

Competitive ELISA Kit for Measuring m¹A (N1-methyladenosine)

CircuLex m¹A (N1-methyladenosine) Competitive ELISA Kit

Cat# CY-8124

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Intended Use

The MBL Research Product **CircuLex m¹A (N1-methyladenosine) Competitive ELISA Kit** is used for the quantitative measurement of m¹A-adducts in urine and cell lysate.

Individual users should determine appropriate conditions when using other types of samples.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Do not expose reagents to excessive light.

Introduction

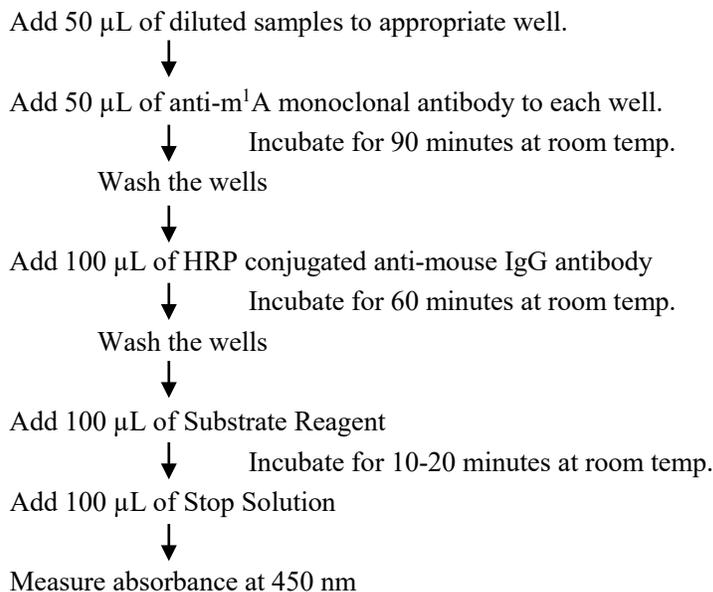
In eukaryote, newly synthesized RNA is chemically modified at specific nucleotide position after transcription.¹⁻³⁾ Over 100 types of chemical modifications have been identified in RNAs, not only in mRNA, tRNA, rRNA but also in non-coding RNA such as miRNA.¹⁻⁵⁾

N1-methyladenosine (m¹A) is one of major modified nucleosides present in most eukaryotic tRNAs and also found in yeast rRNAs.⁶⁾ The m¹A affects structural stability of tRNA and its binding to polysome in translation. Urinary excretion of modified nucleosides including m¹A is known to be increased in the patients with various cancers and AIDS.^{7, 8)} It is reported that the amount of urinary m¹A correlates with m¹A level in tissue from same patients with esophageal carcinoma.⁹⁾ Moreover, elevated serum levels of m¹A are also detected under stress conditions.¹⁰⁾

Principle of the Assay

The CircuLex m¹A (N1-methyladenosine) Competitive ELISA Kit employs the quantitative competitive enzyme immunoassay technique. The m¹A-BSA has been pre-coated onto a microplate. Standards or samples and anti-m¹A monoclonal antibody AMA-2 are pipetted into the wells. Any free anti-m¹A monoclonal antibody present is bound by the immobilized m¹A-BSA. After washing away any unbound substances, an HRP conjugated polyclonal antibody specific for mouse IgG is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H₂O₂-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is inversely proportional to the concentration of m¹A-adduct. A standard curve is constructed by plotting absorbance values versus m¹A monomer concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure



Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Antigen coated Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with m¹A-BSA.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing Tween[®]-20

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for m¹A standard and sample dilution. Ready to use.

m¹A Standard: One vial containing X* µg of lyophilized m¹A monomer.

***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

100X Primary Antibody: One bottle containing 150 µL of anti-m¹A monoclonal antibody AMA-2.

100X HRP conjugated Detection Antibody: One bottle containing 150 µL of HRP (horseradish peroxidase) conjugated anti-mouse IgG antibody.

Conjugate Dilution Buffer: One bottle containing 20 mL of 1X buffer; used for dilution of 100X Primary Antibody and 100X HRP conjugated Detection Antibody. Ready to use

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle supplied ready to use, containing 20 mL of 1 N H₂SO₄. Ready to use.

Materials Required but not Provided

- **Sample preparation microplate:** 96 wells microplate for mixing Primary Antibody and diluted samples.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Sample Collection and Storage

Please see page 7 for more information on "Sample Preparation".

Urine: Remove any particulates by centrifugation and assay immediately or aliquot and store samples below -70°C. Avoid repeated freeze-thaw cycles.

Cell lysate:

1. Harvest and pellet cells by centrifugation using standard methods.
2. Resuspend the cell pellet with an appropriate extraction buffer (for example; 20 mM HEPES-KOH, pH 7.5, 250 mM NaCl, 0.1 % NP-40, 1 mM EDTA, 10% Glycerol, protease inhibitor) and lyse the resuspended cells using either a Dounce Homogenizer, sonication, three cycles of freezing and thawing, or pipetting on ice.
3. Transfer extracts to microcentrifuge tubes and centrifuge at 15,000 rpm for 10 minutes at 4°C.
4. Aliquot cleared lysate to a clean microcentrifuge tube.
5. Assay immediately or store the samples on ice for a few hours before assaying. Aliquots of the samples may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Other biological samples: MBL has not tested. Please see page 7 for more information.

(e.g. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles. Individual users should determine appropriate conditions when using other types of samples.)

Detailed Protocol

The MBL Research Product **CircuLex m¹A (N1-methyladenosine) Competitive ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the **m¹A Standard** within the kit, should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer**, **100X Primary Antibody**, **100X HRP conjugated Detection Antibody** and **m¹A Standard**.

1. Prepare a working solution of Wash Buffer by adding **100 mL** of the **10X Wash Buffer** to **900 mL** of deionized (distilled) water (**ddH₂O**). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.
2. Reconstitute **m¹A Standard** with **X* µL** of **ddH₂O** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 100 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles. The concentration of the reconstituted m¹A Standard should be **4,000 ng/mL**, which is referred to as the **Master Standard** of m¹A.

***The amount is changed depending on lot. See the real “User’s Manual” included in the kit box.**

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1,000 ng/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Standard Dilution Buffer	Concentration
Std.1	150 µL of Master Standard	450 µL	1,000 ng/mL
Std.2	300 µL of Std. 1 (1,000 ng/mL)	300 µL	500 ng/mL
Std.3	300 µL of Std. 2 (500 ng/mL)	300 µL	250 ng/mL
Std.4	300 µL of Std. 3 (250 ng/mL)	300 µL	125 ng/mL
Std.5	300 µL of Std. 4 (125 ng/mL)	300 µL	62.5 ng/mL
Std.6	300 µL of Std. 5 (62.5 ng/mL)	300 µL	31.25 ng/mL
Std.7	300 µL of Std. 5 (31.25 ng/mL)	300 µL	15.625 ng/mL
Blank	-	300 µL	0 ng/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with **Dilution Buffer** before dispensing.

3. Prepare **1X Primary Antibody** by diluting the **100X Primary Antibody** (provided) 100-fold with **Dilution Buffer** at the time of use and discard any unused portion after use.
4. Prepare **1X HRP conjugated Detection Antibody** by diluting the **100X HRP conjugated Detection Antibody** (provided) 100-fold with **Conjugate Dilution Buffer** at the time of use and discard any unused portion after use.

Sample Preparation

Dilute samples with **Dilution Buffer**. Optimal dilution ratio should be determined by the end user.

Samples-confirmed by MBL

- Urine samples: may require 50-fold dilution.
- Cell lysates: may require appropriate dilution.

Samples-expected

- Plasma samples: may require preprocessing of ultrafiltration.*
e.g. Amicon® Ultra-0.5 Centrifugal Filter Concentrator with Ultracel® 50 Regenerated Cellulose Membrane (Merck; code no. UFC505024). 500 µL of sample is put in a tube and centrifuged at 14,000 x g for 20 minutes at 4°C.

* Please refer to the reference, Mishima E. et al. Conformational Change in Transfer RNA Is an Early Indicator of Acute Cellular Damage. J Am Soc Nephrol. 25, 2316-26 (2014) PMID: 24833129

Assay Procedure

1. Remove the appropriate number of microtiter wells of **Antigen coated Microplate** from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.
2. Dilute samples with **Dilution Buffer**. (See “Sample Preparation” above.)
3. Pipette **50 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate well.
4. Add **50 µL** of **1X Primary Antibody** into each well.
5. Incubate the wells **at room temperature (ca.25°C) for 90 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.**
6. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
7. Add **100 µL** of **1X HRP conjugated Detection Antibody** into each well.
8. Incubate the wells **at room temperature (ca.25°C) for 60 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.**
9. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
10. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed
11. Incubate the wells **at room temperature (ca. 25°C) for 10-20 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.** The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.

12. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.
13. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 2.5 units for the blank (zero concentration).

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the blank (zero concentrations), perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine the concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control and sample, and subtract the optical density of the average zero standard. Plot the optical density versus the concentration of standards and draw the best curve. Most microtiter plate readers perform automatic calculations of analyte concentration. The standard curve fits best to a sigmoidal four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a **four-parameter logistic function**.

A standard curve is also to be constructed by plotting the absorbance (Y) versus log of the known concentration (X) of standards, using a cubic function. Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of optical density (Y) is plotted versus log of the known concentration (X) of standards). To determine the concentration of each sample, first find the optical density on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Measurement Range

The measurement range is 15.6 ng/mL to 1,000 ng/mL. Any sample reading lower than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the m¹A concentration.

Troubleshooting

1. All samples and standards should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.
2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.
3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

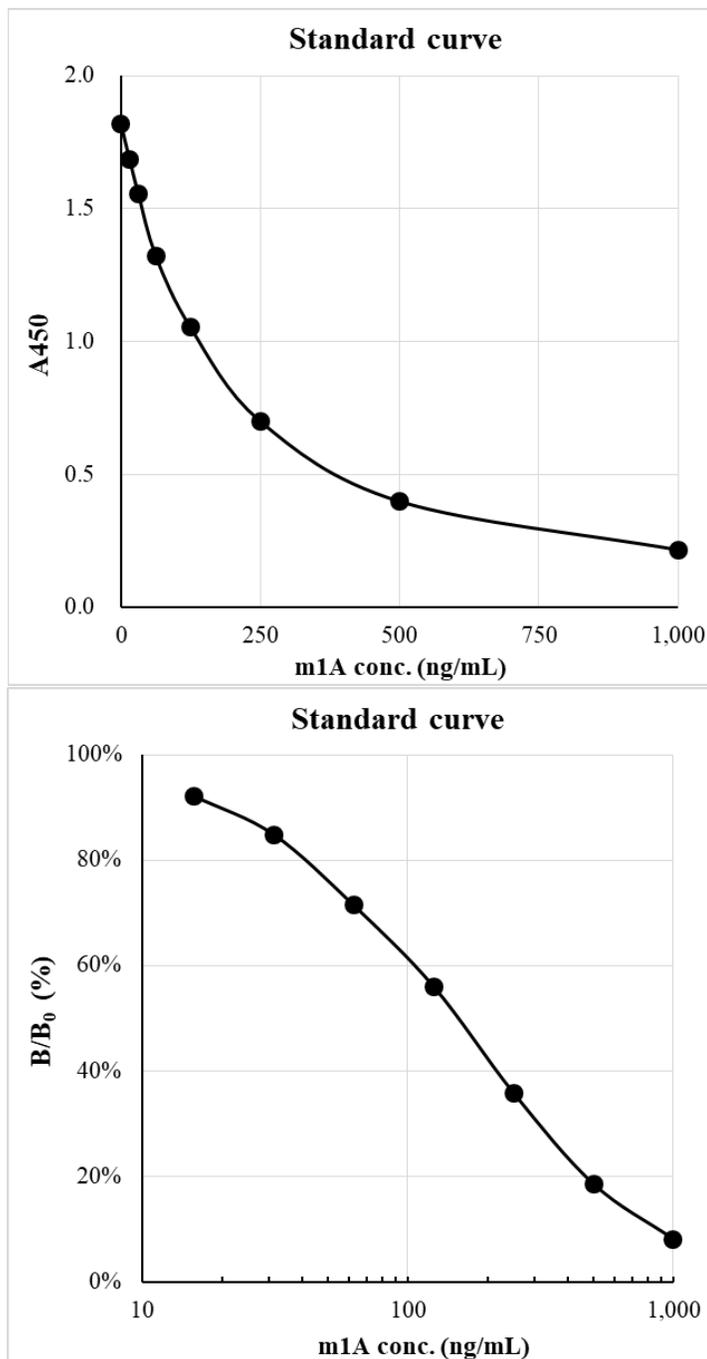
All of the reagents included in the **CircuLex m¹A (N1-methyladenosine) Competitive ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of m¹A monomer giving absorbance lower than mean absorbance of Blank minus three standard deviations of the absorbance of Blank: Blank - 3SD Blank) is better than 14.6 ng/mL of sample.

Typical Standard Curve



2. Precision

Intra-assay Precision (Precision within an assay)

Three urine samples of known concentration were tested 16 times on one plate to assess intra-assay precision.

- Intra-assay (Within-Run, n=16) CV=3.3-9.4 %

	m¹A conc. (ng/mL)		
	Sample 1	Sample 2	Sample 3
1	35.7	146.0	469.5
2	40.0	148.8	489.7
3	38.9	156.0	497.6
4	41.6	155.7	508.5
5	36.8	149.3	490.6
6	30.7	155.2	491.8
7	43.1	156.2	510.6
8	39.4	151.3	506.0
9	39.6	143.6	455.9
10	40.7	161.2	471.6
11	46.4	150.0	470.4
12	41.6	158.2	488.1
13	43.3	159.7	464.7
14	37.3	158.4	485.3
15	44.3	156.5	495.6
16	39.9	153.8	488.0
MAX.	46.4	161.2	510.6
MIN.	30.7	143.6	455.9
MEAN	40.0	153.7	486.5
S.D.	3.75	5.05	16.11
C.V.	9.4%	3.3%	3.3%

Inter-assay Precision (Precision between assays)

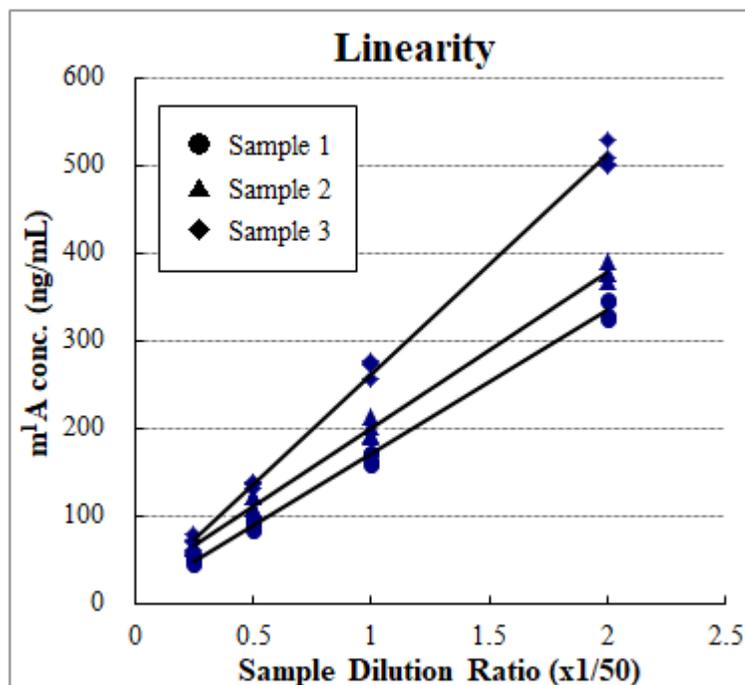
Three urine samples of known concentration were tested in five separate assays to assess inter-assay precision.

- Inter-assay (Run-to-Run, n=5) CV=4.2-12.8 %

	m¹A conc. (ng/mL)		
	Sample 1	Sample 2	Sample 3
1	38.6	139.1	417.8
2	45.8	143.5	439.1
3	40.1	152.2	415.0
4	45.7	162.0	449.9
5	33.3	146.1	455.1
MAX.	45.8	162.0	455.1
MIN.	33.3	139.1	415.0
MEAN	40.7	148.6	435.4
S.D.	5.23	8.85	18.29
C.V.	12.84%	5.96%	4.20%

3. Linearity

To assess the linearity of the assay, three urine samples were serially diluted with the Dilution Buffer from 25 to 200-fold and assayed (n=4).



	Dilution	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample 1	1/25	337.4	333.9	101.0%
	1/50	166.9	166.9	100.0%
	1/100	92.0	83.5	110.2%
	1/200	50.5	41.7	121.1%
Sample 2	1/25	378.6	333.9	113.4%
	1/50	166.9	166.9	100.0%
	1/100	115.6	83.5	138.5%
	1/200	65.1	41.7	155.9%
Sample 3	1/25	509.9	333.9	152.7%
	1/50	166.9	166.9	100.0%
	1/100	136.5	83.5	163.5%
	1/200	71.3	41.7	170.7%

50-fold dilution is recommended.

4. Spiking Recover

Urine samples were spiked with different amounts of m¹A monomer and assayed. The recovery of m¹A monomer spiked to levels throughout the range of the assay was evaluated.

Sample Average % Recovery Range: human urine (n=4), 81.7 – 138.0 %

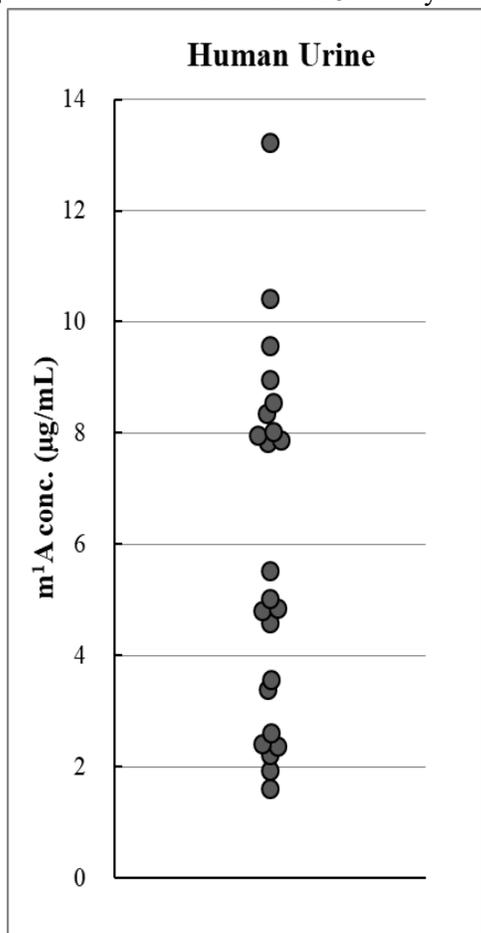
Sample 1				
	None	+ 400 ng/mL	+ 125 ng/mL	+ 40 ng/mL
Average (ng/mL)	245.4	588.9	420.6	309.2
Recovery rate (%)	-	95.3%	114.6%	138.0%

Sample 2				
	None	+ 400 ng/mL	+ 125 ng/mL	+ 40 ng/mL
Average (ng/mL)	166.8	509.5	319.8	204.6
Recovery rate (%)	-	95.0%	100.1%	81.7%

Sample 3				
	None	+ 400 ng/mL	+ 125 ng/mL	+ 40 ng/mL
Average (ng/mL)	62.2	405.8	208.5	107.1
Recovery rate (%)	-	95.3%	95.8%	97.4%

Example of Test Results

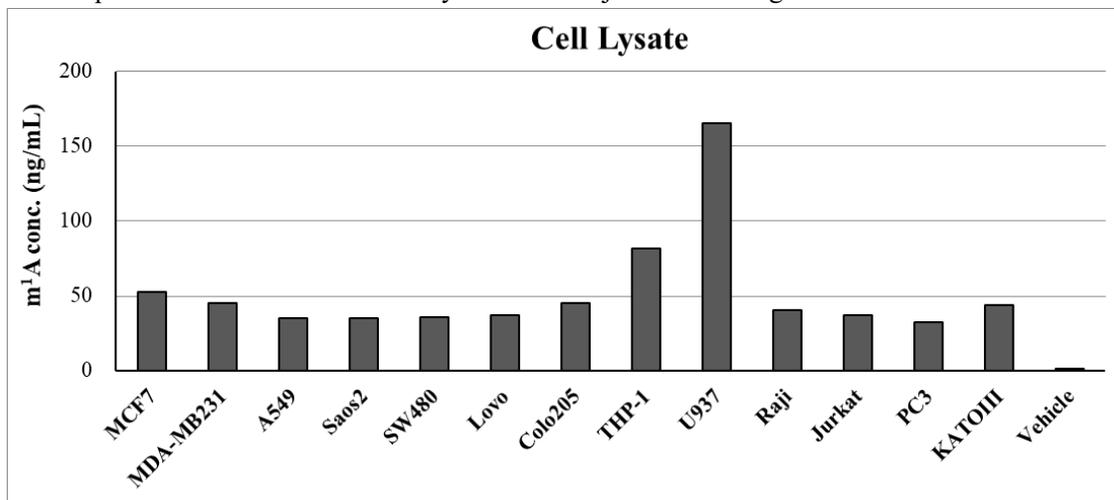
Fig.1 Concentrations of m¹A in 23 healthy volunteer's urine.



m ¹ A conc. (µg/mL)	
MAX.	13.22
MIN.	1.60
MEAN	5.89

Fig.2 Concentrations of m¹A in cell lysate.

The protein concentration of these lysates were adjusted to 0.2 mg/mL.



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