.
	Incubation time with **Diluted DAb** is too long.	The incubation time at Step 3a should not exceed 90 min.
	Over-development of color.	Decrease the development time in Step 4a before adding **SS** in Step 4b.

No signal or weak signal only in sample wells or for some of H3 modification patterns.	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of plasma or serum is used as indicated in Step 2d.
	Sample was not stored properly or has been stored for too long.	Ensure plasma or serum is stored in aliquots at proper temperature, for no more than 6 months.
	Little or no modified H3 at specific sites in the sample.	This problem may be a result of many factors. If the affecting factors cannot be determined, use new or re-prepared samples to make sure that the sample does indeed contain little or no modified H3.
Uneven color development.	Insufficient washing of the wells.	Ensure the wells are washed according to the guidance of washing, and that the residual washing buffer is removed as much as possible.
	Delayed color development or delayed stopping of color development in the wells.	Ensure **DS** or **SS** is added sequentially and is consistent with the order you added the other reagents (e.g., from well A to well H 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** and **SS** 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** and **SS**, 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 µl) are completely added into the wells.
	Solutions or antibodies were not actually added into the wells.	Do not allow the pipette tip to touch the outer edges or inner sides of the wells to prevent solutions from sticking to the surface.
	SS was not evenly distributed in the wells in 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.

RELATED PRODUCTS

Histone Extract Preparation

OP-0006	EpiQuik™ Total Histone Extraction Kit
OP-0007	EpiQuik™ Total Histone Extraction HT Kit

Singleplex Histone Modification Quantification

P-3091	EpiQuik™ Circulating Total Histone H3 Quantification Kit (Colorimetric)
P-3097	EpiQuik™ Circulating Histone H3 Citrullination ELISA Kit (Colorimetric)

110 Bi County Blvd. Ste. 122, Farmingdale, NY 11735
Tel: 1-877-374-4368 ■ Fax: 1-718-484-3956 ■ E-mail: info@epigentek.com ■ Web: www.epigentek.com