JMJD2 Demethylase Activity/Inhibition Assay Kit (Colorimetric)

Catalog Number KA1526

48 assays

Version: 01

Intended for research use only

www.abnova.com

Introduction and Background

A. Product Use Information

Uses

The JMJD2 Demethylase Activity/Inhibition Assay Kit (Colorimetric) is suitable for measuring activity or inhibition of total JMJD2 using nuclear extracts or JMJD2 subtypes (JMJD2A-JMJD2F) using purified enzymes from a broad range of species such as mammals, plants, fungi, and bacteria, in a variety of forms including cultured cells and fresh tissues. Nuclear extracts can be prepared by using your own successful method.

Input Material

Input materials can be nuclear extracts or purified JMJD2 enzymes. The amount of nuclear extracts for each assay can be 1 ug to 20 ug with an optimal range of 5 to 10 ug. The amount of purified enzymes can be 10 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.

Internal Control

The JMJD2 assay standard (demethylated hsitone H3-K9) is provided in this kit for the quantification of JMJD2 enzyme activity. Because JMJD2 activity 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.

Precautions

To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosolbarrier 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.

B. General Product Information

Quality Control

Each lot of the JMJD2 Demethylase Activity/Inhibition Assay Kit (Colorimetric) is tested against predetermined specifications to ensure consistent product quality.

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

Abnova 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.

Usage Limitation

The JMJD2 Demethylase Activity/Inhibition Assay Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application.

C. Overview

Lysine histone methylation is one of the most robust epigenetic marks, and is essential for the regulation of multiple cellular processes. The methylation of H3-K9 seems to be of particular significance, as it is associated with repression regions of the genome. H3-K9 methylation was considered irreversible until the identification of a large number of histone demethylases indicating that demethylation events play an important role in histone modification dynamics. So far at least 2 classes of H3-K9 specific histone demethylase, JMJD1(JHDM2), and JMJD2 (JHDM3) have been identified. The JMJD1 family, including JMJD1A, JMJD1B, and JMJD1C can remove di- and mono-methylation from H3-K9 while the JMJD2 family, including JMJD2A, JMJD2B, JMJD2C, and JMJD2D, JMJD2E, and JMJD2F can remove tri-methylation from H3-K9 and H3-K36. JMJD2 demethylases are Jumonji domain proteins and catalyze the removal of methylation by using a hydroxylation reaction with iron and a-ketoglutarate required as cofactors.

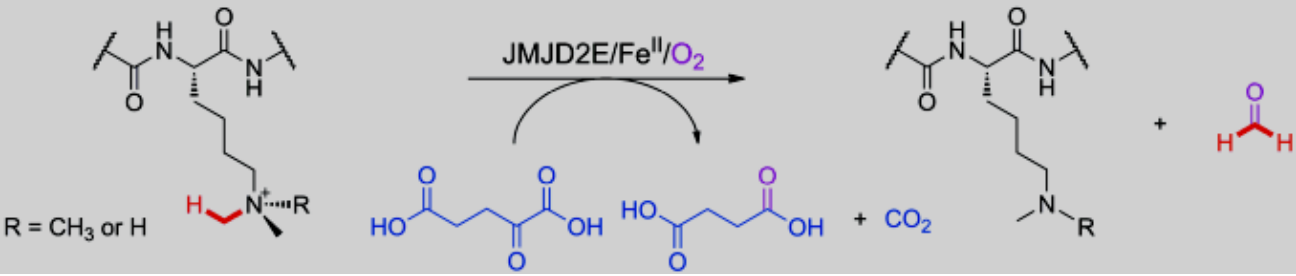


Fig 1. Histone H3-K9 demethylation reaction catalyzed by JMJD2 demethylase. (Hopkinson et al: Chembiochem, 4: 506-510, 2010)

JMJD2 demethylases are found to have potential oncogenic functions. For example, JMJD2A is amplified in prostate cancer and JMJD2C overexpression is observed in oesophageal carcinoma. Detection of activity and inhibition of JMJD2 would be important in elucidating mechanisms of epigenetic regulation of gene activation and silencing, as well as benefiting cancer diagnostics and therapeutics. There are only a couple of methods used for detecting JMJD2 activity and inhibition. These methods are based on the measurement of formaldehyde release, a by-product of JMJD2 enzymatic reaction, and have significant weaknesses: (1) Large amounts (at :g level) of substrate and enzyme are required; (2) Nuclear extracts from cell/tissues cannot be used; (3) Redox-sensitive JMJD2 inhibitiors are not suitable for testing with these methods; (4) High interference by SDS, DMSO, thiol-containing chemicals, and ions, which are often contained in enzyme solutions, tested compound solvents, and assay buffers; and (5) Less accuracy than a direct measurement of JMJD2-converted demethylated product. The JMJD2 Demethylase Activity/Inhibition Assay Kit (Colorimetric) is designed to address these issues. Compared to a formaldehyde release-based method, this kit has the following advantages:

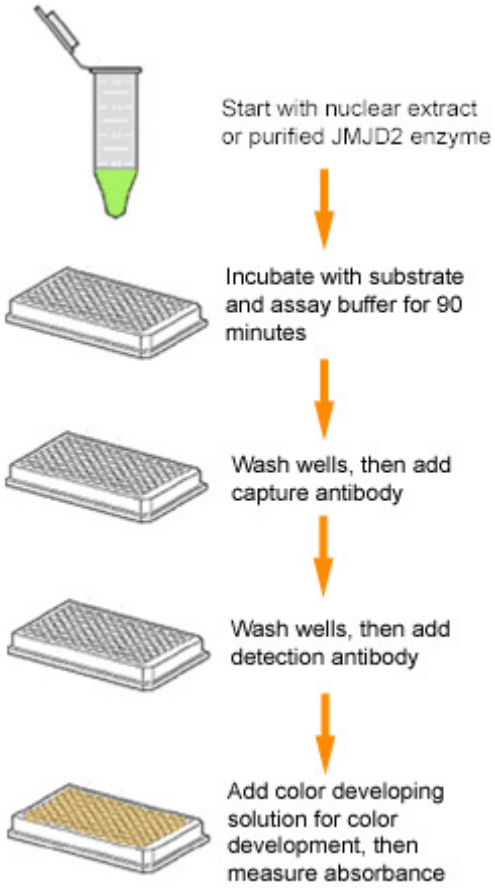
- 3 hour colorimetric procedure in a 96 stripwell microplate format allows for either manual or high throughput analysis.
- Directly measures JMJD2 activity via a straightforward detection of JMJD2-converted demethylated products, rather than byproducts, thus eliminating assay interference caused by thiolcontaining chemicals such as DTT, GSH and 2-mercaptoethanol, or caused by detergents/ions such as tween-20, SDS, triton X-100, Fe, and Na.
- Both cell/tissue extracts and purified JMJD2 can be used, which allows for the detection of inhibitory

effects of JMJD2 inhibitors in vivo and in vitro.

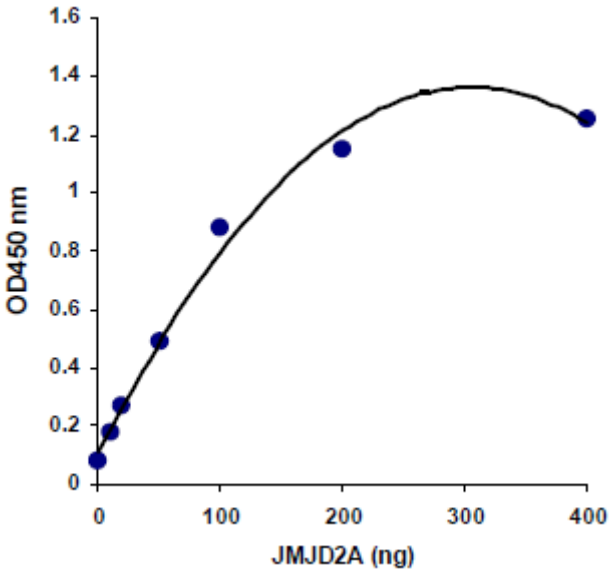
- Sensitivity is up to 1,000 times higher than formaldehyde release-based JMJD2 assays, allowing activity to be colorimetrically detected from as low as 10 ng of purified JMJD2 enzyme.
- Demethylated H3-K9 standard is included, allowing specific activity of JMJD2 to be quantified.
- Accurate, reliable, and consistent with extremely low background signals.

D. Test Principle

In this assay, a tri-methylated histone H3-K9 substrate is stably coated onto microplate wells. Active JMJD2s bind to the substrate and remove methyl groups from the substrate. The JMJD2-demethylated products can be recognized with a specific antibody. The ratio or amount of demethylated products, which is proportional to enzyme activity, can then be colorimetrically measured by reading the absorbance in a microplate spectrophometer at a wavelength of 450 nm. The activity of the JMJD2 enzyme is in turn proportional to the optical density intensity measured.



Schematic procedure of JMJD2 Demethylase Activity/Inhibition Assay Kit (Colorimetric)



Demonstration of high sensitivity of the JMJD2 activity assay achieved by using recombinant JMJD2

Material and Method

A. List of component

Component	**48 assays**	**Storage Upon Receipt**
JA1 (10X Wash Buffer)	14 ml	4℃
JA2 (JMJD2 Assay Buffer)	4 ml	RT
JA3 (JMJD2 Substrate, 50 ug/ml)*	60 ul	-20℃
JA4 (JMJD2 Assay Standard, 50 ug/ml)*	10 ul	-20℃
JA5 (Capture Antibody, 1000 ug/ml*)	5 ul	4℃
JA6 (Detection Antibody, 400 ug/ml)*	6 ul	-20℃
JA7 (Developer Solution)	5 ml	4℃
JA8 (Stop Solution)	5 ml	RT
Co-factor 1*	30 ul	4℃
Co-factor 2*	30 ul	4℃
Co-factor 3*	30 ul	4℃
8-Well Assay Strips (With Frame)	6	4℃
Adhesive Covering Film	1	RT

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

B. Additional Required Materials But Not Provided

1. Adjustable pipette or multiple-channel pipette
2. Multiple-channel pipette reservoirs
3. Aerosol resistant pipette tips
4. Microplate reader capable of reading absorbance at 450 nm

5. 1.5 ml microcentrifuge tubes
6. Incubator for 37℃ incubation
7. Distilled water
8. Nuclear extract or purified enzymes
9. Parafilm M or aluminum foil

C. Stability and storage

The kit is shipped in three parts: the first part at ambient room temperature and the second and third part on frozen ice packs at 4℃. Upon receipt: (1) Store **JA3**, **JA4** and **JA6** at –20℃ away from light; (2) Store **JA1**, **JA5**, **JA7**, **Co-factor 1**, **Co-factor 2**, **Co-factor 3**, and **8-Well Assay Strips** at 4℃ away from light; and (3) Store remaining components (**JA2**, **JA8**, and **Adhesive Covering Film**) at room temperature away from light. *Note: (1) Check if **JA1** (10X Wash Buffer) contains salt precipitates before use. If so, warm (at room temperature or 37℃) and shake the buffer until the salts are re-dissolved; and (2) check if a blue color present in **JA7** (Developer Solution), which would indicate contamination of the solution and should not be used. To avoid contamination, transfer the amount of **JA7** required into a secondary container (tube or vial) before adding **JA7** into the assay wells.*

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

D. Protocol

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 1 ug to 20 ug with optimized range of 5-10 ug. The amount of purified enzymes can be 5 ng to 500 ng, depending on the purity and catalatic activity of the enzymes.

Nuclear Extraction: You can use your method of choice for preparing nuclear extracts.

Nuclear Extract or Purified JMJD2 Storage: Nuclear extract or purified JMJD2 enzyme should be stored in aliquots at -80℃ until use.

1. **Working Buffer and Solution Preparation**
   a. Prepare **Diluted JA1** 1X Wash Buffer: 48-Assay Kit: Add 13 ml of **JA1** 10X Wash Buffer to 117 ml of distilled water and adjust pH to 7.2-7.5. This **Diluted JA1** 1X Wash Buffer can now be stored at 4℃ for up to six months.
   b. Prepare **CJA2** Completed Assay Buffer: Add **Co-factor 1**, **Co-factor 2**, and **Co-factor 3** to **JA2** Assay Buffer at a ratio of 1:100 for each Cofactor (i.e., add 1 ul of each Co-factor to 100 ul of **JA2** for a total of 103 ul).
   c. Prepare **Diluted JA5** Capture Antibody Solution: Dilute **JA5** Capture Antibody with **Diluted JA1** 1X Wash Buffer at a ratio of 1:1000 (i.e., add 1 ul of capture antibody to 1000 ul of 1X Wash Buffer). 50 ul of **Diluted JA5** will be required for each assay well.

d. Prepare **Diluted JA6** Detection Antibody Solution: Dilute **JA6** Detection Antibody with **Diluted JA1** 1X Wash Buffer at a ratio of 1:2000 (i.e., add 1 ul of **JA6** Detection Antibody to 2000 ul of **Diluted JA1** 1X Wash Buffer). 50 ul of **Diluted JA6** will be required for each assay well.

e. Prepare **Diluted JA4** Standard Solution: Suggested Standard Curve Preparation: First, dilute **JA4** Assay Standard with **JA2** Assay Buffer to 5 ng/ul by adding 1 ul of **JA4** to 9 ul of **JA2**. Then, further prepare five concentrations by combining the 5 ng/ul **Diluted JA4** with **JA2** into final concentrations of 0.2, 0.5, 1.0, 2.0, and 5.0 ng/ul according to the following dilution chart:

Tube	**JA4 (5 ng/ul)**	**JA2**	**Resulting JA4 Concentration**
1	1.0 ul	24.0 ul	0.2 ng/ul
2	1.0 ul	9.0 ul	0.5 ng/ul
3	1.0 ul	4.0 ul	1.0 ng/ul
4	2.0 ul	3.0 ul	2.0 ng/ul
5	4.0 ul	0.0 ul	5.0 ng/ul

*Note: Keep each of diluted solutions except **Diluted JA1** 1X Wash Buffer on ice until use. Any remaining diluted solutions other than **Diluted JA1** should be discarded if not used within the same day.*

2. **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℃).

b. Blank Wells: Add 49 ul of **CJA2** and 1 ul of **JA3** to each blank well.

c. Standard Wells: For a standard curve, add 49 ul of **CJA2** and 1 ul of **Diluted JA4** to each standard well with a minimum of five wells, each at a different concentration between 0.2 to 5 ng/ul (based on the dilution chart in Step 1e)

d. Sample Wells Without Inhibitor: Add 45 to 48 ul of **CJA2**, 1 ul of **JA3**, and 1 to 4 ul of your nuclear extracts or 1 to 4 ul of your purified JMJD2 enzyme. Total volume should be 50 ul per well.

e. Sample Well With Inhibitor: Add 40 to 43 ul of **CJA2**, 1 ul of **JA3**, 1 to 4 ul of your nuclear extracts or 1 to 4 ul of your purified JMJD2 enzyme, and 5 ul of inhibitor solution. Total volume should be 50 ul per well.

*Note: (1) Follow the suggested well setup diagrams; (2) It is recommended to use 2 ug to 10 ug of nuclear extract per well or 20 ng to 100 ng of purified enzyme per well; (3) The concentration of inhibitors to be added into the sample wells can be varied (e.g., 1 uM to 1000 uM). However, the final concentration of the inhibitors before adding to the wells should be prepared with **JA2** at a 1:10 ratio (e.g., add 0.5 ul of inhibitor to 4.5 ul of **JA2**), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less. The Jumonji demethylase general inhibitor, N-Oxalylglycine can be used as a control inhibitor.*

f. Tightly cover strip-well microplate with **Adhesive Covering Film** to avoid evaporation and incubate at 37℃ for 60-120 min.

*Note: (1) The incubation time may depend on intrinsic JMJD2 activity. However, in general, 60-90 min incubation is suitable for active purified JMJD2 enzymes and 90-120 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 with 150 ul of the **Diluted JA1** 1X Wash Buffer each time.

3. **Antibody Binding and Signal Enhancing**

a. Add 50 ul of the **Diluted JA5** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.

b. Remove the **Diluted JA5** solution from each well.

c. Wash each well with 150 ul of the **Diluted JA1** each time.

d. Add 50 ul of the **Diluted JA6** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

e. Remove the **Diluted JA6** solution from each well.

f. Wash each well with 150 ul of the **Diluted JA1** 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 ul of **JA7** 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 **JA7** solution will turn blue in the presence of sufficient demethylated products.

b. Add 100 ul of **JA8** 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 **JA8** 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. **JMJD2 Activity Calculation**

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

b. Calculate JMJD2 activity or inhibition using the following formulas:

For simple calculation: $\text{JMJD2 Activity (OD/min/mg)} = \frac{(\text{Sample OD} - \text{Blank OD})}{(\text{Protein Amount (ug)} * \text{ x min} * *)} \times 1000$

* Protein amount (ug) added into the reaction at step 2d.

** Incubation time (minutes) at step 2f.

Example calculation:

Average OD450 of sample is 0.65

Average OD450 of blank is 0.05

Protein amount is 5 ug

Incubation time is 120 minutes (2 hours)

$$\text{JMJD2 activity} = \frac{(0.65 - 0.05)}{(5 \times 120)} \times 1000 = 1\,\text{OD} / \text{min} / \text{mg}$$

For accurate or specific activity calculation:

1. Generate a standard curve and plot OD value versus amount of **JA4** 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 JMJD2-converted demethylated product using the following formulas:

$$\text{Demethylated product (ng)} = \frac{(\text{Sample OD} - \text{Blank OD})}{\text{Slope}}$$

$$\text{JMJD2 Activity (ng/min/mg)} = \frac{\text{Demethylated Product (ng)}}{(\text{Protein Amount (ug)} \times \text{min*})} \times 1000$$

* Incubation time (minutes) at Step 2f.

For inhibition calculation:

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

E. 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**	**1 strip (8 wells)**	**2 strips (16 wells)**	**6 strips (48 wells)**
Diluted JA1	2.5 ml	20 ml	40 ml	120 ml
CJA2	50 ul	400 ul	800 ul	2400 ul
JA3	1 ul	8 ul	16 ul	50 ul
JA4	NA	NA	1 ul (optional)	2 ul
Diluted JA5	50 ul	400 ul	800 ul	2400 ul
Diluted JA6	50 ul	400 ul	800 ul	2400 ul
Developer Solution	0.1 ml	0.8 ml	1.6 ml	4.8 ml
Stop Solution	0.1 ml	0.8 ml	1.6 ml	4.8 ml

F. **Suggested Strip Well Setup**

Table 2. The suggested strip-well plate setup for JMJD2 activity assay in a 48-assay format. The controls and samples can be measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	JA4 0.2 ng	JA4 0.2 ng	Sample	Sample	Sample	Sample
C	JA4 0.5 ng	JA4 0.5 ng	Sample	Sample	Sample	Sample
D	JA4 1.0 ng	JA4 1.0 ng	Sample	Sample	Sample	Sample
E	JA4 2.0 ng	JA4 2.0 ng	Sample	Sample	Sample	Sample
F	JA4 5.0 ng	JA4 5.0 ng	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

Appendix

Troubleshooting

Problem	Possible Cause	Suggestion
No Signal for the No Inhibitor Control	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 enzyme reaction.	Ensure the well is not washed prior to adding the positive 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) 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 standard curve wells	The standard amount is insufficiently added to the well in Step 2c.	Ensure a sufficient amount of standard is added.
	The standard is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance of this User Guide for storage of **JA4** (JMJD2 Assay Standard).
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 standard.	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips.
	Incubation time with **Diluted JA6** is too long.	The incubation time at Step 3d should not exceed 2 hours.
	Over-development of color.	Decrease the development time in Step 4a before adding **JA8** Stop Solution in Step 4b.
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for JMJD2 protein extraction. Also, use fresh cells 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 -80℃, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing.
	Little or no activity of JMJD2 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 guidance of washing and residue washing buffer is removed as much as possible.
	Delayed color development or delayed stopping of color development in the wells.	Ensure color development solution or stop solution is added sequentially and is consistent with the order you added the other reagents (e.g., from well A to well G or from well 1 to well 12).