m^{6}A RNA Methylation Quantification Kit

—— GK- 4048

Product Manual

(Colorimetric)

*PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures

Uses: The Genelily™ m^{6}A RNA Methylation Quantification Kit (Colorimetric) is suitable for detecting N 6-methyladenosine (m^{6}A) RNA me thylation status directly using total RNA isolated from any species such as mammals, plants, fungi, bacteria, and viruses.

Input RNA: The amount of RNA for each assay can be 100 ng to 300 ng. For optimal quantification, the input RNA amount should be 200 ng, as the abundance of m^{6}A is generally less than 0.1% of total RNA.

Starting Material: Starting materials can include various tissue or cell samples such as cells from flask or microplate cultured cells, fresh and frozen tissues, paraffin-embedded tissues, blood, body fluid samples, etc.

Internal Control: Both negative and positive RNA controls are provided in this kit. A standard curve can be performed (range: 0.02 to 1 ng of m^{6}A) or a single quantity of m6A can be used as a positive control. Becaus e m6A content can vary from tissue to tissue, and from normal and dise ased states, or vary under treated and untreated conditions, it is advised to run replicate samples to ensure that the signal generated is validated. This kit will allow the user to quantify an absolute amount of m6A and determine the relative m^{6}A RNA methylation states of two different RN A samples.

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GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the Genelily™ m^{6}A RNA Methylation Quantification Kit (Colorimetric) istested against predetermined specifications to ensure consistent product quality. Genelily 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: Genelily 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. Thus, only use the User Guide that was supplied with the kit when using that kit.

Usage Limitation: The Genelily™ m^{6}A RNA Methylation Quantification Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The Genelily™m^{6}A RNA Methylation Quantification Kit (Colorimetric) and methods of use contain proprietary technologies by Genelily.

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KIT CONTENTS

Component	48 Reactions	96 Reactions	Storage Upon Receipt
WB (10XWash Buffer)	14ml	28ml	4°C
BS (Binding Solution)	5ml	10ml	RT
NC (Negative Control, 100μg/ml) *	10 μl	20 μl	-20°C
PC(Positive Control, m^6A 2 μg/ml) *	10 μl	20 μl	-20°C
CA (Capture Antibody, 1000X) *	5 μl	10 μl	4°C
DA (Detection Antibody,1000X)	6 μl	12 μl	-20°C
ES (Enhancer Solution) *	5 μl	10 μl	-20°C
DS (Developer Solution) *	5ml	10ml	4°C
SS (Stop Solution)	5 ml	10ml	RT
8-Well Assay Strips (With Frame)	6	12	4°C
User Guide	1	1	RT

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

Note: *The NC (Negative Control) is an RNA containing no m^6A. The PC (Positive Control) is m^6A oligos and is normalized to have 100% of m^6A.*

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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 NC, PC, DA, and ES at – 20°C away from light; (2) Store WB, CA, DS, and 8 -Well Assay Strips at 4°C away from light; (3) Store remaining components (BS and SS) at room temperature away from light.

Note: Check if wash buffer, WB, contains salt precipitates before using. If so, warm (at room temperature or 37°C) and shake the buffer until the salts are re-dissolved.

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

MATERIALS REQUIRED (BUT NOT SUPPLIED)

- Adjustable pipette
- Aerosol resistant pipette tips
- Distilled water
- 1X TE buffer pH 7.5 to 8.0
- 1.5 ml microcentrifuge tubes
- Plate seal or Parafilm M
- Isolated RNA of interest
- Incubator for 37°C incubation
- Microplate reader capable of reading absorbance at 450 nm

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A BRIEF OVERVIEW

N^6-methyl-adenosine (m^6A) is the most common and abundant modification on RNA molecules present in eukaryotes. The m^6A modification is catalyzed by a methyltransferase complex METTL3 and removed by the recently discovered m^6A RNA demethylases FTO and ALKBH5, which catalyze m^6A demethylation in an α-ketoglutarate (α-KG)- and Fe^{2+}-dependent manner.

It was shown that METTL3, FTO and ALKBH5 play important roles in many biological processes, ranging from development and metabolism to fertility. m^6A accounts for more than 80% of all RNA base methylations and exists in various species. m^6A is mainly distributed in mRNA and also occurs in noncoding RNA such as tRNA, rRNA and snRNA. The relative abundance of m^6A in mRNA transcripts has been shown to affect RNA metabolism processes such as splicing, nuclear export, translation ability and stability and RNA transcription.

Abnormal m^6A methylation levels induced by defects in m^6A RNA methylase and demethylase could lead to dysfunction of RNA and cause disease.

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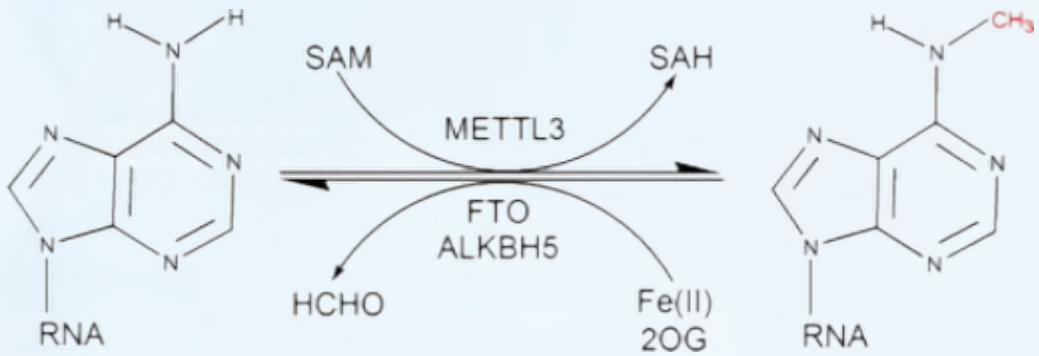
For example, abnormally low levels of m^{6}A in target mRNAs due to increased FTO activity in patients with FTO mutations, through an as-yet undefined pathway, contributes to the onset of obesity and related diseases. The dynamic and reversible chemical m^{6}A modification on RNA may also serve as a novel epigenetic marker of profound biological significance. Therefore, more useful information for better understanding of m^{6}A RNA methylation levels and distribution on RNA transcripts could benefit diagnostics and therapeutics of disease.



Reversible m^{6}A methylation in mRNA

(Niu Y et al: Genomics, Proteomics & Bioinformatics, 11: 2013)

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Several chromatography-based techniques such as HPLC-ECD and LC-MS are used for detecting m^{6}A in tissues and cells. However, these methods are time consuming and have low throughput with high costs. To address these problems,We offers the Genelily™ m^{6}A RNA Methylation Quantification Kit (Colorimetric) which uses a unique procedure to directly quantify m^{6}A RNA methylation status using total RNA isolated from cells/tissues. As the first commercially available product used for quantification of m^{6}A RNA methylation, the kit has the following advantages and features:

- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be completed within 3 hours and 45 minutes.
- High sensitivity, of which the detection limit can be as low as 10 pg of m^{6}A.
- Unique binding solution allows that RNA >70 nts can be tightly bound to the wells, which enables quantification of m6A from both mRNA and nc-RNA such as tRNA , rRNA and snRNA.
- Optimized antibody and enhancer solutions allow high specificity to m^{6}A, with no cross-reactivity to unmethylated adenosine within the indicated concentration range of the sample RNA.
- Universal positive and negative controls are included, which are suitable for quantifying m^{6}A fromany species.
- Strip-well microplate format makes the assay flexible for manual or high throughput analysis.
- Simple, reliable, and consistent assay conditions.

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PRINCIPLE & PROCEDURE

The Genelily™ m^{6}A RNA Methylation Quantification Kit (Colorimetric) contains all reagents necessary for the quantification of m^{6}A in RNA. In this assay, total RNA is bound to strip wells using RNA high binding solution. m^{6}A is detected using capture and detection antibodies. The detected signal is enhanced and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of m^{6}A is proportional to the OD intensity measured.

Total RNA isolation

↓

Bind RNA Assay Wells

↓

Wash Wells，
add capture antibody

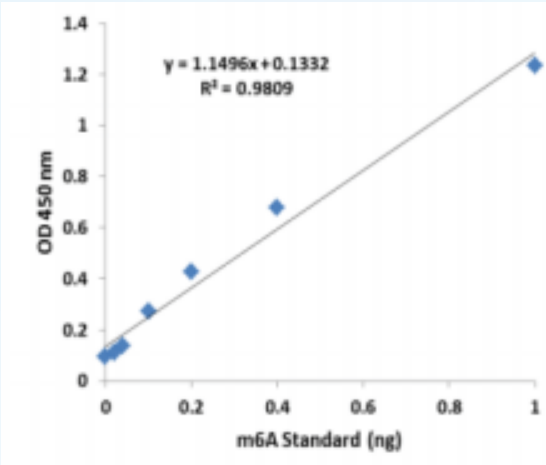
↓

Wash Wells,add detection antibodyand enhancer solution

↓

add color developing solution for color development then measure absorbance

Schematic procedure of the Genelily™ m^{6}A RNA Methylation Quantification Kit (Colorimetric)



m^{6}A standard control was added into the assay wells at different concentrations and then measured with the Genelily™m^{6}A RNA Methylation Quantification Kit (Colorimetric)

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ASSAY PROTOCOL

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

1.Starting Materials

Input RNA Amount: Total RNA amount can range from 100 ng to 300 ng per reaction. An optimal amount is 200 ng per reaction. Starting RNA may be in water or in a buffer such as TE. You can use your method of choice for RNA isolation.

RNA Storage: Isolated total RNA can be stored at -20°C (short term) or -80°C (long term) until use.

2.Buffer and Solution Preparation

a. Preparation of 1X Wash Buffer:

48-Assay Kit: Add 13 ml of WB (10X Wash Buffer) to 117 ml of distilled water (final pH 7.2-7.5).

96-Assay Kit: Add 26 ml of WB (10X Wash Buffer) to 234 ml of distilled water (final pH 7.2-7.5).

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

b. Prepare Diluted CA (Capture Antibody) Solution:

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

c. Prepare Diluted DA (Detection Antibody) Solution:

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

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d. Prepare Diluted ES (Enhancer Solution):

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

e. Preparation of Diluted Positive Control:

Single Point Control Prep:

Dilute PC (Positive Control) with 1X TE to 0.5 ng/µl (1 µl PC + 3 µl TE).

Suggested Standard Curve Prep:

First, dilute PC to 0.5 ng/µl (ex: 3 µl of PC + 9 µl of 1X TE).
Then, further prepare 6 different concentrations with the 0.5 ng/µl PC and 1X TE into 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 ng/µl according to thefollowing dilution chart:

Tube	PC (0.5ng/µl)	1X TE	Resulting PC Concentration
1	1.0 µl	49.0 µl	0.01ng/µl
2	1.0 µl	24.0 µl	0.02ng/µl
3	1.0 µl	9.0 µl	0.05ng/µl
4	1.0 µl	4.0 µl	0.1ng/µl
5	2.0 µl	3.0 µl	0.2ng/µl
6	4.5 µl	0.0 µl	0.5ng/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.*

3.RNA Binding

a. Predetermine the number of strip wells required for your experiment. 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. Add 80 µl of BS (Binding Solution) to each well.
c. Add 2 µl of NC, 2 µl of Diluted PC (see note below), and 200 ng of your sam ple RNA (1-8 µl) into the designated wells depicted in Table 1 or Table 2. Mix s olution by gently tilting from side to side or shaking the plate several times. Ens ure the solution coats the bottom of the well evenly.

Note: (1) For a single point control, add 2 µl of PC at a concentration of 0.5 ng/ µl as prepared in Step 2e; For the standard curve, add 2 µl of Diluted PC at concentrations of 0.01 to 0.5 ng/µl (see the chart in Step 2e). The final amounts should be 0.02, 0.04, 0.1, 0.2, 0.4 and 1 ng per well.
(2) For optimal binding, sample RNA volume added should not exceed 8 µl.
(3) To ensure that NC, Diluted PC, and sample RNA are completely added into the wells, the pipette tip should be placed into the BS solution in the well and aspirated in/out 1-2 times.

d. Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 90 min.
e. Remove the BS (Binding Solution) from each well. Wash each well with 150 µl of Diluted WB by pipetting Diluted WB into the wells and then removing it using a pipette. Repeat the wash two times for a total of three washes.

4.m^{6}A RNA Capture

a. Add 50 µl of Diluted CA to each well, then cover and incubate at room temp erature for 60 min.
b. Remove the Diluted CA solution from each well using a pipette.
c. Wash each well with 150 µl of Diluted WB each time for three times.
d. Add 50 µl of Diluted DA to each well, then cover and incubate at room temp erature for 30 min.
e. Remove the Diluted DA solution from each well using a pipette.

f. Wash each well with 150 µl of Diluted WB each time for four times.
g. Add 50 µl of Diluted ES to each well, then cover and incubate at room temperature for 30 min.
h. Remove the Diluted ES solution from each well.
i. Wash each well with 150 µl of Diluted WB each time for five times

5. 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 m^6A.
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 at 450 nm within 2 to 15 min.

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

6. m^6A Calculation

Relative Quantification: To determine the relative m6A RNA methylation status of two different RNA samples, a simple calculation for the percentage of m6A in your total RNA can be carried out using the following formula:

$$m^6A\% = \frac{(\text{Sample OD} - \text{NC OD}) \div S}{(\text{PC OD} - \text{NC OD}) \div P} \times 100\%$$

S is the amount of input sample RNA in ng.
P is the amount of input positive control (PC) in ng.

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Example calculation:
Average OD450 of NC is 0.1
Average OD450 of PC is 0.4
Average OD450 of Sample is 0.16
S is 200 ng
P is 1 ng

$$m^6A\% = \frac{(0.16 - 0.1) \div 200}{(0.4 - 0.4) \div 1} \times 100\% = 0.1\%$$

Absolute Quantification: To quantify the absolute amount of m^6A using an accurate calculation, first generate a standard curve and plot the OD values (background (NC)-subtracted) versus the amount of PC at each concentration point. Next, determine the slope (OD/ng) of the standard curve using linear regression (Microsoft Excel's linear regression functions are suitable for such calculation). Use the most linear part of the standard curve (include at least 4 concentration points) for optimal slope calculation. Now calculate the amount and percentage of m^6A in your total RNA using the following formulas:

$$m^6A\% = \frac{m^6A \text{ Amount (ng)}}{S} \times 100\% \qquad m^6A\,(ng) = \frac{\text{Sample OD - NC OD}}{\text{Slope}}$$

S is the amount of input sample RNA in ng.

Example calculation:
Average OD450 of NC is 0.10
Average OD450 of sample is 0.16
Slope is 0.3 OD/ng
S is 200 ng

$$m^6A(ng) = \frac{0.16-0.1}{0.3} = 0.2 \text{ ng}$$

$$m^6A\% = \frac{0.2}{200} \times 100\% = 0.1\%$$

SUGGESTED STRIP WELL SETUP

Table 1. The suggested strip-well plate setup using a single point positive control in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample).

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The controls and samples can be measured in duplicate.

Well#	Strip 1	Stirp 2	Stirp 3	Stirp 4	Stirp 5	Stirp 6
A	NC	NC	Sample	Sample	Sample	Sample
B	PC	PC	Sample	Sample	Sample	Sample
C	Sample	Sample	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

Table 2. 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 be measured in duplicate.

Well#	Strip 1	Stirp 2	Stirp 3	Stirp 4	Stirp 5	Stirp 6
A	NC	NC	Sample	Sample	Sample	Sample
B	PC 0.02 ng/well	PC 0.02 ng/well	Sample	Sample	Sample	Sample
C	PC 0.04 ng/well	PC 0.04 ng/well	Sample	Sample	Sample	Sample
D	PC 0.1ng/well	PC 0.1ng/well	Sample	Sample	Sample	Sample
E	PC 0.2 ng/well	PC 0.2 ng/well	Sample	Sample	Sample	Sample
F	PC 0.4 ng/well	PC 0.4 ng/well	Sample	Sample	Sample	Sample
G	PC 0.1 ng/well	PC 0.1 ng/well	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

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SUGGESTED WORKING BUFFER AND SOLUTION SETUP

Table 3. Approximate amount of required buffers and solutions for defined ass ay wells based on the protocol.

Reagents	1 well	8 well (1 strip)	16 well (2 strip)	48 well (6 strip)	96 well (12 strip)
Diluted WB	2.5ml	20ml	40ml	120ml	240ml
BS	80 µl	640 µl	1300 µl	3900 µl	8000 µl
Diluted CA	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted DA	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted ES	50 µl	400 µl	800 µl	2400µl	4800 µl
DS	0.1ml	0.8ml	1.6ml	4.8ml	9.6ml
NC	N/A	0.5µl - 1µl	0.5µl - 2µl	1µl - 4µl	2µl - 8µl
PC	N/A	0.5µl - 1µl	0.5µl - 2µl	1µl - 4µl	2µl - 8µl
SS	0.1ml	0.8ml	1.6ml	4.8ml	9.6ml

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal in both the positive control and sample wells	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 RNA binding.	Ensure the well is **NOT** washed prior to adding the positive control and sample.
	The bottom of the well is not completely covered by the **BS** (Binding Solution).	Ensure the solution coats the bottom of the well by gently tilting from side to side or shaking the plate several times.

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	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol is followed correctly.
	Insufficient input materials.	Ensure that a sufficient amount of positive control (> 0.2 ng) and sample (200 ng) is added into the wells.
	Incorrect absorbance reading.	Check if the 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 secured after each opening or use.
No signal or weak signal in only the **PC** (Positive control) wells	The **PC** (Positive Control) is insufficiently added to the well in Step 3c.	Ensure a sufficient amount of **PC** (Positive Control) is added.
	The **PC** (Positive Control) is degraded due to improper storage conditions.	Follow the Shipping & Storage guidelines of this User Guide for storage of **PC** (Positive Control).
No signal or weak signal only in sample wells	RNA sample is not properly extracted or purified.	Ensure the RNA sample is good quality . The 260/280 ratio should be >1.9 with no or minimal DNA contamination.
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of RNA is used as indicated in Step 3c.
	Little or no m6A contained in the sample.	N/A
High background present in the negative control wells	Insufficient washing of wells.	Check if washing recommendations at each step are performed according to the protocol.

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	Contaminated by sample or positive control.	Ensure the well is not contaminated by the sample or positive control or from the use of contaminated tips.
	Incubation time is too long.	The incubation time at Step 3d should not exceed 2 h.
	Over development of color.	Decrease the development time in Step 5a before adding **SS** (Stop Solution) in Step 5b.
Uneven color development	Insufficient washing of the wells.	Ensure the wells are washed according to the washing guidelines. Make sure the residue of the 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 ad 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).