ab139433 –

Universal Methyltransferase Activity Assay Kit

Instructions for Use

A complete kit for the screening of candidate compounds that may alter normal Methyltransferase activity.

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

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1. Introduction

The Methyltransferase activity kit is a complete kit for the screening of candidate compounds that may alter normal Methyltransferase activity. Please read the complete kit insert before performing this assay.

Methylation of proteins, nucleic acids and oligosaccharides is an important post-translational regulatory event. Activities that are methylation-related include meiosis, biosynthesis, development, signal transduction, chromatin regulation, and gene silencing. The enzymes that mediate the covalent transfer of a methyl group from a donor to an acceptor molecule are Methyltransferases. Methyltransferases have structurally unrelated acceptors as diverse as proteins and DNA, however frequently use S-adenosyl-L-Methionine as a universal donor. Part of the acceptor diversity of this enzyme family relates to the flexible structural folds that bind these molecules in proximity of the donor. The side- chains of lysine, arginine, glutamate, glutamine, asparagines, and isoprenylated residues serve as methylation sites in proteins like histones. Changes in methylation patterns have been tightly linked to disease states such as cancer and vascular disease.

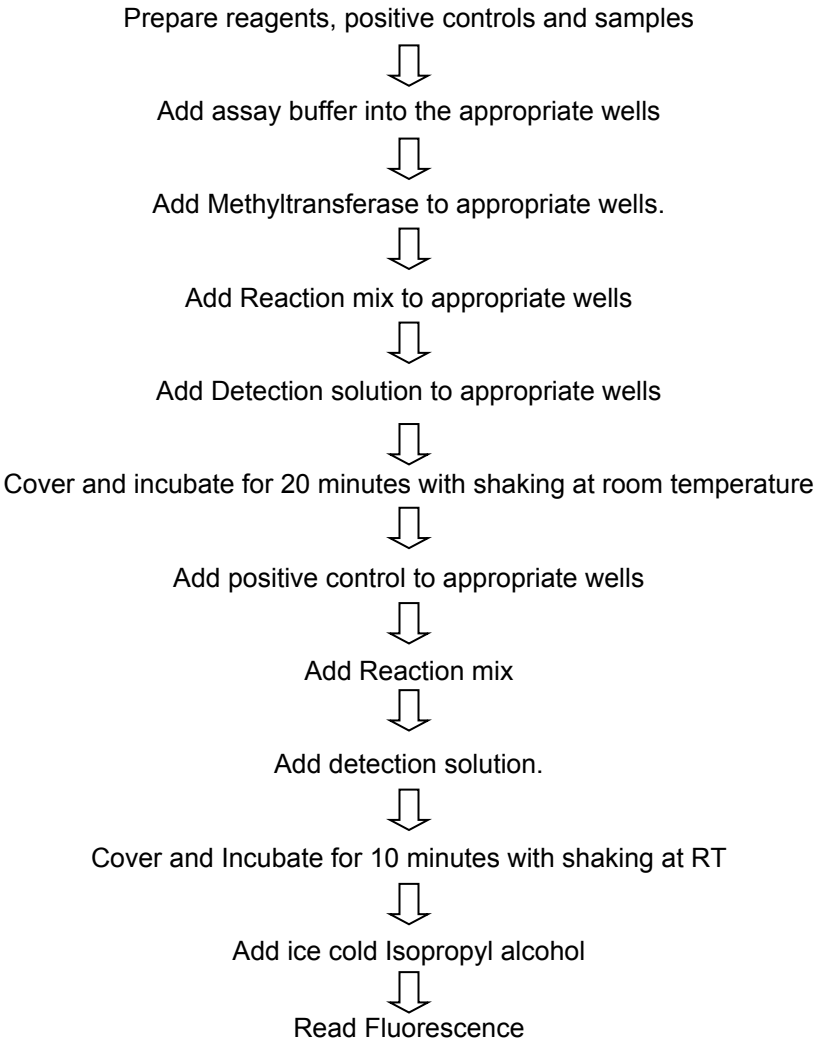
The principle of the assay is as follows:

- Enzyme, reaction mix spiked with acceptor substrate and detection solution are added to well of a 96-wp. Incubate plate.
- Ice cold isopropyl alcohol is added to stop the reaction.
- Plate is transferred to a plate reader and fluorescence is measured at Ex/ Em = 380/520 nm

Acceptor Substrate + Reaction Buffer + Detection Solution $\xrightarrow{\text{Methyltransferase}}$ Fluorescence

2. Assay Summary

Endpoint/ Kinetic assays:



3. Components and Storage

A. Kit Contents

Item	Quantity	Storage Temp
Black 96 well Microtitre plate	1	≤ -20°C
Transferase Assay Buffer Concentrate	15 ml	+ 4°C
Methyltransferase Reaction Buffer Concentrate	0.3 ml	≤ -20°C
Transferase Detection Solution Concentrate	0.15 ml	≤ -20°C
Methyltransferase Positive Control (A buffered solution of 1mM S-(5'-Adenosyl)-L-homocysteine)	0.2 ml	≤ -20°C
Foil Plate Sealer	3	+ 4°C

B. Storage and Handling

All reagents should be stored at -20°C, with the exception of Transferase Assay Buffer Concentrate which should be stored at 4°C. The recommended storage temperature does not necessarily reflect shipping conditions.

C. Additional Materials Required

- Deionized or distilled water
- S-adenosyl-L-methionine dependent Methyltransferase
- Appropriate Methyltransferase acceptor substrate
- Inhibitor/ activator compounds to be screened
- Precision pipets for volumes between 5 µl – 100 µl
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- A microplate shaker
- Disposable microtubes, 0.5 and 1.5 ml
- Microplate reader capable of measuring fluorescence at Ex/Em = 380/520 nm
- Crushed ice and container
- Isopropyl alcohol (ice cold)

4. Pre-Assay Preparation

A. Reagent Preparation

1. Transferase Assay Buffer

Prepare 150ml of 1X Transferase Assay Buffer by diluting 15 ml of Transferase Assay Buffer Concentrate with 135 ml of deionized water. This buffer will be used to prepare dilutions of Methyltransferase Reaction Mix, Transferase Detection Solution, enzymes, substrates, and compounds to be screened.

2. Transferase Detection Solution

To prepare enough Detection solution, count the total number of wells needed for screening and add 6 (for the zero, positive control, and blank wells in duplicate). Use the following formula to calculate the volume of 1X Detection solution required:

Total volume required:

$$[\text{ Total number wells needed} + 6\ ] \text{ x } 100\ \mu\text{l} = \text{X } \mu\text{l}$$

Transferase Detection Solution Concentrate Volume:

$$[\text{ X } \mu\text{l }] \text{ x } 0.01 = \text{Y } \mu\text{l}$$

1X Transferase Assay Buffer volume:

$$[\text{ X } \mu\text{l }] \text{ x } 0.99 = \text{Z } \mu\text{l}$$

Prepare 1X Detection Solution by combining the appropriate Y and Z reagent volumes calculated above: for example, to prepare 2 ml of 1X Transferase Detection Solution, combine 20 µl of the supplied Transferase Detection Solution Concentrate + 1980 µl 1X Transferase Assay Buffer. Diluted Detection Solution should be kept on ice and used within 4 hours of preparation. Any unused 1X Detection Solution should be discarded.

3. Methyltransferase Reaction Mix

Count the total number of wells needed for compound screening and add 6 (for the zero, positive control, and blank wells in duplicate). Use the following formula to calculate the volume of 1X Methyltransferase Reaction Mix required.

Total volume required:

$$[\ \text{Total number wells needed} + 6\ ]\ \text{x}\ 25\ \mu\text{l} = \text{X}\ \mu\text{l}$$

Methyltransferase Reaction Buffer Concentrate volume:

$$[\ \text{X}\ \mu\text{l}\ ]\ \text{x}\ 0.1 = \text{Y}\ \mu\text{l}$$

Acceptor substrate volume:

$$[\ \text{Determined empirically based on enzyme used}\ ] = \text{Z}\ \mu\text{l}$$

1X Transferase Assay Buffer volume:

$$[\ \text{X}\ \mu\text{l}\ ]\ \text{x}\ 0.90 - \text{Z}\ \mu\text{l} = \text{W}\ \mu\text{l}$$

Prepare 1X Reaction Mix on ice by combining the appropriate Y, Z and W reagent volumes calculated above: for example, to prepare 2 ml of Reaction Mix spiked with 22 µl of acceptor substrate, combine 200µl Methyltransferase Reaction Buffer Concentrate + 22 µl Substrate + 1778 µl 1X Transferase Assay Buffer. Diluted Reaction Mix should be kept on ice and used within 8 hours of preparation. Any unused 1X Reaction Mix should be discarded.

4. Positive Control

A positive control is included in the kit to verify the activity of the kit components, and it should not be used to calculate the concentration of Methyltransferase activity in samples. Prepare enough Positive Control to test in duplicate. Use the following formula to calculate the volume of Positive Control required. A minimum of 2 wells of the positive control are recommended per assay.

Total volume required:

$$[\text{ Total number wells needed }] \text{ x } 25 \text{ µl} = \text{X µl}$$

Positive Control volume:

$$[\text{ X µl }] \text{ x } 0.1 = \text{Y µl}$$

1X Transferase Assay Buffer volume:

$$[\text{ X µl }] \text{ x } 0.9 = \text{Z µl}$$

Prepare Positive Control on ice by combining the appropriate Y and Z reagent volumes calculated above: for example, to prepare 200 µl of Positive Control, combine 20 µl of Positive Control + 180 µl of 1X Transferase Assay Buffer. Diluted Positive Control should be kept on ice and used within 8 hours of preparation. Any unused 1X Positive Control should be discarded.

B. Sample Handling

This assay is suitable for use with all S-adenosyl-L-methionine dependent Methyltransferases. It is necessary to titrate each enzyme/substrate system in the assay to determine optimal conditions. This assay should only be used to screen purified *in vitro* samples in buffer systems without reductants. It is recommended that an end-point assay is performed to determine the optimal concentration of enzyme/ substrate to use prior to screening candidate compounds. Make serial dilutions of the Methyltransferase of interest in the assay buffer. Initial concentrations of 100 nM are recommended. A kinetic assay format is also an available option. The positive control provided may also be used to test colored compounds for interference in the assay.

5. Assay Protocol

1. End point / Kinetic Assay Procedure

Determine the number of wells to be used. Cover unused wells tightly with a plate sealer. DO NOT REUSE WELLS.

1. Pipet 25 µl of 1X Transferase Assay Buffer into the blank wells.
2. Pipet 25 µl of Methyltransferase dilutions into the bottom of the appropriate wells.
3. Pipet 25 µl of 1X Reaction Mix into each well.
4. Pipet 100 µl 1X Detection Solution into each well.
5. Cover plate with foil plate sealer. Incubate for 20 minutes (for end point format) shaking at room temperature.

NOTE: Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120-700 rpm.

For Kinetic measurements, incubate identical reaction wells for the desired periods of time. The duplicate wells for each time point can be stopped as in step 10 below.

6. Pipet 25 µl of 1X Positive Control into the bottom of the appropriate wells.
7. Pipet 25 µl of 1X Reaction Mix into Positive Control wells.
8. Pipet 100 µl Detection Solution into Positive Control wells.

9. Cover plate with foil plate sealer. Incubate for 10 min.
10. Pipet 50 µl of ice cold isopropyl alcohol into each well.
11. Read fluorescence at 380ex/520em.

2. Inhibition Assay Procedure

Determine the number of wells to be used. Cover unused wells tightly with a plate sealer. DO NOT REUSE WELLS.

1. Pipet 25 µl of 1X Transferase Assay Buffer into the blank wells.
2. Pipet 10 µl of 1X Transferase Assay Buffer into the zero wells.
3. Pipet 10 µl of inhibitor dilution into the bottom of the appropriate wells.
4. Pipet 15 µl of Methyltransferase at chosen working concentration into appropriate wells.
5. Cover plate with foil plate sealer. Incubate for 10 minutes at room temperature without shaking.
6. Pipet 25 µl of 1X Reaction Mix into each well.
7. Pipet 100 µl of 1X Detection Solution into each well.
8. Cover plate with foil plate sealer. Incubate for 30 min, shaking at room temperature.

NOTE: Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120-700 rpm.

9. Pipet 50 µl of ice cold isopropyl alcohol into each well.
10. Read fluorescence at 380ex/520em.

3. Enzyme Interference Assay Procedure

1. Pipet 25 µl of 1X Transferase Assay Buffer into the blank wells.
2. Pipet 10 µl of 1X Transferase Assay Buffer into the zero wells.
3. Pipet 10 µl of colored compound dilution into appropriate wells
4. Pipet 15 µl of 1X Positive Control into the zero wells, and wells containing colored compound dilutions.
5. Pipet 25 µl of 1X un-spiked reaction mix into each well.
6. Pipet 100 µl 1X Detection Solution into each well.
7. Cover plate with foil plate sealer; incubate for 10 min at room temperature without shaking.
8. Pipet 50 µl of ice cold isopropyl alcohol into each well.
9. Read fluorescence at Ex/Em = 380/ 520nm.

To determine whether or not colored compounds will interfere with the assay, calculate and compare the signal to noise ratio of the colored compound dilutions to the signal to noise ratio of the zero wells.

Assay Layout Sheet:

A1 Blank	A2 Blank	A3 Dil.1	A4 Dil.1	A5 Dil.2	A6 Dil.2	A7 Dil.3	A8 Dil.3	A9 Dil.4	A10 Dil.4	A11 Pos. Control	A12 Pos. Control
B1 Blank	B2 Blank	B3 Inhib. Dil.1	B4 Inhib Dil.1	B5 Inhib Dil. 2	B6 Inhib Dil. 2	B7 Inhib Dil. 3	B8 Inhib Dil. 3	B9 Inhib Dil. 4	B10 Inhib Dil.4	B11 D0	B12 D0
C1 Kinetic Blank	C2 Kinetic Blank	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1 Kinetic D1	D2 Kinetic D1	D3 Kinetic D1	D4 Kinetic D1	D5 Kinetic D1	D6 Kinetic D1	D7	D8	D9	D10	D11	D12
E1 Kinetic D2	E2 Kinetic D2	E3 Kinetic D2	E4 Kinetic D2	E5 Kinetic D2	E6 Kinetic D2	E7	E8	E9	E10	E11	E12
F1 Kinetic D3	F2 Kinetic D3	F3 Kinetic D3	F4 Kinetic D3	F5 Kinetic D3	F6 Kinetic D3	F7	F8	F9	F10	F11	F12
G1 Kinetic D4	G2 Kinetic D4	G3 Kinetic D4	G4 Kinetic D4	G5 Kinetic D4	G6 Kinetic D4	G7	G8	G9	G10	G11	G12
H1 Kinetic D5	H2 Kinetic D5	H3 Kinetic D5	H4 Kinetic D5	H5 Kinetic D5	H6 Kinetic D5	H7	H8	H9	H10	H11	H12

6. Data Analysis

1. End point/Kinetic Assay Results

- Plot the mean of the duplicate relative fluorescence units (RFU) at 380ex/520em versus Enzyme concentration.
- Calculate the signal to noise ratio:

$$\frac{\text{Mean RFU for enzyme dilution}}{\text{Mean RFU Blank}}$$

Figure 1 shows a titration assay of an engineered portion of the Human lysine specific histone methyltransferase SET7/9 using 15 µM of the peptide substrate TAF-10. Serial dilutions of SET7/9 were prepared in Transferase Assay Buffer. Average relative fluorescence was plotted against SET7/9 concentration to generate the following graph. This is for illustration purposes only. The investigator must titrate their own enzyme substrate system in the assay.

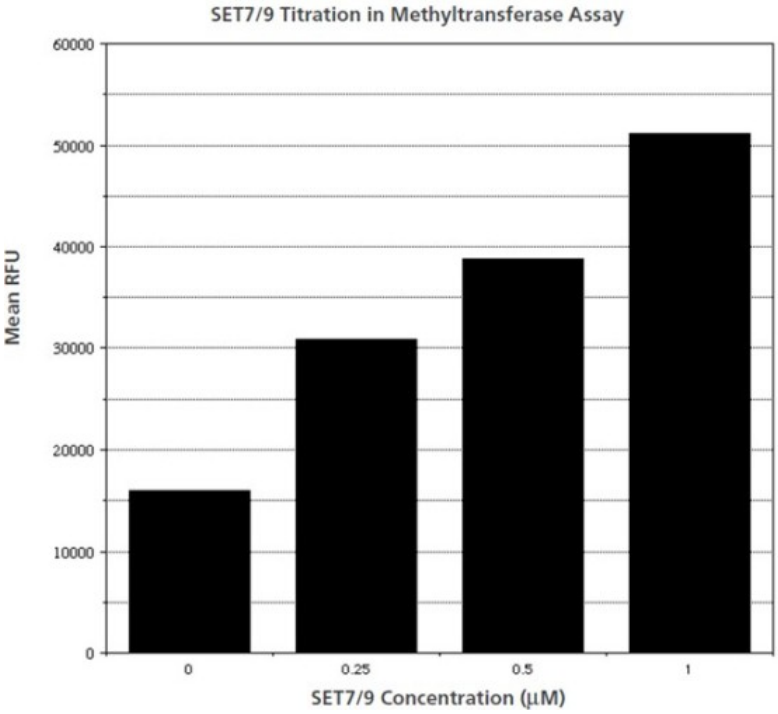


Figure 1: Titration assay of SET7/9

Based on this titration data, 0.5 µM of SET7/9 produces a maximum signal within the detection range of the plate reader, with a signal to noise ratio sufficient for easy detection of altered enzyme activity.

Example of Kinetic Assay Results:

Based on the data obtained in the end point assay (figure 1), 0.5 µM SET7/9 was tested with 15 µM TAF-10 substrate in the kinetic assay format. Mean relative fluorescence was plotted against the stop time interval to generate figure 2. This graph is for illustration purposes only. The kinetic assay must be optimized by the investigator, with their enzyme / substrate system.

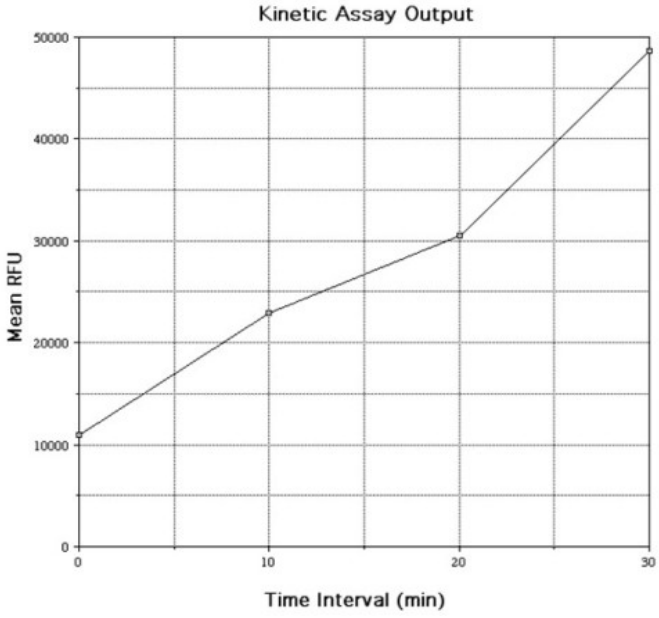


Figure 2: Kinetic assay using 0.5 µM SET7/9 with 15 µM TAF-10 substrate

2. Inhibition Assay Results

Several options are available for the calculation of the inhibition of Methyltransferase. We recommend that the data be handled by a software package utilizing a suitable curve fitting program to determine the percent inhibition. If data reduction software is not readily available, the data can be calculated as follows:

i. Calculate the mean net RFU for each sample by subtracting the mean blank RFU from the mean RFU for the samples:

$$\text{Mean Net RFU} = \text{Mean Sample RFU} - \text{Mean Blank RFU}$$

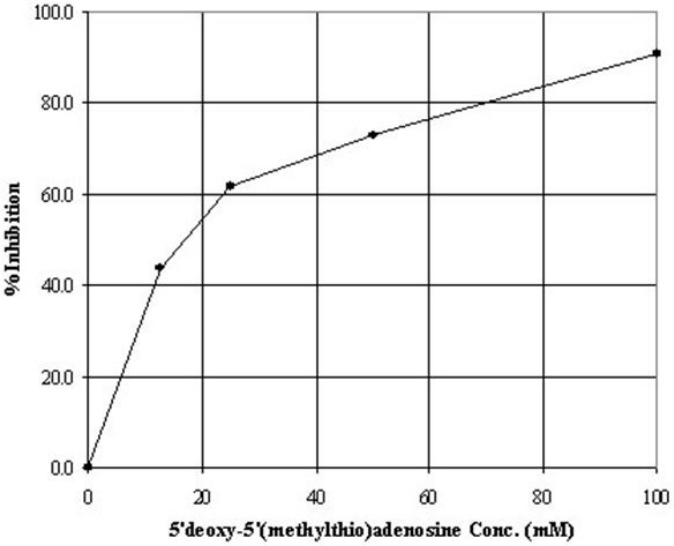
ii. Percent inhibition should be calculated using the following formula for each inhibitor dilution:

$$\% \text{ Inhibition} = \frac{(\text{Mean Net Zero (non-inhibited enzyme) RFU} - \text{Mean Net Inhibited enzyme RFU})}{\text{Mean Net Zero(non-inhibited enzyme) RFU}} \text{x100}$$

Typical Inhibition Assay Results:

Using SET7/9 and TAF-10 as an enzyme substrate system, percent inhibition for dilutions of 5'deoxy-5'(methylthio)-adenosine was tested. The results shown below are for illustration only and should not be used to calculate results from another assay.

Dilution	Inhibition Conc (mM)	Mean RFU	Mean Net RFU	% Inhibition
1	100	12828	1135	90.7
2	50	15030	3337	72.7
3	25	16411	4718	61.4
4	12.5	18575	6882	43.7
0	0	23910	12217	0
Blank		11693	0	-



3. Interference Assay

The following solvents were tested for interference with the fluorescent signal generated in the assay. The table lists the percentage of signal in the presence of interferant relative to the zero for each solvent.

% Interferant	DMSO	DMF	Acetonitrile
12.5	108	68	49
6.25	107	82	71
3.12	107	88	86

1.56	112	98	96
0.78	114	104	95
0.39	115	109	100
0	100	100	100

*Percent interferant is relative to a 50 µl total reaction volume.

Diluents containing Bovine Serum Albumin (BSA) or other Thiol containing reagents, should be treated with N-Ethylmaleimide (NEM) prior to use in the assay. As general rule, we recommend reacting 10% BSA with 1 mM NEM for 1 hour at room temperature; however this procedure should be optimized for each reagent.

7. Troubleshooting

Problem	**Reason**	**Solution**
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled – needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly

	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, protocol will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Ensure you are using appropriate reader and filter settings
	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Too many freeze/thaw cycles	Aliquot samples to reduce the number of freeze/thaw cycles
	Samples are too old or incorrectly stored	Use fresh made samples and store at recommended temperature until use
Lower/higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently m ix prior use
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use

	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)
Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently m ix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when setting up the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates

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