ab156913 – TET Hydroxylase Activity Quantification Kit (Fluorometric)

Instructions for Use

For the measurement of total 5mC hydroxylase TET enzyme activity/inhibition using nuclear extracts or purified TET isoforms (TET 1-3) in various samples

This product is for research use only and is not intended for diagnostic use.

Version 1 Last Updated 1 February 2019

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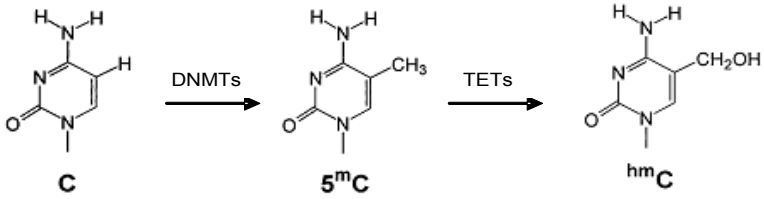
1. BACKGROUND

DNA methylation occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNA methyltransferases, resulting in 5-methylcytosine (5mC). In somatic cells, 5mC is found almost exclusively in the context of paired symmetrical methylation of the dinucleotide CpG, whereas in embryonic stem (ES) cells, a substantial amount of 5mC is also observed in non-CpG contexts. The biological importance of 5mC as a major epigenetic modification in phenotype and gene expression has been recognized widely.

5-hydroxymethylcytosine (5hmC), as a sixth DNA base with functions in transcription regulation, has been detected to be abundant in human and mouse brain and embryonic stem (ES) cells. In mammals, it can be generated by oxidation of 5mC, a reaction mediated by the ten-eleven translocation (TET) family of 5mC-hydroxylases.



Unmethylated DNA	Methylated DNA	Hydroxymethylated DNA
T-C-G-T-C-G-A-C-G	T-^{m}C-G-T-^{m}C-G-A-^{m}C-G	T-^{hm}C-G-T-^{hm}C-G-A-^{hm}C-G

The TET family of 5mC hydroxylases includes TET1, TET2 and TET3. These TET proteins may promote DNA demethylation by binding to CpG-rich regions to prevent unwanted DNA methyltransferase activity, and by converting 5mC to 5hmC and further to 5-carboxylcytosine (5-caC) through hydroxylase activity. It was shown that genomic 5hmC level correlates to TET hydroxylase activity. In addition, TET1 was shown to have dual functions in transcription activation and repression by binding different target genes in ES cells. TET1 is also a fusion partner of the MLL gene in acute

myeloid leukemia and is considered an oncoprotein. TET2 is found to be frequently mutated in leukemia and considered to act as tumor suppressor. TET3 has been demonstrated to play a unique role for DNA methylation reprogramming processes in the mammalian zygote. Thus, activating tumor suppressor TET enzymes such as TET2 or inhibiting oncoprotein TET enzymes such as TET1 would be important in benefiting cancer diagnostics and developing new target-based cancer therapeutics. However there are few methods available for detecting TET hydroxylase activity/inhibition using both nuclear extracts and purified enzymes. To address this issue, Abcam offers the TET Hydroxylase Activity Quantification Kit (Fluorometric).

The kit has the following advantages and features:

- 5 hour fluorometric assay with easy-to-follow steps for convenience and speed. The entire procedure can be finished within 5 hours
- Directly measures TET hydroxylase activity via a straightforward detection of TET-converted hydroxymethylated products
- Innovative kit composition enables background signals to be extremely low and allows the assay to be simple, accurate, reliable, and consistent
- Both cell/tissue extracts and purified TET proteins can be used, which allows detection of inhibitory effects of TET hydroxylase inhibitor in vivo and in vitro
- Novel assay principle allows high sensitivity to be achieved. The activity can be detected from as low as 10 ng of purified TET1 hydroxylase
- A hydroxymethylated standard is included, which allows the specific activity of TET hydroxylases to be quantified.
- Strip microplate format makes the assay flexible: manual or high throughput analysis (96 assays)

Abcam's TET Hydroxylase Activity Quantification Kit (Fluorometric) is suitable for measuring the activity/inhibition of total 5mC hydroxylase TET enzyme using nuclear extracts or purified TET isoforms (TET 1-3) from a

broad range of species such as mammalian, plant, fungal, and bacterial, in a variety of forms including, but not limited to cultured cells, fresh and frozen tissues.

In this assay, a methylated substrate is stably coated onto microplate wells. Active TETs bind to the substrate and convert methylated substrate to hydroxymethylated products. The TET-converted hydroxymethylated products can be recognized with a specific antibody. The ratio or amount of hydroxymethylated products, which is proportional to enzyme activity, can then be fluorometrically measured by reading the fluorescence in a fluorescent microplate reader at 530 excitation and 590 emission. The activity of the TET enzyme is in turn proportional to the relative fluorescent units measured.

2. ASSAY SUMMARY

Start with nuclear extract or pirified TET enzyme

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Incubate with substrate and assay buffer

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Wash wells, then add capture antibody

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Wash wells, and then add detection antibody and enhancer solution

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Add fluoro developing solution for fluorescence development and measure RFU

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table and away from light upon receipt.

Observe the storage conditions for individual prepared components in sections 9, 10 & 11.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

Check if the 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.

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	14 mL	28 mL	4°C
TET Assay Buffer	3 mL	6 mL	RT
10X TET Substrate	10 µL	20 µL	-20°C
Binding Solution	5 mL	10 mL	RT
TET Assay Standard, 20 µg/mL	10 µL	20 µL	-20°C
Capture Antibody, 1000 µg/mL	4 µL	8 µL	4°C
Detection Antibody, 400 µg/mL	8 µL	16 µL	-20°C
Enhancer Solution	8 µL	16 µL	-20°C
Fluoro Developer	10 µL	20 µL	-20°C
Fluoro Enhancer	10 µL	20 µL	4°C
Dilution Buffer	4 mL	8 mL	RT
Co-factor 1	25 µL	50 µL	4°C
Co-factor 2	25 µL	50 µL	4°C
Co-factor 3	25 µL	50 µL	4°C
8-Well Assay Strips (With Frame)	6	12	4°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Aerosol resistant pipette tips
- Fluorescent microplate reader capable of reading fluorescence at 530ex/590em nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Nuclear extract or purified enzymes
- Parafilm M or aluminum foil

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Prepare fresh reagents immediately prior to use.

9.1 1X Wash Buffer

Add the volume specified in the table below of 10X Wash Buffer to distilled water and adjust to pH 7.2-7.5.

	Volume to Dilute (mL)	Volume distilled water (mL)	Total Volume (mL)
48 Tests	13	117	130
96 Tests	26	234	260

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

9.2 Final TET Assay Buffer

Add Co-Factor 1, Co-Factor 2, and Co-Factor 3 to TET Assay Buffer at a ratio of 1:100 (e.g. add 1 µL of each Co-Factor to 100 µL of TET Assay Buffer for a total of 103 µL). About 50 µL of Final TET Assay Buffer will be required for each assay well.

9.3 0.5X TET Substrate

Add 1 µL of 10X TET Substrate to 19 µL of TET Assay Buffer. About 2 µL of 0.5X TET Substrate will be required for each assay well.

9.4 Diluted Capture Antibody

Dilute the Capture Antibody with 1X Wash Buffer at a ratio of 1:1000 (e.g. add 1 µL of Capture Antibody to 1000 µL of 1X Wash Buffer). About 50 µL of Diluted Capture Antibody will be required for each assay well.

9.5 Diluted Detection Antibody

Dilute the Detection Antibody with 1X Wash Buffer at a ratio of 1:2000 (e.g. add 1 µL of Detection Antibody to 2000 µL of 1X Wash Buffer). About 50 µL of Diluted Detection Antibody will be required for each assay well.

9.6 **Diluted Enhancer Solution**

Dilute the Enhancer Solution with 1X Wash Buffer at a ratio of 1:5000 (e.g. add 1 µL of Enhancer Solution to 5000 µL of 1X Wash Buffer). About 50 µL of Diluted Enhancer Solution will be required for each assay well.

9.7 **Fluorescence Development Solution**

Add 1 µL of Fluoro Developer and 1 µL of Fluoro Enhancer to every 500 µL of Dilution Buffer. About 50 µL of the Fluorescence Development Solution will be required for each well to be developed.

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

10. SAMPLE PREPARATION

Input Amount: The amount of nuclear extracts for each assay can be 2-20 µg with an optimal range of 5-10 µg. The amount of purified enzymes can be 10-500 ng, depending on the purity and catalytic activity of the enzymes.

Nuclear Extraction: You can use your method of choice for preparing nuclear extracts. Abcam offers a Nuclear Extraction Kit (ab113474) optimized for use with this kit. Nuclear extract or purified TET enzyme should be stored in aliquots at -80°C until use.

11. STANDARD PREPARATION

Suggested Standard Curve Preparation:

11.1 First, dilute TET Assay Standard with Final TET Assay Buffer to 2 ng/µL by adding 1 µL of TET Assay Standard to 9 µL of Final TET Assay Buffer.

11.2 Then, further prepare five concentrations by combining the 2 ng/µL Diluted TET Assay Standard with Final TET Assay Buffer into final concentrations of 0.05, 0.2, 0.5, 1.0, and 2.0 ng/µL according to the following dilution chart:

Tube	TET Assay Standard (2 ng/µL) (µL)	TET Assay Buffer (µL)	Resulting TET Assay Standard Concentration (ng/µL)
1	1.0	39.0	0.05
2	1.0	9.0	0.20
3	1.0	3.0	0.50
4	2.0	2.0	1.00
5	4.0	0.0	2.00

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

12. PLATE PREPARATION

The suggested strip-well plate setup for standard curve preparation in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	TET Assay Standard 0.05 ng/well	TET Assay Standard 0.05 ng/well	Sample	Sample	Sample	Sample
C	TET Assay Standard 0.20 ng/well	TET Assay Standard 0.20 ng/well	Sample	Sample	Sample	Sample
D	TET Assay Standard 0.50 ng/well	TET Assay Standard 0.50 ng/well	Sample	Sample	Sample	Sample
E	TET Assay Standard 1.00 ng/well	TET Assay Standard 1.00 ng/well	Sample	Sample	Sample	Sample
F	TET Assay Standard 2.00 ng/well	TET Assay Standard 2.00 ng/well	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

13. ASSAY PROCEDURE

- Internal Control: The TET assay standard (5-hydroxymethylcytosine) is provided in this kit for the quantification of TET enzyme activity. Because TET 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

13.1 Enzymatic Reaction

13.1.1 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).

13.1.2 Add 80 µL of Binding Solution to each well.

13.1.3 Add 2 µL of 0.5X TET Substrate into each blank well and each sample well. Add 1 µL of Diluted TET Assay Standard into the standard curve wells (see the designated wells depicted in Section 12 – Plate Preparation). Mix solution by gently tilting from side to side or shaking the plate several times. Ensure the solution coats the bottom of the well evenly.

Note: *For the standard curve, add 1 µL of Diluted TET Assay Standard at concentrations of 0.05-2 ng/µL (see Section 11 - Standard Preparation). The final concentrations should be 0.05, 0.2, 0.5, 1, and 2 ng per well.*

13.1.4 Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 90 minutes.

13.1.5 Remove the Binding Solution from each well.

13.1.6 Wash each well three times with 150 µL of 1X Wash Buffer each time.

13.1.7 Blank Wells: Add 50 µL of Final TET Assay Buffer to each blank well.

13.1.8 Standard Wells: Add 50 µL of Final TET Assay Buffer to each standard well.

13.1.9 Sample Wells without Inhibitor: Add 46-49 µL of Final TET Assay Buffer and 1-4 µL of nuclear extracts or purified TET enzyme to each sample well without inhibitor. Total volume should be 50 µL per well.

13.1.10 Sample Wells with Inhibitor: Add 41-44 µL of Final TET Assay Buffer, 1-4 µL of nuclear extracts or purified TET enzyme, and 5 µL of inhibitor solution. Total volume should be 50 µL per well.

Note: *(1) Follow the suggested well setup in Section 12 – Plate Preparation (2) It is recommended to use 5-10 µg of nuclear extract per well or 50-500 ng of purified enzyme per well; (3) The concentration of inhibitor to be added into the sample wells can be varied (1 µM to 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with the Final TET Assay Buffer at a 1:10 ratio (e.g. add 0.5 µL of inhibitor to 4.5 µL of the Final TET Assay Buffer) so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.*

13.1.11 Tightly cover strip plate with Parafilm M to avoid evaporation and incubate at 37°C for 60-90 minutes.

Note: *(1) The incubation time may depend on intrinsic TET activity. However, in general, 60 minutes incubation is suitable for active purified TET enzyme and 90 minutes incubation is required for nuclear extract*

13.1.12 Remove the reaction solution from each well. Wash each well three times with 150 µL of 1X Wash Buffer each time.

13.2 Antibody Binding and Signal Enhancing

13.2.1 Add 50 µL of the Diluted Capture Antibody to each well, then cover Parafilm M or aluminum foil and incubate at room temperature for 60 minutes.

13.2.2 Remove the Diluted Capture Antibody solution from each well.

13.2.3 Wash each well three times with 150 µL of 1X Wash Buffer each time.

13.2.4 Add 50 µL of the Diluted Detection Antibody to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 minutes.

13.2.5 Remove the Diluted Detection Antibody solution from each well.

13.2.6 Wash each well four times with 150 µL of 1X Wash Buffer each time.

13.2.7 Add 50 µL of the Diluted Enhancer Solution to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 minutes.

13.2.8 Remove the Diluted Enhancer Solution from each well.

13.2.9 Wash each well five times with 150 µL of 1X Wash Buffer each time.

Note: *Ensure any residual wash buffer in the wells is thoroughly removed at each wash step. The wash can be carried out by simply pipetting the wash buffer into the wells and then pipetting the buffer out from the wells (discard the buffer).*

13.3 **Signal Detection**

13.3.1 Add 50 µL of Fluorescence Development Solution to each well and incubate at room temperature Continue to monitor the development for approximately 2-4 minutes) until the Fluorescence Development Solution turns pink in the presence of sufficient demethylated products.

13.3.2 Read the fluorescence on a fluorescence microplate reader within 2-10 minutes at 530ex/590em nm.

Note: *If the stripwell microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

13.3.3 Calculate TET activity or inhibition using the formulae provided in Section 14 - Data Analysis.

14. ANALYSIS

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

Calculate TET activity or inhibition using the following formula:

$$\text{TET Activity (RFU/min/mg)} = \frac{\text{Sample RFU} - \text{Blank RFU}}{\text{Protein Amount (µg)* x Incubation Time**}} \times 1000$$

* Protein amount added into the reaction at step 13.1.9.
**Incubation time at step 13.1.10 (in minutes).

Example calculation:

Average RFU of sample is 6800
Average RFU of blank is 800
Protein amount is 5 µg
Incubation time is 1 hour (60 minutes)

$$\text{TET activity} = \frac{6800 - 800}{5 \times 60} \times 1000 = 20000 \text{ RFU/min/mg}$$

For an accurate calculation, generate a standard curve and plot RFU versus the amount of TET Assay Standard at each concentration point and determine the slope as delta RFU/ng.

Calculate the amount TET Assay Standard converted Hydroxmethylated product using the following formulae:

$$\text{Hydroxymethylated product (ng)} = \frac{\text{Sample RFU} - \text{Blank RFU}}{\text{Slope}}$$

$$\text{TET Activity (ng/min/mg)} = \frac{\text{Hydroxymethylated product (ng)}}{\text{Protein Amount (µg)* x Incubation Time**}} \times 1000$$

* Protein amount added into the reaction at step 13.1.9.
**Incubation time at step 13.1.10 (in minutes).

$$\text{Inhibition \%} = 1 - \left(\frac{\text{Inhibitor RFU} - \text{Blank RFU}}{\text{No Inhibitor Sample RFU} - \text{Blank RFU}} \right) \times 100\%$$

Typical Results

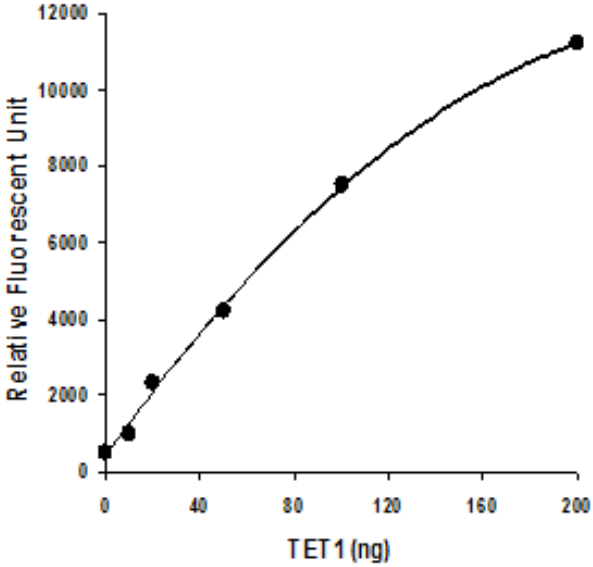

Figure 1. Demonstration of high sensitivity and specificity of the TET1 activity/inhibition assay achieved by using recombinant TET1 with the TET Hydroxylase Activity Quantification Kit (Fluoroimetric)

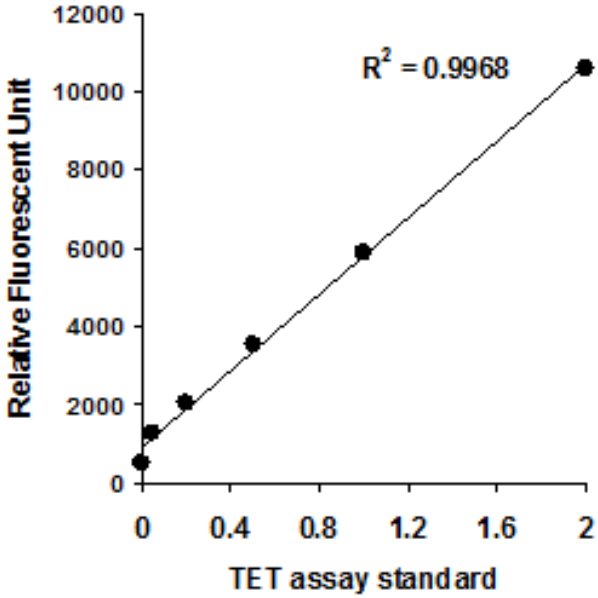

Figure 2. Illustrated standard curve generated with the TET assay standard.

15. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in both the standard 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 substrate and standard are not properly bound to the wells	Ensure that (1) the 0.5X TET Assay Buffer and Diluted TET Assay Standard are added into the wells; (2) the wells are completely covered with sufficient Binding Solution; and (3) binding time is sufficient (90 minutes)
	Incubation time and temperature are incorrect	Ensure the incubation time and temperature described in the protocol are followed correctly
	Incorrect fluorescence reading	Check if the appropriate fluorescence filters (530ex/590em nm filter) are used
	Kit was not stored or handled properly	Ensure all components of the kit were stored at the appropriate temperatures 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 13.1.3	Ensure a sufficient amount of standard is added
	The standard is degraded due to improper storage conditions	Follow the Storage and Stability guidance of this User Guide for storage of TET Assay Standard
High background present in the blank wells	Insufficient washing of wells	Check if washing 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 detection antibody is too long	The incubation time at step 13.2.4 should not exceed 45 minutes
	Over development of fluorescence	Decrease the development time in step 13.3.1
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified	Ensure your protocol is suitable for TET protein extraction. For the best results, it is advised to use Abcam's Nuclear Extraction Kit (ab113474). 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 steps 13.1.9 and 13.1.10. 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°C, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing
	Little or no activity of TET 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 fluorescent development	Insufficient wash 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 fluorescent development in the wells	Ensure fluorescence development is added sequentially and consistent with the order you added the other reagents (e.g. from well A to G or from well 1 to 12)

16. NOTES

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