Product Manual

DNMT Colorimetric Drug Discovery Kit

Catalog #: ENZ-45017
1 x 96 rxns

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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INTRODUCTION

DNA methylation occurs by a covalent addition of a methyl group at the 5-carbon of the cytosine ring, resulting in 5-methylcytosine. These methyl groups project into the major grooves of DNA and inhibit transcription. In human DNA, 5-methylcytosine is found in approximately 1.5% of genomic DNA, primarily at CpG sites. There are clusters of CpG sites at 0.3 to 2 kb stretches of DNA known as CpG islands that are typically found in or near promoter regions of genes, where transcription is initiated. In the bulk of genomic DNA, most CpG sites are heavily methylated. However, CpG islands in germ-line tissue and promoters of normal somatic cells remain unmethylated, allowing gene expression to occur. When a CpG island in the promoter region of a gene is methylated, the expression of the gene is repressed. The repression can be caused by directly inhibiting the binding of specific transcription factors, and indirectly by recruiting methyl-CpG-binding proteins and their associated repressive chromatin remodeling activity. In addition to the effect on gene transcription, DNA methylation is also involved in genomic imprinting, which refers to a parental origin specific expression of a gene, and the formation of a chromatin domain.

DNA methylation is controlled at several different levels in normal and diseased cells. The addition of methyl groups is carried out by a family of enzymes, DNA methyltransferases (DNMTs). Chromatin structure in the vicinity of gene promoters also affects DNA methylation and transcriptional activity. Three DNMTs (DNMT1, DNMT3A, and DNMT3B) are required for the establishment and maintenance of DNA methylation patterns. Two additional enzymes (DNMT2 and DNMT3L) may also have more specialized but related functions. DNMT1 appears to be responsible for the maintenance of established patterns of DNA methylation, while DNMT3A and DNMT3B seem to mediate the establishment of new or de novo DNA methylation patterns. DNMT3L is found to be a catalytically inactive regulatory factor of DNA methyltransferases, which is essential for the function of DNMT3A and DNMT3B. Diseased cells such as cancer cells may be different in that DNMT1 alone is not responsible for maintaining abnormal gene hypermethylation and both DNMT1 and DNMT3B may be cooperative for this function. The local chromatin structure also contributes to the control of DNA methylation.

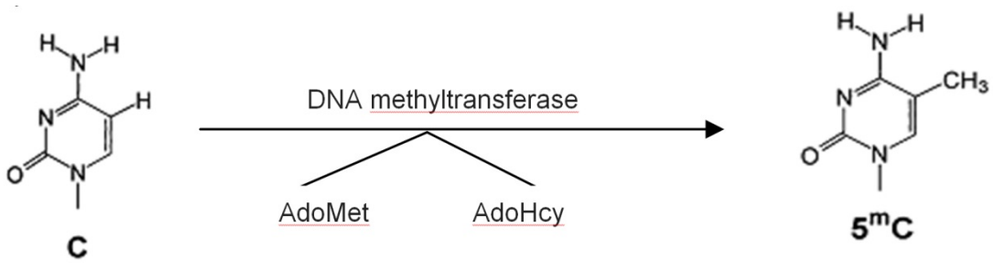


Fig 1. Methylation of cytosine in DNA via DNA methyltransferase and S-adenosylmethionine

The importance of DNA methylation is emphasized by the growing number of human diseases that are known to occur when DNA methylation information is not properly established and/or maintained. Abnormal DNA methylation associated with increased expression or the activity of DNMTs has been found in many different diseases, especially in cancer. Inhibition of DNMTs may lead to demethylation and expression of silenced genes. DNMT inhibitors are currently being developed as potential anticancer agents.

The DNMT Colorimetric Drug Discovery Kit is suitable for measuring total DNMT activity or inhibition using nuclear extracts or purified enzymes from a broad range of species such as mammalians, plants, fungi, bacteria, and viruses in a variety of forms including, but not limited to, cultured cells and fresh and frozen tissues. Nuclear extracts can be prepared by using your own successful method or using the EpiXtract™ Nuclear Protein Isolation Kit (Prod. No. ENZ-45016). Purified enzymes can be active DNMTs from recombinant proteins or isolated from cell/tissues.

Conventional DNMT activity/inhibition assay methods are time consuming, labor-intensive, have low throughput, and/or produce radioactive waste. The DNMT Colorimetric Drug Discovery Kit is a sensitive, simple 96-well plate format that has enhanced sample signals and minimized background signals.

- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be completed within 3 hours and 45 minutes.
- Safe and innovative colorimetric assay without radioactivity, extraction, and chromatography.
- The ultra-sensitive detection limit can be as low as 0.5µg of nuclear extract or 0.5ng of purified enzymes.

- Optimized antibody & enhancer solutions allow high specificity to 5-mC without cross-reactivity to unmethylated cytosine.
- 96 strip well microplate format allows for either low or high throughput analysis.

REAGENTS PROVIDED AND STORAGE

Upon receipt: (1) Store **Adomet (50X)**, **DNMT Enzyme Control**, **Detection Antibody**, and **Enhancer Solution** at –20°C away from light; (2) Store **10X Wash Buffer**, **Capture Antibody**, **Developer Solution**, and the **8-Well Assay Strips** at 4°C away from light; (3) Store all remaining components (**DNMT Assay Buffer**, **Stop Solution**, and the **Adhesive Covering Film**) at room temperature away from light.

***Note**: (1) Check if 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) transfer the amount of **Developer Solution** required into a secondary container (tube or vial) before adding **Developer Solution** into the assay wells in order to avoid contamination. Check if a blue color is present in **Developer Solution** before each use, as this would indicate contamination of the solution and should not be used.*

Reagent	Volume	Storage Temperature
10X Wash Buffer	28ml	4°C
DNMT Assay Buffer	8ml	RT
Adomet, 50X*	120µl	–20°C
DNMT Enzyme Control, 50µg/ml*	12µl	–20°C
Capture Antibody, 1000µg/ml*	10µl	4°C
Detection Antibody, 400µg/ml*	12µl	–20°C
Enhancer Solution*	12µl	–20°C
Developer Solution	10ml	4°C
Stop Solution	10ml	RT
8-Well Assay Strips (With Frame)	12	4°C
Adhesive Covering Film	1	RT

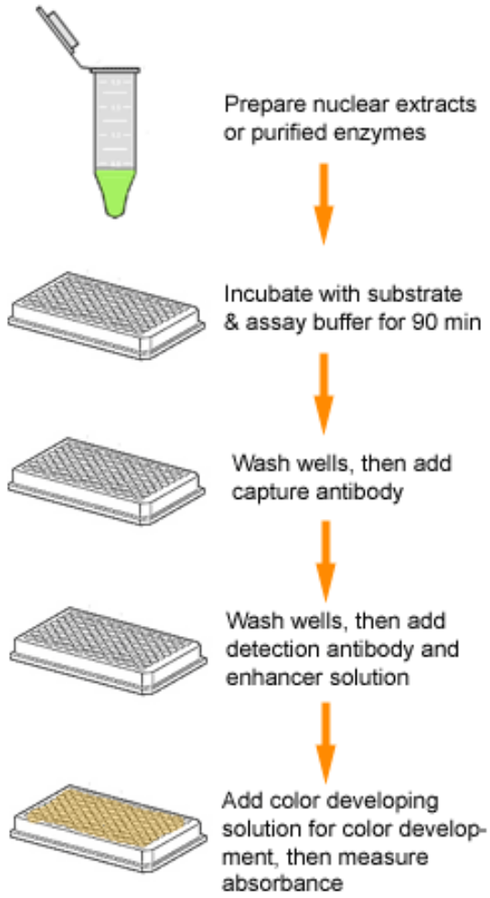
* Spin the solution down to the bottom prior to use.

ADDITIONAL MATERIALS REQUIRED

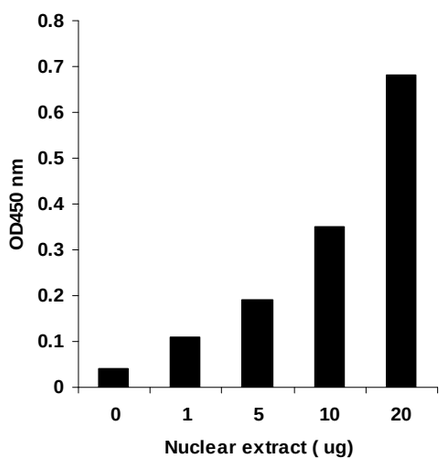
- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Aerosol resistant pipette tips
- Microplate reader capable of reading absorbance at 450 nm
- 1.5ml microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Nuclear extract or purified enzyme samples containing DNMT activity
- DNMT inhibitors (optional)
- Parafilm M or aluminium foil

PRINCIPLE

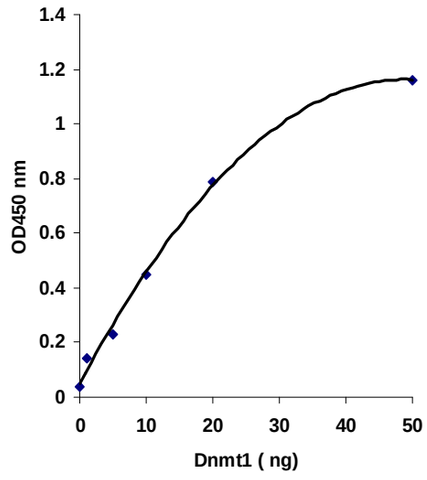
The DNMT Colorimetric Drug Discovery Kit contains all reagents necessary for the measurement of DNMT activity or inhibition. In this assay, a universal DNMT substrate is stably coated onto microplate wells. DNMT enzymes transfer methyl group to cytosine from Adomet to methylate DNA substrate and the methylated DNA can be recognized with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be measured through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The activity of DNMT enzymes is proportional to the optical density intensity measured.



Schematic procedure of the DNMT Colorimetric Drug Discovery Kit.



Demonstration of high sensitivity and specificity of the DNMT activity assay achieved by using nuclear extracts with the DNMT Colorimetric Drug Discovery Kit. Nuclear extracts were prepared from MCF-7 cells by using the EpiXtract™ Nuclear Protein Isolation Kit (Prod No. ENZ-45016).



Demonstration of high sensitivity and specificity of DNMT activity / inhibition assay achieved by using recombinant DNMT1 with the DNMT Colorimetric Drug Discovery Kit.

METHODS AND PROCEDURE

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

Starting Materials

Input Amount: The amount of nuclear extracts for each assay can be between 0.5µg and 20µg with an optimal range of 5µg to 10µg. The amount of purified enzymes can be 0.5ng to 200ng, depending on the purity and catalytic activity of the enzymes.

Nuclear Extraction: You can use your own method of choice for preparing nuclear extracts. EpiXtract™ Nuclear Protein Isolation Kit (Prod. No. ENZ-45016) is optimized for use with this kit.

Nuclear Extract or Purified DNMT Storage: Nuclear extract or purified DNMT enzymes should be stored at –80°C until use

Buffer Solution & Preparation

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

   Add 26ml of 10X Wash Buffer to 234ml of distilled water and adjust pH to 7.2-7.5.

   This **Diluted** 1X Wash Buffer can now be stored at 4°C for up to six months.

b. Prepare **Diluted Adomet** Working Buffer:

   Freshly prepare the **Diluted Adomet** Working Buffer required for the assay by adding 2µl of **Adomet, 50X** into 98µl of DNMT Assay Buffer. About 50µl of this **Diluted Adomet** will be required for each assay well.

c. Prepare Diluted **Capture Antibody** Solution:

   Dilute **Capture Antibody** with **Diluted Wash Buffer** at a ratio of 1:1000 (i.e., add 1µl of **Capture Antibody** to 1000µl of **Diluted Wash Buffer**). About 50µl of this **Diluted Capture Antibody** will be required for each assay well.

d. Prepare **Diluted Detection Antibody** Solution:

   Dilute **Detection Antibody** with **Diluted Wash Buffer** at a ratio of 1:2000 (i.e., add 1µl of **Detection Antibody** to 2000µl of **Diluted Wash Buffer**). About 50µl of this **Diluted Detection Antibody** will be required for each assay well.

e. Prepare **Diluted Enhancer Solution**:

   Dilute Enhancer Solution with **Diluted Wash Buffer** at a ratio of 1:5000 (i.e., add 1µl of **Enhancer Solution** to 5000µl of **Diluted Wash Buffer**). About 50µl of this **Diluted Enhancer Solution** will be required for each assay well.

f. About the **DNMT Enzyme Control**:

   The **DNMT Enzyme Control** is an enzyme with activity of both maintenance and *de novo* DNMTs and is used as the positive control of this assay. We do not recommend using this enzyme control to generate a standard curve for quantifying the activity of your samples, as the amount of the enzyme is limited and catalytic activity/unit is different.

   ***Note**: Keep each of the diluted solutions (except **Diluted** 1X Wash Buffer) on ice until use. Any remaining diluted solutions, other than **Diluted Wash Buffer**, should be discarded if not used within the same day.*

Enzymatic Reaction

a. Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (include blank and positive control) 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).

b. Blank Wells: Add 50µl of **Diluted Adomet** per well.

c. Positive Control Wells: Add 50µl of **Diluted Adomet** and 1µl of **DNMT Enzyme Control** per well.

d. Sample Wells Without Inhibitor: Add 45µl to 49µl of **Diluted Adomet**, and 1µl to 5µl of nuclear extracts or 1 to 5µl of purified DNMT enzymes per well. The total volume should be 50µl per well.

e. Sample Wells With Inhibitor: Add 40µl to 44µl of **Diluted Adomet**, 1 to 5µl of nuclear extracts or 1 to 5µl of purified DNMT enzymes, and 5µl of inhibitor solution per well. The total volume should be 50µl per well.

***Note**: (1) Follow suggested well setup diagrams; (2) It is recommended to use 5µg to 10µg of nuclear extract per well or 10ng to 100ng of purified enzyme per well; (3) The concentration of inhibitors to be added into the sample wells can be varied (e.g., 1µM to 1000µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with **DNMT Assay Buffer** at a 1:10 ratio (e.g., add 0.5µl of inhibitor to 4.5µl of **DNMT Assay Buffer**), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.*

f. Tightly cover the strip-well microplate with **Adhesive Covering Film** to avoid evaporation, and incubate at 37°C for 90-120 min.

***Note**: (1) The incubation time may depend on intrinsic DNMT activity. In general, 90 min incubation is suitable for active purified DNMT enzymes and 120 min incubation is required for nuclear extracts; (2) The **Adhesive Covering Film** can be cut to the required size to cover the strips based on the number of strips to be used.*

g. Remove the reaction solution from each well. Wash each well three times with 150µl of **Diluted** 1X Wash Buffer each time. This can be done by simply pipetting **Diluted Wash Buffer** in and out of the wells.

Antibody Binding & Signal Enhancing

a. Add 50µl of the **Diluted Capture Antibody** to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.

b. Remove the **Diluted Capture Antibody** solution from each well.

c. Wash each well with 150µl of the **Diluted Wash Buffer** each time for three times.

d. Add 50µl of the **Diluted Detection Antibody** to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

e. Remove the **Diluted Detection Antibody** solution from each well.

f. Wash each well with 150µl of the **Diluted Wash Buffer** each time for four times.

g. Add 50µl of the **Diluted Enhancer Solution** to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

h. Remove the **Diluted Enhancer Solution** from each well.

i. Wash each well with 150µl of the **Diluted Wash Buffer** each time for five times.

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

Signal Detection

a. Add 100µl of **Developer Solution** to each well and incubate at room temperature for 1 to 10 min away from direct light. Monitor color change in the sample wells and control wells. The **Developer Solution** will turn blue in the presence of sufficient methylated DNA.

b. Add 100µl of **Stop Solution** to each well to stop enzyme reaction when the color in the positive control wells turns medium blue. Mix the solution by gently shaking the frame and wait 1-2 min to allow the color reaction to be completely stopped. The color will change to yellow after adding **Stop Solution** and 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 the 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 stripwell microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

DNMT Activity Calculation

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

b. Calculate DNMT activity using the following formula:

$$DNMT\ Activity\ (OD/h/mg) = \frac{(Sample\ OD\ -\ Blank\ OD)}{(Protein\ amount\ (\mu g)^{*} \times hour^{**})} \times 1000$$

* Protein amount added into the reaction at step 2d inµg.

** Incubation time at step 2f.

Example calculation:

Average OD450 of sample is 0.55

Average OD450 of blank is 0.05

Protein amount is 5µg

Incubation time is 2 hours (120 min)

$$\text{DNMT activity} = \frac{(0.55 - 0.05)}{(5 \times 2)} \times 1000 = 50\ \text{OD/h/mg}$$

c. Calculate DNMT inhibition using the following formula:

$$DNMT\ Inhibition\ \% = \left[1 - \frac{Inhibitor\ Sample\ OD - Blank\ OD}{No\ Inhibitor\ Sample\ OD - Blank\ OD}\right] \times 100\%$$

SUGGESTED WORKING BUFFER AND SOLUTION SETUP

Table 1. 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 Wash Buffer	2.5ml	20ml	40ml	120ml	240ml
Diluted Adomet	50µl	400µl	800µl	2400µl	4800µl
Diluted Capture Antibody	50µl	400µl	800µl	2400µl	4800µl
Diluted Detection Antibody	50µl	400µl	800µl	2400µl	4800µl
Diluted Enhancer Solution	50µl	400µl	800µl	2400µl	4800µl
Developer Solution	0.1ml	0.8ml	1.6ml	4.8ml	9.6ml
Stop Solution	0.1ml	0.8ml	1.6ml	4.8ml	9.6ml
DNMT Enzyme Control	N/A	0.25µl – 1µl	0.5µl – 2µl	1µl – 4µl	2µl – 8µl

SUGGESTED STRIPWELL SETUP

Table 2. The suggested strip-well plate setup for the DNMT activity assay, using 6 strips. Strips 7 to 12 can be configured as samples. The controls and samples can be measured in duplicates.

Well#	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	DNMT Enzyme Control 0.5µl	DNMT Enzyme Control 0.5µl	Sample	Sample	Sample	Sample
C	DNMT Enzyme Control 1µl	DNMT Enzyme Control 1.0µl	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

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal in both the positive 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.
	The well is incorrectly washed before enzyme reaction.	Ensure the well is not washed prior to adding the standard control and sample.
	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 filter) 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 DNMT enzyme control is insufficiently added to the well in Step 2c.	Ensure a sufficient amount of DNMT enzyme control is added.
	The quality of the DNMT enzyme control has been degraded due to improper storage conditions.	Follow the Shipping & Storage guidance for storage instructions of **DNMT Enzyme Control**.
High background present in the blank wells	Insufficient washing of wells.	Check if washing recommendations at each step is 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 contaminated tips.
	Incubation time with detection antibody is too long.	The incubation time at Step 3d should not exceed 45 min.
	Over development of color.	Decrease the development time in Step 4a before adding **Stop Solution** in Step 4b.

Problem	Possible Cause	Suggestion
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for DNMT protein extraction. For the best results, it is advised to use EpiXtract™ Nuclear Protein Isolation Kit (Prod. No. ENZ-45016). Also, use fresh cell or tissues for protein extraction, as frozen cells or tissues could lose enzyme activity.
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of purified enzymes or nuclear extracts is used as indicated in step 2. The sample can be titrated to determine the optimal amount to use in the assay.
	Sample was not stored properly or has been stored for too long.	Ensure sample is stored in aliquots at –80ºC, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing.
	Little or no activity of DNMT contained in the sample.	This problem may be a result of many factors. If the affecting factors cannot be determined, use new or re-prepared nuclear extracts or purified enzymes.
Uneven color development	Insufficient washing of the wells.	Ensure the wells are washed according to the protocol. Ensure any residues from the wash buffer are removed as much as possible.
	Delayed color development or delayed stopping of color development in the wells.	Ensure color development solution is added sequentially and consistent with the order you added the other reagents (e.g., from well A to well G or from well 1 to well 12).

Problem	Possible Cause	Suggestion
Large variation between replicate wells	Color reaction is not evenly stopped due to an inconsistency in pipetting time.	Ensure **Developer Solution** and **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 **Developer Solution** and **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 evenly after adding **Stop Solution** 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.
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.

NOTES

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