



- (51) **International Patent Classification:**
A61K 38/48 (2006.01) *C12N 9/78* (2006.01)
- (21) **International Application Number:**
PCT/US2016/049407
- (22) **International Filing Date:**
30 August 2016 (30.08.2016)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
62/211,948 31 August 2015 (31.08.2015) US
- (71) **Applicant:** **THE UNIVERSITY OF CHICAGO**
[US/US]; 5801 S. Ellis Ave., Chicago, IL 60637 (US).
- (72) **Inventors:** **HE, Chuan**; c/o The University of Chicago,
5801 S. Ellis Ave., Chicago, IL 60637 (US). **CHEN, Kai**;
c/o The University of Chicago, 5801 S. Ellis Ave., Chicago,
IL 60637 (US). **DAI, Qing**; c/o The University of
Chicago, 5801 S. Ellis Ave., Chicago, IL 60637 (US).
- (74) **Agent:** **STELLMAN, Laurie**; Norton Rose Fulbright US
LLP, 98 San Jacinto Blvd, Suite 1100, Austin, TX 78701
(US).
- (81) **Designated States** (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*

(54) **Title:** COMPOSITION AND METHODS FOR DETECTING ADENOSINE MODIFICATIONS

(57) **Abstract:** The current disclosure relates to methods, compositions and kits for detecting modified adenosine in a target RNA molecule. Aspects relate to a method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising contacting the target RNA with an adenosine deaminase enzyme (adenosine deaminase, RNA-specific) to generate a target RNA with deaminated adenosines and sequencing the target RNA with deaminated adenosines; wherein the modified adenosine is detected when the nucleotide sequence is adenosine.



WO 2017/040477 A1

COMPOSITION AND METHODS FOR DETECTING ADENOSINE MODIFICATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. Provisional Patent Application
5 No. 62/211,948, filed August 31, 2015, which is hereby incorporated by reference in its
entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under contract R01 HG006827
awarded by the National Institutes of Health. The government has certain rights in the
10 invention.

BACKGROUND OF THE INVENTION

I. FIELD OF THE INVENTION

[0003] The present invention relates generally to the field of molecular biology. More
particularly, it concerns methods and compositions for detecting, evaluating, sequencing,
15 and/or mapping modified adenosines.

II. BACKGROUND

[0004] A central question of biology is how the flow of genetic information from DNA to
RNA to protein is regulated. While transcriptional regulation—the production of messenger
RNA (mRNA)—plays major roles, and has been extensively studied, protein expression
20 ultimately determines biological phenotypes. Protein production is augmented by various
post-transcriptional regulations such as mRNA structure, microRNA, and mRNA translation;
each of these processes fundamentally affects the protein levels and localizations that
eventually impact every biological process.

[0005] Reversible and dynamic mRNA and long non-coding RNA (lncRNA) modifications
25 were recently discovered as being a fundamental mechanism that broadly controls protein
expression at the post-transcriptional level (Jia G, *et al.*, Nat Chem Biol. 2011;7(12):885-7; Liu J,
et al., Nat Chem Biol. 2014;10(2):93-5; Wang X, *et al.*, Nature. 2014;505(7481):117-20; Zheng G, *et al.*,
Mol Cell. 2013;49(1):18-29; and Fu Y, *et al.*, Nat Rev Genet. 2014;15(5):293-306).

[0006] Since then, there has been extensive research interests in profiling various mRNA/lncRNA modifications such as *N*⁶-methyladenosine (m⁶A) based on antibodies or pseudouridine (Ψ) based on a chemical reaction (Carlile TM, *et al.*, Nature. 2014; 515(7525):143-6; Schwartz S, *et al.*, Cell. 2014;159(1):148-62; Dominissini D, *et al.*, Nature. 2012;485(7397):201-6; and Meyer K, Cell. 2012;149(7):1635-46). These studies have identified the presence of a very large number of modification sites, leading to the current high interests in the epitranscriptome field. Functional explorations of RNA modifications in various biological processes have so far uncovered several new gene expression regulatory mechanisms (Liu J, *et al.*, Nat Chem Biol. 2014;10(2):93-5; Wang X, *et al.*, Nature. 2014;505(7481):117-20; Zheng G, *et al.*, Mol Cell. 2013;49(1):18-29; Batista PJ, *et al.*, Cell Stem Cell. 2014;15(6):707-19; Chen T, *et al.*, Cell Stem Cell. 2015;16(3):289-301; Geula S, *et al.*, Science. 2015;347(6225):1002-6; Ping X-L, *et al.*, Cell Res. 2014;24(2):177-89; Schwartz S, *et al.*, Cell. 2013;155(6):1409-21; and Wang Y, *et al.*, Nat Cell Biol. 2014;16(2):191-8). RNA modification is a highly fertile ground where additional regulatory mechanisms will be discovered. In particular, mRNA/lncRNA modifications are expected to be increasingly associated with human health and diseases as the field progresses.

[0007] Despite the functional significances and potential associations with human diseases, mRNA/lncRNA modifications have been studied with methods confined to either antibody-based immunoprecipitations or applications of decades-old chemical approaches; all these methods are significantly limited in resolution and sensitivity. Therefore, there is a need in the art for new methods of detecting RNA modifications.

SUMMARY OF THE INVENTION

[0008] The current disclosure addresses the aforementioned need in the art and describes a new generation of sequencing technology that can be applied generally in order to obtain highly sensitive and single-base-resolution mapping of different RNA modifications. Described herein is a method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising contacting the target RNA with an adenosine deaminase enzyme to generate a target RNA with deaminated adenosines and sequencing the target RNA with deaminated adenosines; wherein any modified adenosine in the target RNA is read as an adenosine in the sequencing of the target RNA with deaminated adenosines. In some embodiments of any of the methods, kits, and compositions described herein, the adenosine deaminase enzyme is ADAR (adenosine deaminase, RNA-specific). In some embodiments, the adenosine deaminase enzyme is RNA specific. In some embodiments, the adenosine deaminase enzyme lacks significant sequence specificity or is non-sequence specific. In

some embodiments, the adenosine deaminase enzyme works on double-stranded nucleic acids.

[0009] Adenosine deaminase enzymes include, for example, adenosine deaminases from any organisms such as humans (ADA, GenBank Accession: NP_000013), mouse (ADA, 5 GenBank Accession: NP_001258981), *Drosophila melanogaster* (SEQ ID NO:1); RNA adenosine deaminase from humans (GenBank Accession: AAB97118), cows (GenBank Accession: XP_010801274.1), rat (GenBank Accession: EDM00617). The sequences associated with each of these is herein incorporated by references in their entirety.

[0010] In some embodiments, the method further comprises contacting target RNA with a 10 demethylating enzyme; contacting the demethylated target RNA with the adenosine deaminase enzyme to produce control RNA; and sequencing the control RNA; wherein any modified adenosine in the target RNA is read as a guanosine in the sequencing of the control RNA with deaminated adenosines. In further embodiments, the demethylated target RNA is made by methods and steps described herein for generating controls. In some embodiments, 15 the method further comprises comprising comparing the sequence of the target RNA with dominated deaminated adenosines to the sequence of the demethylated target RNA. In some embodiments, the demethylating enzyme is an N6-methyladenosine-specific demethylating enzyme. In some embodiments, the demethylating enzyme is ALKBH5 or FTO. FTO (fat mass and obesity associated) and ALKBH5 (alkB homolog 5, RNA demethylase or alkB, 20 alkylation repair homolog 5) are demethylating enzymes specific for *N*⁶-methyladenosine. FTO and ALKBH5 are known in the art. The enzyme may be recombinantly made or synthetic, and may be from any species. In some embodiments, the enzyme is the mammalian enzyme. The human ALKBH5 is represented by GenBank Accession Nos: NM_017758.3 (mRNA) and NP_060228.3 (protein). The mouse ALKBH5 is represented by 25 GenBank Accession Nos.: NM_172943.4 (mRNA) and NP_766531.2 (protein). The human FTO is represented by GenBank Accession Nos.: XM_011523313.1 (mRNA), XP_011521615.1 (protein), XM_011523316.1 (mRNA), XP_011521618.1 (protein), XM_011523314.1 (mRNA) XP_011521616.1 (protein), XM_011523315.1 (mRNA), and XP_011521617.1 (protein). In some embodiments, the demethylating enzyme is ALKBH5. 30 The sequences associated with each of these GenBank accession numbers is herein incorporated by reference for all purposes. In some embodiments, the demethylating enzyme

is from insects. In some embodiments, the demethylating enzyme is from *Drosophila melanogaster*.

[0011] In some embodiments, the target and/or demethylated target RNA is in a duplex with a complementary strand of RNA or DNA. In some embodiments, the complementary strand is DNA. In some embodiments, the complementary strand comprises modified adenosine. The modified adenosine may be one known in the art and/or described herein. In some embodiments, the modified adenosine is *N*⁶-methyladenosine or *N*¹-methyladenosine.

[0012] ADAR encodes the enzyme responsible for RNA editing by site-specific deamination of adenosines. This enzyme destabilizes double-stranded RNA through conversion of adenosine to inosine. The ADAR may be from any organisms such as human, mouse, insect, etc... In some embodiments, the ADAR is from *Drosophila melanogaster*, which is abbreviated dADAR. In some embodiments, the ADAR is from insects. The ADAR may be synthetically made or recombinantly made. Methods of producing and purifying enzymes are known in the art.

[0013] The target RNA may be any type of RNA in a cell. In some embodiments, the target RNA is mRNA, lncRNA, pri-microRNA, pre-piRNA, rRNA, tRNA, snoRNA, or snRNA. In some embodiments, the target RNA is mRNA or lncRNA. In some embodiments, the method further comprises isolating RNA. In some embodiments, the method comprises isolating a specific RNA. The term isolating, in this context, refers to the separation of one type of RNA from other types of RNA. Therefore, the isolated RNA fraction may contain at least, at most, or exactly about 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% (or any derivable range therein) of a specific RNA type. In some embodiments, the term isolated also refers to something that is separated from cellular components and/or is free of cellular materials.

[0014] In some embodiments, the method further comprises generating a nucleic acid strand that is complementary with the target and/or demethylated target RNA and hybridizing the complementary nucleic acid strand with the target RNA and/or demethylated target RNA. Methods of generating complementary nucleic acid strands are known in the art and described herein. In some embodiments, generating the nucleic acid strand that is complementary with the target and/or demethylated target RNA comprises synthesis of a nucleic acid strand complementary to the target and/or demethylated target RNA. In some

embodiments, the synthesis of the nucleic acid strand comprises a synthesis reaction composition comprising adenosine triphosphate, wherein all of the adenosine triphosphate is modified adenosine triphosphate. In some embodiments, the complementary nucleic acid strand comprises modified adenosines.

5 **[0015]** When the term “modified adenosines” is used herein, the modified adenosine may be any modified adenosine known in the art and/or described herein. In some embodiments of the methods, compositions, and kits of the disclosure, the modified adenosine is N^6 -methyladenosine. In some embodiments, the modified adenosine is N^1 -methyladenosine. In some embodiments, the nucleic acid may comprise more than one type of adenosine
10 modification. In some embodiments, the method further comprises determining the type of modification in a type of RNA.

[0016] In some embodiments, the complementary nucleic acid strand is RNA. In some embodiments, the complementary nucleic acid strand is DNA.

[0017] In some embodiments, the target and/or demethylated target RNA is contacted with
15 an RNA polymerase to synthesize a complementary RNA strand. The RNA polymerase may be any RNA polymerase known in the art. In some embodiments, the method further comprises contacting the target and/or demethylated target RNA with a RNA replicase to synthesize a complementary RNA strand. In some embodiments, the RNA replicase is Phi6. In further embodiments, the RNA replicase is one known in the art. In some embodiment,
20 the method comprises synthesis of a complementary nucleic acid from a cDNA library. In some embodiments, the complementary nucleic acid strand is RNA.

[0018] In some embodiments, the target RNA is immobilized on a solid support. Solid supports are known in the art and include, for example, glass, plastics, polymers, metals, metalloids, ceramics, organics, beads, agarose, cellulose, dextran (commercially available as,
25 i.e., Sephadex, Sepharose) carboxymethyl cellulose, polystyrene, polyethylene glycol (PEG), filter paper, nitrocellulose, ion exchange resins, plastic films, polyaminemethylvinylether maleic acid copolymer, glass beads, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc.

[0019] In some embodiments, the target RNA strand is labeled. As used herein, the term
30 “label” intends a directly or indirectly detectable compound or reactable functional group useful for attachment of nucleic acids to solid supports. The label may also be conjugated

directly or indirectly to the composition to be detected, e.g., polynucleotide or protein such as an antibody so as to generate a "labeled" composition. The term also includes sequences conjugated to the polynucleotide that will provide a signal upon expression of the inserted sequences, such as green fluorescent protein (GFP) and the like. The label may be detectable
5 by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. The labels can be suitable for small scale detection or more suitable for high-throughput screening. As such, suitable labels include, but are not limited to radioisotopes, fluorochromes, chemiluminescent compounds, dyes, and proteins, including enzymes. The
10 label may be simply detected or it may be quantified. A response that is simply detected generally comprises a response whose existence merely is confirmed, whereas a response that is quantified generally comprises a response having a quantifiable (e.g., numerically reportable) value such as an intensity, polarization, and/or other property. In luminescence or fluorescence assays, the detectable response may be generated directly using a luminophore
15 or fluorophore associated with an assay component actually involved in binding, or indirectly using a luminophore or fluorophore associated with another (e.g., reporter or indicator) component.

[0020] In some embodiments, the label is a compound or reactable functional group useful for attachment of a nucleic acid to a solid support. In some embodiments, the label is a
20 phosphorothioate group. In some embodiments, the target RNA is immobilized by reaction of the phosphorothioate group with a thiol-reactive group. In some embodiments, the thiol-reactive group is iodoacetamide, maleimide, or methanethiosulfonate.

[0021] In some embodiments, the target RNA is fragmented prior to immobilization on the solid support. In some embodiments, the RNA is fragmented into RNA molecules 50-300
25 nucleotides in length. In some embodiments, the RNA is fragmented into RNA molecules 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 350, 400, 450, or 500 to 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 350, 400, 450, 500, 1000, 1500, or 2000 (or any range derivable therein) nucleotides in length. In some
30 embodiments, the average RNA molecule fragment is about 25, 50, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 220, 240, 260, 280, 300, 325, 350, 375, 400, 450, 500, or 600 nucleotides in length. In some embodiments, the target RNA is fragmented prior to immobilization.

[0022] In some embodiments, the method comprises one or more of the following steps: fragmenting the target RNA; contacting the target and/or demethylated target RNA with RNA replicase to synthesize a complementary RNA strand that forms a duplex with the target RNA or demethylated target RNA; denaturing of the RNA duplex to create mobilized complementary RNA; further comprises removal of the mobilized complementary RNA; and/or mobilizing the target RNA by separating it from the solid support. In some embodiments, mobilizing the target RNA is done by silver (I)-mediated cleavage (for phosphothiolate linkage). In some embodiments, mobilizing the target RNA further comprises proteinase K digestion and/or biotin completion (for biotin-based linkage). In some embodiments, sequencing of the target RNA comprises construction of a library of nucleic acid molecules comprising the target RNA sequence.

[0023] In some embodiments, the method comprises the steps of a) immobilizing the target RNA, b) contacting the target and/or demethylated target RNA with RNA replicase to synthesize a complementary RNA strand that forms a duplex with the target RNA or demethylated target RNA, c) contacting the target RNA with an adenosine deaminase enzyme to generate a target RNA with deaminated adenosines, d) denaturing of the RNA duplex to create mobilized complementary RNA; e) removal of the mobilized complementary RNA; and f) sequencing of the target RNA. In some embodiments, the steps a-f are sequential. In some embodiments, step a is performed followed by iterative rounds of the ordered steps of b-e, followed by step f. In some embodiments, steps b-e are repeated in iterative cycles for one or more times prior to step f. For example, one method includes three iterative rounds of steps b-e, which includes performing the following steps in the following order: step a, b, c, d, e, b, c, d, e, b, c, d, e, f. In some embodiments, steps b-e are repeated at least, at most, or exactly 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 50, 75, or 100 times (or any derivable range therein).

[0024] In some embodiments, the method further comprises contacting the target and/or demethylated target RNA with reverse transcriptase to synthesize a complementary DNA strand.

[0025] The target and/or demethylated target RNA may be of any length. In some embodiments, the target RNA is 10-1000 nucleic acids in length. In some embodiments the target RNA is at least, at most, or exactly about 5, 10, 20, 30, 40, 50, 60, 70, 80, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 7500, or

10000 nucleic acids in length, or any derivable range therein. In some embodiments, nucleic acid molecules may be DNA, RNA, or a combination of both. Nucleic acids may be recombinant, genomic, or synthesized. In additional embodiments, methods involve nucleic acid molecules that are isolated and/or purified. The nucleic acid may be isolated from a cell
5 or biological sample in some embodiments. Certain embodiments involve isolating nucleic acids from a eukaryotic, mammalian, or human cell. In some cases, they are isolated from non-nucleic acids. In some embodiments, the nucleic acid molecule is eukaryotic; in some cases, the nucleic acid is mammalian, which may be human. This means the nucleic acid molecule is isolated from a human cell and/or has a sequence that identifies it as human. In
10 particular embodiments, it is contemplated that the nucleic acid molecule is not a prokaryotic nucleic acid, such as a bacterial nucleic acid molecule. In additional embodiments, isolated nucleic acid molecules are on an array. In particular cases, the array is a microarray. In some cases, a nucleic acid is isolated by any technique known to those of skill in the art, including, but not limited to, using a gel, column, matrix or filter to isolate the nucleic acids. In some
15 embodiments, the gel is a polyacrylamide or agarose gel.

[0026] In some embodiments, the sequence of the target and/or demethylated target RNA is known. The term sequence as used herein refers to the nucleotide sequence such as “A” for adenosine, “G” for guanine, “C” for cytosine, “T” for thymine, and “U” for uracil. Eventhough the sequence is known, it may not be known whether the nucleic acid bases are
20 modified or unmodified.

[0027] In some embodiments, the method further comprises comparing the known sequence of the target RNA with the sequence of the target RNA with deaminated adenosines.

[0028] In some embodiments, contacting the target and/or demethylated target RNA with
25 ADAR is done in the presence of GTP. In some embodiments, the GTP is in a concentration of 0.5-5 mM. In some embodiments the concentration of GTP in the solution is at least, at most, or exactly about 0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, 10, 15, 20, 30, 40, 50, 100, 200, 300, 400, or 500 mM (or any derivable range therein).

[0029] In some embodiments, the target RNA molecule is in a duplex with a DNA
30 molecule. In some embodiments, the method comprises contacting the RNA-DNA duplex

with a DNA digesting agent prior to sequencing the RNA. The DNA digesting enzyme may be sequence specific or non-specific. In some embodiments, the DNA digesting enzyme is DNase.

[0030] In some embodiments, the method further comprises the steps of: a) generating a DNA strand that is complementary to the target and/or demethylated target RNA and hybridizing the complementary DNA strand with the target and/or demethylated target RNA to generate an RNA-DNA duplex; b) contacting the RNA-DNA duplex with the adenosine deaminase enzyme; and c) contacting the RNA-DNA duplex with a DNA digesting agent; wherein all the steps are done prior to DNA target RNA sequencing. In some embodiments, the method further comprises repeating steps a, b, and/or c one or more times. In some embodiments, the steps are repeated at least two more times. In some embodiments, the steps a, b, and/or c are repeated at least, at most, or exactly 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 more times, or any derivable range therein. In some embodiments, the method further comprises step of strand separation after step b). In some embodiments, the method further comprises purification of the target and/or demethylated target RNA after step c) and prior to DNA target RNA sequencing.

[0031] In some embodiments, the method further comprises providing a quantification RNA control comprising a known percentage of modified adenosine; contacting the quantification control RNA with the adenosine deaminase enzyme; and sequencing the deaminated quantification control RNA. In some embodiments, 0, 25, 50, 75, or 100% of the adenosine in the quantification RNA control is modified. In some embodiments, the method comprises at least, at most, or exactly 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 quantification controls (or any derivable range therein). In each of the quantification controls, at least, at most, or exactly 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100% (or any derivable range therein) of the adenosines may be modified.

[0032] Further aspects of the disclosure relate to a kit comprising: an adenosine deaminase enzyme and instructions for detecting modified adenosines in target RNA. In some embodiments, the kit further comprises a control nucleic acid. The control nucleic acid may be any embodiment described herein. In some embodiments, the control nucleic acid comprises a non-naturally occurring nucleic acid or non-widely present nucleic acid. In some embodiments, the control nucleic acids are a non-naturally occurring nucleic acid sequence. In some embodiments, the control nucleic acid comprises RNA or DNA

comprising modified adenosines. In some embodiments, the percentage of adenosines that are modified in the control nucleic acid is known. In some embodiments, at least, at most, or exactly 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100% (or any derivable range therein) of the adenosines in the control nucleic acid are modified. In some embodiments, the control nucleic acid comprises a duplex with a complementary nucleic acid strand. In some embodiments, the complementary nucleic acid strand is DNA. In some embodiments, the complementary nucleic acid strand is RNA with modified adenosines. In some embodiments, the kit further comprises an adenosine demethylase. The adenosine demethylase may be one known in the art and/or described herein. In some embodiments, the adenosine demethylase is specific for *N*⁶-methyladenosine. In some embodiments, the adenosine demethylase is specific for *N*¹-methyladenosine. In some embodiments, the kit further comprises an adenosine deaminase enzyme reaction composition comprising GTP. In some embodiments, the kit further comprises a DNase. In some embodiments, the kit further comprises a reverse transcriptase. In some embodiments, the kit further comprises a molecule or embodiment described herein in the methods and compositions. In some embodiments, the kit further comprises reagents to perform the method steps described herein, such as immobilization reagents, enzymes, and buffers described throughout the disclosure.

[0033] Further aspects relate to a method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising contacting a double-stranded nucleic acid molecule comprising the target RNA with the adenosine deaminase enzyme to generate a target RNA with deaminated adenosines and sequencing the target RNA with deaminated adenosines; wherein the modified adenosine is detected when the sequence of the target RNA with deaminated adenosines is adenosine.

[0034] A further aspect relates to a method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising: a) providing a target RNA; b) generating a DNA strand that is complementary with the target RNA and hybridizing the complementary DNA strand with the target RNA to generate a RNA-DNA duplex comprising the target RNA; c) contacting the RNA-DNA duplex with the adenosine deaminase enzyme to generate target RNA with deaminated adenosines; and d) contacting the RNA-DNA duplex with a DNA digesting agent; and e) sequencing the target RNA with the deaminated adenosines.

[0035] A further aspect relates to a method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising: a) providing a target RNA; b) generating a DNA strand that is complementary with the target RNA and hybridizing the complementary DNA strand with the target RNA to generate a RNA-DNA duplex comprising the target RNA; c) contacting the RNA-DNA duplex with the adenosine deaminase enzyme to generate target RNA with deaminated adenosines; and d) contacting the RNA-DNA duplex with a DNA digesting agent; e) repeating steps b, c, and d one or more times; and f) sequencing the target RNA with the deaminated adenosines.

[0036] A further aspect relates to a method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising: a) providing a target RNA; b) generating an RNA strand with modified adenosine that is complementary with the target RNA to generate an RNA-RNA duplex comprising the target RNA; c) contacting the RNA-RNA duplex with the adenosine deaminase enzyme to generate target RNA with deaminated adenosines; and d) sequencing the target RNA with the deaminated adenosines.

[0037] In certain embodiments, the enzymes and/or nucleic acids used in the methods, kits, and compositions described herein may comprise one or more detectable moieties and/or modification. A detectable moiety refers to a chemical compound or element that is capable of being detected. In certain embodiments, a detectable moiety is fluorescent, radioactive, enzymatic, electrochemical, or colorimetric. In some embodiments, the detectable moiety is a fluorophore or quantum dot. In some embodiments, a modification moiety may be a linker that allows one or more functional or detectable moieties or isolation tags to be attached to the molecules. In some embodiments the linker is an azide linker or a thiol linker. In further embodiments, the modification moiety may be an isolation tag, which means the tag can be used to isolate a molecule that is attached to the tag. In certain embodiments, the isolation tag is biotin, Flag, or a histidine tag. In some cases, the tag is modified, such as with a detectable moiety. It is contemplated that the linker allows for other chemical compounds or substances to be attached to the molecule.

[0038] Methods and compositions may also involve one or more enzymes. In some embodiments, the enzyme is a restriction enzyme or a polymerase. In certain cases, embodiments involve a restriction enzyme. The restriction enzyme may be methylation-insensitive. In other embodiments, the enzyme is polymerase.

[0039] Methods may involve identifying adenosine modifications in the nucleic acids by comparing modified nucleic acids with unmodified nucleic acids or to nucleic acids whose modification state is already known. Detection of the modification can involve a wide variety of recombinant nucleic acid techniques. In some embodiments, a modified nucleic acid molecule is incubated with polymerase, at least one primer, and one or more nucleotides under conditions to allow polymerization of the modified nucleic acid. In additional embodiments, methods may involve sequencing a modified nucleic acid molecule. In other embodiments, a modified nucleic acid is used in a primer extension assay.

[0040] Methods and compositions may involve a control nucleic acid. In addition to the controls described herein, control may also be used to evaluate whether modification or other enzymatic or chemical reactions are occurring. Alternatively, the control may be used to compare modification states. The control may be a negative control or it may be a positive control. It may be a control that was not incubated with one or more reagents in the modification reaction. Alternatively, a control nucleic acid may be a reference nucleic acid, which means its modification state (based on qualitative and/or quantitative information related to modification at adenosines, or the absence thereof) is used for comparing to a nucleic acid being evaluated. In some embodiments, multiple nucleic acids from different sources provide the basis for a control nucleic acid. In some embodiments, the control is a pool of target RNA that has undergone demethylation. Moreover, in some cases, the control nucleic acid is from a normal sample with respect to a particular attribute, such as a disease or condition, or other phenotype. In some embodiments, the control sample is from a different patient population, a different cell type or organ type, a different disease state, a different phase or severity of a disease state, a different prognosis, a different developmental stage, etc.

[0041] Embodiments also concern kits, which may be in a suitable container, that can be used to achieve the described methods. In further embodiments, a kit may include one or more buffers, such as buffers for nucleic acids or for reactions involving nucleic acids. Other enzymes may be included in kits in addition to the adenosine deaminase enzyme. In some embodiments, an enzyme is a polymerase. Kits may also include nucleotides for use with the polymerase. In some cases, a restriction enzyme, (e.g. DNase) is included in addition to or instead of a polymerase.

[0042] Other embodiments also concern an array or microarray containing nucleic acid molecules that have been modified at adenosines.

[0043] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[0044] It is contemplated that any embodiment discussed herein can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

[0045] Throughout this application, the term “about” is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[0046] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” It is also contemplated that anything listed using the term “or” may also be specifically excluded.

[0047] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0048] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

[0049] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0050] **FIG. 1.** The relatively abundances of known internal modifications in mammalian mRNA and lncRNA as measured by LC-MS/MS.

[0051] **FIG. 2.** The m⁶A modification affects a wide range of different properties and functions associated with mRNA and lncRNA.

5 [0052] **FIG. 3.** The general distribution of m⁶A in mammalian mRNA.

[0053] **FIG. 4.** The reversible m¹A58 tRNA methylation controls tRNA utility in translation.

[0054] **FIG. 5A-B.** To differentiate a modified base from the unmodified base in DNA and RNA sequencing, a method to selectively “mutate” the unmodified or modified base is required. In bisulfite sequencing (**A**), the bisulfite treatment selectively converts C to U. We
10 propose strategies to selectively mutate A (but not m⁶A) to I (**B**) by deamination.

[0055] **FIG. 6A-B.** The strategy of ADAR-mediated adenosine deamination sequencing (Deam-m⁶A-seq). (**A**) The drosophila or mammalian RNA adenosine deaminase (ADAR) can effectively convert A to I in duplex RNA; however, its activity towards m⁶A in duplex
15 RNA is <2% of that for A. (**B**) We can employ ADAR to convert A to I while maintaining m⁶A as A in Deam-m⁶A-seq.

[0056] **FIG. 7.** The replacement of A with m⁶A on the “anti-sense” strand significantly improves deamination ratio by recombinant dADAR (SEQ ID NOs: 2 and 3).

[0057] **FIG. 8A-C.** A strategy to couple Deam-m⁶A-seq with ALKBH5-mediated
20 demethylation for accurate m⁶A assignments. (**A**) The isolated mRNA is split into two portions, one treated with ALKBH5, the other untreated. Both groups are then supplied with anti-sense RNA strand to form dsRNA, which is the natural substrate of the dADAR-mediated deamination reaction. The product is subjected to RT-PCR and high-throughput sequencing. The A-to-G transitions observed in the demethylated sample represent
25 methylation sites in RNA. The +demethylation process exemplifies the production of the control RNA by demethylation of a pool of target RNA. (**B**) ALKBH5 catalyzes the demethylation of close to 90% of all m⁶A in isolated mammalian polyA-tailed RNA revealed by LS-MS/MS.(SEQ ID NOs. 2 and 3) (**C**) The tandem demethylation followed by the deamination procedure was applied to a dsRNA substrate with 1:1 mixture of A:m⁶A at every

adenosine position in the target strand. The A-to-G transition ratios of the target strand revealed significant deamination differences before and after the demethylation treatment.

[0058] **FIG. 9A-B.** (A) A new buffer system (buffer 2 – second bar of each series) leads to elevated deamination of A in dsRNA and noticeable deamination of A in a RNA-DNA hybrid duplex by dADAR. (B) Iterative RT-deamination-DNA digestion-RT to improve the deamination efficiency of A using RNA-DNA hybrid duplex as the substrate. An example sequence is shown here (SEQ ID NOs: 4-10).

[0059] **FIG. 10A-B.** Preparation of dADAR substrate by using (A) Phi6 RNA replicase with *N*⁶-methyl-ATP to form dsRNA and (B) SuperScript II Reverse Transcriptase (or other alternative reverse transcriptases) to form RNA-DNA hybrid.

[0060] **FIG. 11A-B.** Depicted is the scheme of the immobilization and iterative Deam-seq strategy (A). ATP γ S is Adenosine 5'-[γ -thio]triphosphate, in which γ -phosphate is modified with a sulfur and transferred by T4 PNK to oligonucleotides for further reaction (B).

[0061] **FIG. 12A-C.** (A) Coomassie brilliant blue staining of recombinant His₆-Phi6 RNA replicase purified from *E. coli*, after Source Q ion exchange. (B) Adapted Bismark analysis workflow demonstrated how A-to-G transition containing read is precisely mapped back to the reference genome. (C) MTSEA-biotin phosphothioate labeling. The reactions were monitored by MALDI-TOF. (C(1)) The RNA probe control. (C(2)) The phosphothioate added probe. (C(3)) The biotinylated probe. (C(4)) The DTT mediated cleaved probe.

DETAILED DESCRIPTION OF THE INVENTION

[0062] Certain embodiments are directed to methods and compositions for detecting modified adenosine in a target ribonucleic acid (RNA) by contacting the target RNA with an adenosine deaminase enzyme (adenosine deaminase, RNA-specific). In some embodiments, the adenosine deaminase enzyme is ADAR. The ADAR enzyme deaminates unmodified adenosines, converting them to inosines. ADAR enzymes, such as those expressed and purified from insect cells, are highly active against unmodified adenosines, but possesses over 50-fold lower activity towards modified adenosines, such as m⁶A, for example, compared to unmodified adenosine (Véliz EA, *et al.*, Journal of the American Chemical Society. 2003;125(36):10867-76). Therefore, after selective deamination of all adenosines in

the transcriptome and subsequent reverse transcriptase (RT)-PCR followed by sequencing, only modified adenosines will be read as “A” (FIG. 5A).

I. RNA modification

[0063] Dynamic chemical modifications of DNA and histone proteins represent
5 fundamental mechanisms of biological regulation. Post-transcriptional modifications are also
ubiquitous in RNA. To date, over 100 different RNA modifications have been identified with
a wide variety of chemical diversities. Examples of such modifications can be found on the
world wide web at rna-mdb.cas.albany.edu/RNAmods/, and the contents of this website
publication are herein incorporated by reference. Unlike genomic DNA that tends to have
10 limited variation of chemical modifications, the wide variety of RNA modifications appears
to be a strategy used by nature to entail and facilitate a much greater diversity of structures
and cellular functions for different RNA species. The m⁶A modification in mRNA/lncRNA
alone is known to modulate the affinity for RNA-binding proteins, control subcellular
localization, lifetime, storage, transport, and translation of mRNAs, switch secondary
15 structures of RNAs, as well as to affect innate immune response.

[0064] The explosive discoveries of functional RNAs in the last decade have changed the
current views on the functions of RNA and biological mechanisms that control RNA.
However, the exact roles of most RNA modifications remain unknown. Certain RNA
modifications are essential for life, with defects of RNA-modifying enzymes known to be
20 associated with diverse human diseases. Most studies before 2011 on RNA modifications
were limited to the abundant RNAs such as rRNA, snRNA, or tRNA. The positions of these
modifications can be studied with RNA digestion followed by traditional liquid
chromatography separation coupled with mass spectrometry or thin-layer chromatography
due to their high abundances. The limit to these methods is their sensitivity; they cannot be
25 applied to map modifications in low abundant mRNAs and lncRNAs, most of which appear
to play critical roles in regulating gene expression. The methods of the current disclosure
provide new opportunities to investigate distributions of not only mRNA and lncRNA
modifications but also dynamic modifications on tRNA, snRNA, and rRNA that could not be
effectively probed transcriptome-wide in the past. The methods and compositions of the
30 disclosure can be readily applied to any class of RNA. The ability to identify all adenosine
modifications at single-base resolution will significantly advance the frontier of

epitranscriptomics and enable transcriptome-wide investigations that associate genomic variations and mutations with human health and diseases.

[0065] Mammalian mRNA and lncRNA can be modified at tens of thousands of sites. Many of these modifications are conserved in almost all eukaryotes. We have measured the relative abundances of all known mammalian internal mRNA modifications that include *N*⁶-methyladenosine (m⁶A), pseudouridine (Ψ), 5-methylcytosine (m⁵C), *N*¹-methyladenosine (m¹A), and 2'-O-methylation (Nm) (**FIG. 1**). Applicant's liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantifications confirmed that these five modifications are the most abundant ones in mammalian mRNA/lncRNA. As previously known, m⁶A is the most prevalent internal modification in eukaryotic mRNA with ~3 per mRNA in mammalian cells. This modification plays essential and broad roles in cell differentiation, cell development, and numerous other cellular processes. The frequency of the other modifications range from 0.2-1 per mRNA in mammalian cells; some of them have been shown to have significant functional implications.

A. *N*⁶-methyladenosine (m⁶A)

[0066] m⁶A occurs at a high frequency in RNA (*e.g.* mRNA or lnc RNA). It is also reversible and dynamically regulated. The m⁶A modification appears to affect almost every phase of mRNA metabolism and function, thereby impacting diverse biological processes. Therefore, m⁶A studies so far embody the concept of "epitranscriptome"; its functional significance and implementation are exerted by three groups of proteins: "writers" that install, "erasers" that remove, and "readers" that bind or recognize m⁶A in order to determine the cellular fate of the modified mRNA/lncRNA (**FIG. 2**).

[0067] In mammals, m⁶A is installed by a three-protein core complex comprised of two catalytic subunits, METTL3/METTL14 and an accessory factor WTAP. Depletion of METTL3 homologs readily leads to developmental arrest or defects in gametogenesis in yeast, flies, and plants. In zebra fish, knockdown of METTL3 leads to smaller head, eyes, and brain ventricle, and curved notochord. The phenotypes in mammals are more severe. Both methyltransferases METTL3 and METTL14 are essential in mammals. m⁶A is a critical regulator in the differentiation of mouse embryonic stem cells (mESCs).

[0068] The m⁶A modification plays a key role in facilitating transition of mESCs from the naïve state to the primed state upon differentiation. Mettl3-depleted mESCs preserved their

“naïve” pluripotent identity, but failed to proceed into the “primed” EpiSC-like state, hence blocking the subsequent differentiation; they also failed to differentiate into normal embryoid bodies (EBs) and mature neurons upon corresponding inductions. In naïve ESCs and primed EBs, the m⁶A modification was detected in 80% of transcripts of naïve pluripotency genes (e.g., *Nanog*, *Klf4*, *Sox2*, *Esrrb*), as well as multiple lineage commitment regulators (e.g., *Foxa2*, *Sox17*). In general, m⁶A deposition in mESCs decreases the expression of methylated transcripts and directly reduces their stability. Therefore, m⁶A loss increased the abundance and prolonged the lifetimes of naïve pluripotency transcripts. Silencing METTL3 also leads to mRNA processing delay and circadian period elongation. m⁶A depletion prolongs nuclear retention and delays nuclear exit of mature mRNAs of clock genes *Per2* and *Arntl*. This result reveals an important physiological function of m⁶A in setting the pace of the circadian cycle and determining the clock speed and stability by controlling nuclear RNA export. A recent study described that microRNAs (miRNAs) could regulate m⁶A modification via a sequence-pairing mechanism in order to modulate the binding of METTL3 to mRNA substrates. Another recent study uncovered that m⁶A on pri-miRNA plays critical roles in miRNA maturation. The inhibition of pri-miRNA methylation induced delayed maturation of 70% of all miRNA, resulting in >30% changes of mature miRNA levels.

[0069] m⁶A on mRNA can be reversed by two RNA demethylases, FTO and ALKBH5. Defects of FTO and AlkBH5 lead to altered metabolism, neural development retardation, and compromised spermatogenesis. A common variant of the *FTO* gene (an intron mutation) has been shown to generate a predisposition to obesity. Knockout mouse models revealed that FTO is important to development: most knockout mice die at the embryo state or within the first month of birth; those that survived tended to lose body weight and were smaller compared to the control mice. A mutation of the human *FTO* coding region has also been linked to mental retardation. The *Alkbh5* knockout male mice exhibit significant spermatogenesis defects with compromised fertility. The fact that FTO and ALKBH5 show noticeable but very different phenotypes in humans or mice strongly indicates that reversible m⁶A RNA methylation plays important roles in biological regulation.

[0070] m⁶A is recognized by “reader” proteins to exhibit biological functions, just like the interplay between DNA cytosine-methylation and methyl-CpG-binding proteins that regulate gene expression through binding to methylated cytosines. Applicants have identified several m⁶A-specific binding proteins in humans that belong to the YTH family: YTHDF1,

YTHDF2, and YTHDC1. All these proteins bind the m⁶A-containing RNA selectively over unmethylated RNA through direct accommodation of the methyl group in their structures. Functional characterizations revealed that YTHDF2 affects cytoplasmic localization and mediates the decay of methylated mRNA, YTHDF1 promotes translation of methylated mRNA through facilitating translation initiation, and YTHDC1 affects the nuclear export of methylated mRNA. At the organismal level, knockout of *Ythdc1* or *Ythdf2* is embryonically lethal in mouse.

[0071] Applicants have shown that m⁶A methylation can significantly affect the mRNA and lncRNA structure transcriptome-wide. The m⁶A effect on mRNA/lncRNA structure, termed “m⁶A-switch,” can dramatically affect protein-RNA interactions to impact mRNA abundance and alternative splicing of the methylated RNA. Therefore, m⁶A exerts its functions not only through being directly “read,” but also through RNA structural remodeling.

[0072] Using an antibody known to recognize m⁶A to enrich m⁶A-containing RNA fragments, transcriptome-wide profiling has been performed in human cells and mouse tissues. Both studies revealed tens of thousands of m⁶A-containing segments in mRNA and lncRNA. The m⁶A-immunoprecipitation (IP) approach uncovers “m⁶A-peaks” that are on average 100-200 bases wide in the mRNA/lncRNA. Subsequent SCARLET studies by Applicants at a dozen m⁶A sites at single-base resolution revealed sub-stoichiometry at all modification sites in mRNA/lncRNA investigated. Transcriptome-wide profiling of m⁶A during yeast meiosis and sporulation has also uncovered profound mRNA methylation, suggesting functional roles.

[0073] These global mapping studies have unveiled conserved, widespread, and dynamic mRNA methylation in eukaryotes. Three salient features of the m⁶A methylome are conserved in mammals: i) m⁶A sites are mainly confined in the consensus motif Pu[G>A]m⁶AC[U>A>C], consistent with early studies. The overall cellular m⁶A methylation accounts for at most ~15% of all consensus sequences that can be modified; ii) m⁶A marks are not equally distributed across the transcriptome; rather, they are preferentially enriched in a subset of consensus sequences near stop codons, in 3' UTRs, and within long internal exons (**FIG. 3**). This topology is preserved upon endodermal differentiation of stem cells; iii), m⁶A-modified genes are well conserved between human and mouse embryonic stem cells (ESCs) and somatic cells. For instance, ~70% of human ESC genes are also m⁶A modified in

the orthologous mouse gene with ~46% of m⁶A peak sites in common. As expected, higher m⁶A peak intensities were detected in conserved sites compared to those that are not conserved. On the other hand, distinct m⁶A patterns can also be detected among different species or cells in different developmental stages. As expected, certain m⁶A modifications are tissue-specific and dynamically altered in response to different stimuli, again indicating potential functional roles of m⁶A in regulating diverse cellular processes.

[0074] Applicants have performed m⁶A profiling in mRNA from three individuals in each of human, chimpanzee, and rhesus monkey. Results indicate that the newly evolved m⁶A-modified transcripts are noticeably enriched in human disease pathways. In addition, results also indicate the functional significances of m⁶A in mRNA in neurogenesis and neurodevelopment.

B. N¹-methyladenosine (m¹A)

[0075] The most abundant eukaryotic rRNA and tRNA modifications are pseudouridine (Ψ), 2'O-methyls (Nm), N¹-methyladenosine (m¹A), and 5-methylcytidine (m⁵C). These are installed either through the use of guide RNAs bound with protein factors (snoRNP) or through designated protein enzymes. SnoRNA deletion and mutations in snoRNP proteins or rRNA/tRNA modification enzymes have been associated with human diseases including neurodegeneration, diabetes, and cancer. It is unclear in many cases, however, whether these disease phenotypes are truly derived from defects in rRNA/tRNA modifications or other yet-to-be-discovered mechanisms.

[0076] m¹A not only blocks Watson-Crick base pairing but also introduces an extra positive charge under physiological conditions (**FIG. 1**). It can introduce structural changes and dramatically impact protein-RNA interactions due to the presence of the methyl group and the positive charge. Most eukaryotic tRNA species contain N¹-methylation at position 58 (m¹A58). m¹A58 is essential for the stability of initiator tRNA^{iMet}, its absence leads to the decay of tRNA^{iMet}, resulting in impaired cell viability. Other mammalian tRNAs such as tRNA^{Gly}, tRNA^{His}, tRNA^{val}, and tRNA^{Phe} also possess m¹A58 while many mitochondria tRNA species contain m¹A9 methylation. A recent new discovery by Applicants found a new mammalian RNA demethylase that specifically reverses several tRNA m¹A methylation (**FIG. 4**). This enzyme-mediated demethylation affects the association of target tRNAs with polysomes and the use of tRNAs in translation. Increased m¹A methylation on the target tRNA results in increased partition of the tRNA in polysomes, leading to augmented protein

synthesis. Applicants have therefore uncovered that, in addition to m⁶A in mRNA/lncRNA, reversible methylation also acts on m¹A in tRNA and likely mRNA/lncRNA to regulate gene expression. Applicants have indeed discovered relatively abundant m¹A in mammalian mRNA with a unique distribution pattern indicating functional roles.

5 C. Other Adenosine Modifications

[0077] The methods and compositions of the disclosure are useful in the detection of adenosine modifications. It is contemplated that the methods and compositions may be useful for detecting the adenosine modifications listed below.

Symbol	Common Name
m ¹ A	1-methyladenosine
m ² A	2-methyladenosine
m ⁶ A	N ⁶ -methyladenosine
Am	2'-O-methyladenosine
ms ² m ⁶ A	2-methylthio-N ⁶ -methyladenosine
i ⁶ A	N ⁶ -isopentenyladenosine
ms ² i ⁶ A	2-methylthio-N ⁶ -isopentenyladenosine
io ⁶ A	N ⁶ -(<i>cis</i> -hydroxyisopentenyl)adenosine
ms ² io ⁶ A	2-methylthio-N ⁶ -(<i>cis</i> -hydroxyisopentenyl) adenosine
g ⁶ A	N ⁶ -glycinylocarbamoyl-adenosine
t ⁶ A	N ⁶ -threonylcarbamoyl-adenosine
ms ² t ⁶ A	2-methylthio-N ⁶ -threonyl carbamoyl-adenosine
m ⁶ t ⁶ A	N ⁶ -methyl-N ⁶ -threonylcarbamoyl-adenosine
hn ⁶ A	N ⁶ -hydroxynorvalylcarbamoyl-adenosine
ms ² hn ⁶ A	2-methylthio-N ⁶ -hydroxynorvalyl carbamoyl-adenosine
Ar(p)	2'-O-ribosyladenosine (phosphate)
m ⁶ ₂ A	N ⁶ ,N ⁶ -dimethyladenosine
m ⁶ Am	N ⁶ ,2'-O-dimethyladenosine
m ⁶ ₂ Am	N ⁶ ,N ⁶ ,2'-O-trimethyladenosine

m ¹ Am	1,2'-O-dimethyladenosine
ac ⁶ A	N ⁶ -acetyladenosine
m ⁸ A	8-methyladenosine

[0078] To summarize, mammalian mRNA and lncRNA contain many internal modifications with abundances ranging from 0.2-3 modified nucleotides per mRNA. This range of abundance suggests the presence of hundreds to tens of thousands of modified sites for each modification type in mammalian transcriptomes. Further, m⁶A and m¹A are known to be reversible and undergo dynamic regulation. m⁶A is the most abundant and has been best studied with broad and fundamental roles uncovered so far. Other modifications could provide additional tuning of mRNA metabolism and function. The lack of highly sensitive, selective, and robust sequencing approaches for all these modifications presents current technology barriers that significantly hinder biological investigations. Development of single-base resolution and highly sensitive methods will be required in order to move the field forward and also to enable new discoveries on the functions of RNA modifications and their associations with human diseases.

II. Enzyme-catalyzed deamination

[0079] The current disclosure provides methods and compositions comprising enzyme-mediated deamination for base-resolution sequencing of modified adenosines (e.g. m⁶A) in RNA. The current disclosure is based on a method that converts only unmodified A in RNA into a different base, leaving modified A untouched, and thereby allowing differentiation of A from m⁶A in sequencing (FIG. 5B).

A. ADAR (adenosine deaminase, RNA-specific)

[0080] As detailed in the Examples of the application, RNA editing ADAR enzymes that deaminate A to inosine (I) in mRNA using guide RNAs that form duplexes with all target sites can be used. The ADAR enzyme is highly active against A but possesses over 50-fold lower activity towards m⁶A compared to unmodified A. Therefore, after selective deamination of all A in the transcriptome and subsequent RT-PCR followed by sequencing, only m⁶A will be read as A (FIG. 5B).

[0081] The ADAR from *Drosophila melanogaster* (also known as Dmel_CG12598, ADAR, ADAR1, CG12598, Dmel\CG12598, EG:BACN35H14.1, adar, adr, cg12598, dADAR, dAdar, and hypnos-2) is represented by the following GenBank accession Nos:

DNA	Protein	ADAR
NM_001258548.2	NP_001245477.1	adenosine deaminase acting on RNA, isoform F [Drosophila melanogaster]
NM_001297862.1	NP_001284791.1	adenosine deaminase acting on RNA, isoform N [Drosophila melanogaster]
NM_166903.3	NP_726761.2	adenosine deaminase acting on RNA, isoform B [Drosophila melanogaster]
NM_001258547.2	NP_001245476.1	adenosine deaminase acting on RNA, isoform E [Drosophila melanogaster]
NM_001038732.3	NP_001033821.1	adenosine deaminase acting on RNA, isoform C [Drosophila melanogaster]
NM_130584.4	NP_569940.2	adenosine deaminase acting on RNA, isoform A [Drosophila melanogaster]
NM_001258545.2	NP_001245474.1	adenosine deaminase acting on RNA, isoform H [Drosophila melanogaster]
NM_001258546.2	NP_001245475.1	adenosine deaminase acting on RNA, isoform D [Drosophila melanogaster]
NM_001258542.2	NP_001245471.1	adenosine deaminase acting on RNA, isoform K [Drosophila melanogaster]
NM_001258544.2	NP_001245473.1	adenosine deaminase acting on RNA, isoform M [Drosophila melanogaster]
NM_001258543.2	NP_001245472.2	adenosine deaminase acting on RNA, isoform O [Drosophila melanogaster]
NM_001297863.1	NP_001284792.1	adenosine deaminase acting on RNA, isoform P [Drosophila melanogaster]
NM_001258541.2	NP_001245470.1	adenosine deaminase acting on RNA, isoform J [Drosophila melanogaster]
NM_001258540.1	NP_001245469.1	adenosine deaminase acting on RNA, isoform I [Drosophila melanogaster]

[0082] The sequences associated with each of the GenBank Accession numbers is herein incorporated by reference for all purposes. In some embodiments, the ADAR has the sequence associated with NP_001284791.1:

```

5      mlnsannnsp qhpvsapsdi nmngynrklp qkrgyempky sdpkkkmcke
      ripgpkntva mlnelrhgli yklesqtgpv haplftisve vdgqkylgqg
      rskkvariea aatalrsfiq fkdgavlspl kpagnldfts dehlengien
      lssskmfeii qtmlteklsn ptslegptfc msqnvksai tvdgqkcvpd
      kgpvmlllyel fndvnfecin idgaqnnrcf kmtvtinekk fdgtgpskkt
      aknaaakaal aslcnisysp mvvpqknvpl piddksssme lpqihadtig
10     rlvlekfmev ikgqeaysrr kvlagivmte nmnfceakvi svstgtkcvs
      gehmsvngav lndshaeivs rrcllkylya qldlqcnqat ayqsifvrnt
      dgqypyklks gvfhflyint apcgdarifs phendtgvdk hpnrkargql
      rtkiesgegt ipvkssdgiq twdgvlggqr lltmcsdski arwnivgiqg
      slsssiiepv ylhsivlgsl lhpehmyrav cgrieksiqg lpppyhlnkp
15     rlalvtsaep rngakapnfg inwtigdtel evvnsltgrt iggqvsrirk
      qaffvkygfl manlpgilvr kvtttdyggtk anvkdyqiak lelfsafkre
      dlgswlkkpi eqdefglae (SEQ ID NO:1).

```

B. Protein preparation

[0083] A variety of proteins can be purified using methods known in the art. Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterization of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps exploit differences in protein size, physico-chemical properties and binding affinity.

[0084] Evaluating purification yield. The most general method to monitor the purification process is by running a SDS-PAGE of the different steps. This method only gives a rough measure of the amounts of different proteins in the mixture, and it is not able to distinguish between proteins with similar molecular weight. If the protein has a distinguishing spectroscopic feature or an enzymatic activity, this property can be used to detect and

quantify the specific protein, and thus to select the fractions of the separation, that contains the protein. If antibodies against the protein are available then western blotting and ELISA can specifically detect and quantify the amount of desired protein. Some proteins function as receptors and can be detected during purification steps by a ligand binding assay, often using a radioactive ligand.

[0085] In order to evaluate the process of multistep purification, the amount of the specific protein has to be compared to the amount of total protein. The latter can be determined by the Bradford total protein assay or by absorbance of light at 280 nm, however some reagents used during the purification process may interfere with the quantification. For example, imidazole (commonly used for purification of polyhistidine-tagged recombinant proteins) is an amino acid analogue and at low concentrations will interfere with the bicinchoninic acid (BCA) assay for total protein quantification. Impurities in low-grade imidazole will also absorb at 280 nm, resulting in an inaccurate reading of protein concentration from UV absorbance.

[0086] Another method to be considered is Surface Plasmon Resonance (SPR). SPR can detect binding of label free molecules on the surface of a chip. If the desired protein is an antibody, binding can be translated to directly to the activity of the protein. One can express the active concentration of the protein as the percent of the total protein. SPR can be a powerful method for quickly determining protein activity and overall yield. It is a powerful technology that requires an instrument to perform.

[0087] Methods of protein purification. The methods used in protein purification can roughly be divided into analytical and preparative methods. The distinction is not exact, but the deciding factor is the amount of protein that can practically be purified with that method. Analytical methods aim to detect and identify a protein in a mixture, whereas preparative methods aim to produce large quantities of the protein for other purposes, such as structural biology or industrial use.

[0088] Depending on the source, the protein has to be brought into solution by breaking the tissue or cells containing it. There are several methods to achieve this: Repeated freezing and thawing, sonication, homogenization by high pressure, filtration (either via cellulose-based depth filters or cross-flow filtration), or permeabilization by organic solvents. The method of choice depends on how fragile the protein is and how sturdy the cells are. After this

extraction process soluble proteins will be in the solvent, and can be separated from cell membranes, DNA etc. by centrifugation. The extraction process also extracts proteases, which will start digesting the proteins in the solution. If the protein is sensitive to proteolysis, it is usually desirable to proceed quickly, and keep the extract cooled, to slow down proteolysis.

[0089] In bulk protein purification, a common first step to isolate proteins is precipitation with ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$. This is performed by adding increasing amounts of ammonium sulfate and collecting the different fractions of precipitate protein. One advantage of this method is that it can be performed inexpensively with very large volumes.

[0090] The first proteins to be purified are water-soluble proteins. Purification of integral membrane proteins requires disruption of the cell membrane in order to isolate any one particular protein from others that are in the same membrane compartment. Sometimes a particular membrane fraction can be isolated first, such as isolating mitochondria from cells before purifying a protein located in a mitochondrial membrane. A detergent such as sodium dodecyl sulfate (SDS) can be used to dissolve cell membranes and keep membrane proteins in solution during purification; however, because SDS causes denaturation, milder detergents such as Triton X-100 or CHAPS can be used to retain the protein's native conformation during complete purification.

[0091] Centrifugation is a process that uses centrifugal force to separate mixtures of particles of varying masses or densities suspended in a liquid. When a vessel (typically a tube or bottle) containing a mixture of proteins or other particulate matter, such as bacterial cells, is rotated at high speeds, the angular momentum yields an outward force to each particle that is proportional to its mass. The tendency of a given particle to move through the liquid because of this force is offset by the resistance the liquid exerts on the particle. The net effect of "spinning" the sample in a centrifuge is that massive, small, and dense particles move outward faster than less massive particles or particles with more "drag" in the liquid. When suspensions of particles are "spun" in a centrifuge, a "pellet" may form at the bottom of the vessel that is enriched for the most massive particles with low drag in the liquid. Non-compacted particles still remaining mostly in the liquid are called the "supernatant" and can be removed from the vessel to separate the supernatant from the pellet. The rate of centrifugation is specified by the angular acceleration applied to the sample, typically measured in comparison to the g. If samples are centrifuged long enough, the particles in the

vessel will reach equilibrium wherein the particles accumulate specifically at a point in the vessel where their buoyant density is balanced with centrifugal force. Such an "equilibrium" centrifugation can allow extensive purification of a given particle.

[0092] Sucrose gradient centrifugation is a linear concentration gradient of sugar (typically sucrose, glycerol, or a silica based density gradient media, like Percoll™) is generated in a tube such that the highest concentration is on the bottom and lowest on top. A protein sample is then layered on top of the gradient and spun at high speeds in an ultracentrifuge. This causes heavy macromolecules to migrate towards the bottom of the tube faster than lighter material. After separating the protein/particles, the gradient is then fractionated and collected.

[0093] Usually a protein purification protocol contains one or more chromatographic steps. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with various materials. Different proteins interact differently with the column material, and can thus be separated by the time required to pass the column, or the conditions required to elute the protein from the column. Usually proteins are detected as they are coming off the column by their absorbance at 280 nm. Many different chromatographic methods exist:

[0094] Chromatography can be used to separate protein in solution or denaturing conditions by using porous gels. This technique is known as size exclusion chromatography. The principle is that smaller molecules have to traverse a larger volume in a porous matrix. Consequentially, proteins of a certain range in size will require a variable volume of eluent (solvent) before being collected at the other end of the column of gel.

[0095] In the context of protein purification, the eluant is usually pooled in different test tubes. All test tubes containing no measurable trace of the protein to purify are discarded. The remaining solution is thus made of the protein to purify and any other similarly-sized proteins.

[0096] Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The column to be used is selected according to its type and strength of charge. Anion exchange resins have a positive charge and are used to retain and separate negatively charged compounds, while cation exchange resins have a negative charge and are used to separate positively charged molecules. Before the separation begins a buffer

is pumped through the column to equilibrate the opposing charged ions. Upon injection of the sample, solute molecules will exchange with the buffer ions as each competes for the binding sites on the resin. The length of retention for each solute depends upon the strength of its charge. The most weakly charged compounds will elute first, followed by those with successively stronger charges. Because of the nature of the separating mechanism, pH, buffer type, buffer concentration, and temperature all play important roles in controlling the separation.

[0097] Affinity Chromatography is a separation technique based upon molecular conformation, which frequently utilizes application specific resins. These resins have ligands attached to their surfaces which are specific for the compounds to be separated. Most frequently, these ligands function in a fashion similar to that of antibody-antigen interactions. This "lock and key" fit between the ligand and its target compound makes it highly specific, frequently generating a single peak, while all else in the sample is unretained.

[0098] Many membrane proteins are glycoproteins and can be purified by lectin affinity chromatography. Detergent-solubilized proteins can be allowed to bind to a chromatography resin that has been modified to have a covalently attached lectin. Proteins that do not bind to the lectin are washed away and then specifically bound glycoproteins can be eluted by adding a high concentration of a sugar that competes with the bound glycoproteins at the lectin binding site. Some lectins have high affinity binding to oligosaccharides of glycoproteins that is hard to compete with sugars, and bound glycoproteins need to be released by denaturing the lectin.

[0099] A common technique involves engineering a sequence of 6 to 8 histidines into the N- or C-terminal of the protein. The polyhistidine binds strongly to divalent metal ions such as nickel and cobalt. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. While this procedure is generally used for the purification of recombinant proteins with an engineered affinity tag (such as a 6xHis tag or Clontech's HAT tag), it can also be used for natural proteins with an inherent affinity for divalent cations.

[00100] Immunoaffinity chromatography uses the specific binding of an antibody to the target protein to selectively purify the protein. The procedure involves immobilizing an antibody to a column material, which then selectively binds the protein, while everything else flows through. The protein can be eluted by changing the pH or the salinity. Because this method does not involve engineering in a tag, it can be used for proteins from natural sources.

[00101] Another way to tag proteins is to engineer an antigen peptide tag onto the protein, and then purify the protein on a column or by incubating with a loose resin that is coated with an immobilized antibody. This particular procedure is known as immunoprecipitation. Immunoprecipitation is quite capable of generating an extremely specific interaction which usually results in binding only the desired protein. The purified tagged proteins can then easily be separated from the other proteins in solution and later eluted back into clean solution. Tags can be cleaved by use of a protease. This often involves engineering a protease cleavage site between the tag and the protein.

[00102] High performance liquid chromatography or high pressure liquid chromatography is a form of chromatography applying high pressure to drive the solutes through the column faster. This means that the diffusion is limited and the resolution is improved. The most common form is "reversed phase" hplc, where the column material is hydrophobic. The proteins are eluted by a gradient of increasing amounts of an organic solvent, such as acetonitrile. The proteins elute according to their hydrophobicity. After purification by HPLC the protein is in a solution that only contains volatile compounds, and can easily be lyophilized. HPLC purification frequently results in denaturation of the purified proteins and is thus not applicable to proteins that do not spontaneously refold.

[00103] At the end of a protein purification, the protein often has to be concentrated. Different methods exist. If the solution doesn't contain any other soluble component than the protein in question the protein can be lyophilized (dried). This is commonly done after an HPLC run. This simply removes all volatile component leaving the proteins behind.

[00104] Ultrafiltration concentrates a protein solution using selective permeable membranes. The function of the membrane is to let the water and small molecules pass through while retaining the protein. The solution is forced against the membrane by mechanical pump or gas pressure or centrifugation.

[00105] Gel electrophoresis is a common laboratory technique that can be used both as preparative and analytical method. The principle of electrophoresis relies on the movement of a charged ion in an electric field. In practice, the proteins are denatured in a solution containing a detergent (SDS). In these conditions, the proteins are unfolded and coated with negatively charged detergent molecules. The proteins in SDS-PAGE are separated on the sole basis of their size.

[00106] In analytical methods, the protein migrate as bands based on size. Each band can be detected using stains such as Coomassie blue dye or silver stain. Preparative methods to purify large amounts of protein, require the extraction of the protein from the electrophoretic gel. This extraction may involve excision of the gel containing a band, or eluting the band directly off the gel as it runs off the end of the gel.

[00107] In the context of a purification strategy, denaturing condition electrophoresis provides an improved resolution over size exclusion chromatography, but does not scale to large quantity of proteins in a sample as well as the late chromatography columns.

[00108] Methods of the disclosure may involve purification of proteins by any combination of methods known in the art and/or discussed herein. In some embodiments, the protein is purified by a combination of one or more of affinity chromatography, ion exchange chromatograph, and gel filtration chromatography. In some embodiments, the affinity chromatography is anti-FLAG. In some embodiments, the ion exchange chromatography is heparin.

III. Assays Utilizing Adenosine modification

[00109] Nucleic acid analysis and evaluation includes various methods of amplifying, fragmenting, and/or hybridizing nucleic acids that have or have not been modified.

[00110] Methodologies are available for large scale sequence analysis. In certain aspects, the methods described exploit these genomic analysis methodologies and adapt them for uses incorporating the methodologies described herein. In certain instances the methods can be used to perform high resolution adenosine modification analysis on modified adenosines in RNA. Therefore, methods are directed to analysis of the adenosine modification status of a RNA sample, comprising one or more of the steps: (a) contacting the target RNA with an adenosine deaminase enzyme to generate a target RNA with deaminated adenosines, (b)

sequencing the target RNA with deaminated adenosines; wherein the modified adenosine is detected when the nucleotide sequence is adenosine. In some embodiments, the method further comprises steps such as sequencing a control RNA; comparing the sequence of the target RNA with deaminated adenosines to the sequence of the control RNA; generating a
5 nucleic acid strand that is complementary with the target and/or control RNA and hybridizing the complementary nucleic acid strand with the target RNA; comparing the known sequence of the target RNA with the sequence of the target RNA with deaminated adenosines.

[00111] In some embodiments, the assay comprises a cycle of steps as demonstrated in FIG. 9B. For example, the assay may involve a reverse transcriptase reaction with the target RNA
10 to create a complementary DNA strand. The RNA and complementary DNA strand are then annealed together to create RNA-DNA duplexes. Next, the RNA-DNA duplex is contacted with a deaminating enzyme with specificity for unmodified adenosines and no or greatly reduced, such as 10, 15, 20, 25, 30, 40, 50, 100, or 200 fold less activity (compared to unmodified), for modified adenosines. Furthermore, the ADAR only deaminates RNA.
15 Therefore, the RNA unmodified adenosines are converted to inosines in the target RNA strand. The DNA can then be digested using a DNA digesting enzyme, leaving the RNA intact. The RNA can then be purified and another round of reverse transcriptase can be performed, creating a new complementary DNA strand. The new complementary DNA strand is different from the first complementary DNA strand, since cytosines are generated as
20 the complement to the inosine. In contrast, uracils were generated as the complement to the adenosine in the first complementary strand. The deamination reaction is repeated so that unmodified adenosines that did not get deaminated in the previous round could then be deaminated and converted to inosine on a subsequent round. This may be repeated until a high percentage such as at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% of unmodified
25 adenosines are deaminated and converted to inosine.

A. RNA isolation

[00112] RNA may be isolated from an organism of interest, including, but not limited to eukaryotic organisms and prokaryotic organisms, preferably mammalian organisms, such as humans. RNA may be isolated from cells grown *in vitro* or from cells *in vivo*. RNA may be
30 isolated from tissues such as bone, blood, liver, heart, etc... Furthermore, the RNA may be isolated and fractionated by technique that separate different RNA types. Therefore, in some embodiments, RNA is purified, and the purified RNA comprises at least 80, 85, 90, 95,

96, 97, 98, 99, or 100% of a particular RNA such as mRNA, lncRNA, non-coding RNA, microRNA, pri-microRNA, pre-piRNA, rRNA, tRNA, snoRNA, or snRNA.

B. Sequencing

[00113] The sequencing may be done by known methods of sequencing nucleic acids. In certain embodiments, the target nucleic acids molecules are sequenced using any suitable sequencing technique known in the art. In one example, the sequencing is single-molecule sequencing-by-synthesis. Single-molecule sequencing is shown for example in U.S. Pat. Nos.: 7,169,560, 6,818,395, 7,282,337, the contents of each of these references is incorporated by reference herein in its entirety. Other examples of sequencing nucleic acids may include Maxam-Gilbert techniques, Sanger type techniques, Sequencing by Synthesis methods (SBS), Sequencing by Hybridization (SBH), Sequencing by Ligation (SBL), Sequencing by Incorporation (SBI) techniques, massively parallel signature sequencing (MPSS), polony sequencing techniques, nanopore, waveguide and other single molecule detection techniques, reversible terminator techniques, or other sequencing technique now know or may be developed in the future.

[00114] In one embodiment, the sequencing is Illumina sequencing. Illumina sequencing is based on the amplification of DNA on a solid surface using fold-back PCR and anchored primers. Genomic DNA is fragmented, and adapters are added to the 5' and 3' ends of the fragments. DNA fragments that are attached to the surface of flow cell channels are extended and bridge amplified. The fragments become double stranded, and the double stranded molecules are denatured. Multiple cycles of the solid-phase amplification followed by denaturation can create several million clusters of approximately 1,000 copies of single-stranded DNA molecules of the same template in each channel of the flow cell. Primers, DNA polymerase and four fluorophore-labeled, reversibly terminating nucleotides are used to perform sequential sequencing. After nucleotide incorporation, a laser is used to excite the fluorophores, and an image is captured and the identity of the first base is recorded. The 3' terminators and fluorophores from each incorporated base are removed and the incorporation, detection and identification steps are repeated.

[00115] In another embodiment, Ion Torrent sequencing can be used. (See, e.g., U.S. patent application numbers 2009/0026082, 2009/0127589, 2010/0035252, 2010/0137143, 2010/0188073, 2010/0197507, 2010/0282617, 2010/0300559, 2010/0300895, 2010/0301398, and 2010/0304982), the content of each of which is incorporated by reference

herein in its entirety.) Oligonucleotide adaptors are ligated to the ends of target nucleic acid molecules. The adaptors serve as primers for amplification and sequencing of the fragments. The fragments can be attached to a surface and is attached at a resolution such that the fragments are individually resolvable. Addition of one or more nucleotides releases a proton
5 (H⁺), which signal detected and recorded in a sequencing instrument. The signal strength is proportional to the number of nucleotides incorporated.

[00116] In some embodiments, sequencing the target RNA comprises creating a complementary DNA (cDNA) from the target RNA. In some embodiments, sequencing the target RNA comprises reverse transcription. In some embodiments, sequencing the target
10 RNA comprises contacting the target RNA with an enzyme capable of transcribing DNA using the target RNA as a template (e.g. reverse transcriptase). In some embodiments, a cDNA of the target RNA is sequenced. The sequence of the cDNA is determined, and the cDNA sequence is used to determine the sequence of the target RNA. In some embodiments, the target RNA is determined to have a modified adenosine at the corresponding position of
15 the cDNA that is determined by sequencing to be thymine.

[00117] In some embodiments, sequencing the target RNA comprises amplification of nucleic acids. Amplification can be done by techniques known in the art, such as PCR, that uses primers, polymerase, deoxynucleoside triphosphates, buffers, and bivalent and monovalent cations in a reaction that generates copies of a target DNA sequence from a
20 single or few copies of the target DNA sequence.

C. Controls

[00118] In some embodiments, the methods described herein further comprise control samples. The intrinsically present RNA editing product should not influence the *in vitro* deamination (A has already been converted to I). However, an accurate assignment of the
25 methylation fraction at each modification site could be affected if the same site is also intrinsically deaminated to some extent. In addition, the ADAR enzyme may exhibit certain sequence and/or structure biases that need to be corrected. Therefore, a control sample with minimum methylation is required to correct these factors.

[00119] In one embodiment, the control sample is a control transcript with minimum
30 adenosine modification. In one embodiment, the control sample is a control transcript minimum m⁶A methylation (**FIG. 8A**). Cellular mRNA can be isolated and subjected to a

demethylase that is specific for the adenosine modification. For example, the m⁶A modification can be demethylated by way of ALKBH5-catalyzed m⁶A demethylation. ALKBH5 is one of the two known m⁶A RNA demethylases. Recombinant ALKBH5 is highly active and can remove most m⁶A (90%) from mammalian mRNA *in vitro* as shown in

FIG. 8B.

[00120] In some embodiments the method comprises a control sample. To construct a control sample, the RNA preparation comprising the target RNA may be separated into multiple, such as at least, at most, or exactly 2, 3, 4, 5, 6, or more portions (or any derivable range therein). One portion may be subjected to modification-specific demethylation to remove the modification on the RNA. In some embodiment, one portion is contacted with ALKBH5 to catalyze m⁶A demethylation to remove most m⁶A on RNA. Next, both portions of the mRNA samples (after forming duplex RNA) will be subjected to the enzyme-mediated deamination, RT-PCR amplification, and high-throughput sequencing. Because the modified adenosine is resistant to deamination it will be read as A in the sample without modification-specific demethylation (e.g. ALKBH5 treatment). In the demethylation (e.g. ALKBH5-treated) control m⁶A is converted to A, which is deaminated to inosine in the deamination step and will be read as G. A comparison of the two parallel sequencing data will accurately reveal specific modification sites (i.e. m⁶A when ALKBH5 is used as the demethylase) at base resolution and eliminate potential RNA editing at the modification site (unmodified A-to-I) and potential biases of the deamination step.

IV. Kits

[00121] The invention additionally provides kits for detecting modified adenosines in a target RNA. Each kit may also include additional components that are useful for amplifying the nucleic acid, or sequencing the nucleic acid, or other applications of the present invention as described herein. The kit may optionally provide additional components that are useful in the procedure. These optional components include buffers, capture reagents, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information. The kit may also include reagents for RNA isolation and/or purification.

V. Examples

[00122] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods described herein are presently representative of certain embodiments, are provided as an example, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1: Deamination-based sequencing to differentiate modified versus unmodified adenosines and cytosines

[00123] Because the m⁶A modification on mRNA/lncRNA plays the most significant and broad roles in gene expression regulation, the focus of this Example is on sequencing of this modification. However, one skilled in the art would readily understand that parallel techniques could be applied for sequencing other adenosine modifications.

ADAR-mediated adenosine deamination sequencing (Deam-m⁶A-seq)

[00124] Applicants contemplated that adenosine deamination would be useful in methods for obtaining base-resolution sequencing of m⁶A in RNA (**FIG. 6**). Adenosine deamination, leading to adenosine-to-inosine (A-to-I) RNA editing, plays important regulatory roles in post-transcriptional processes including mRNA re-coding and alternative splicing. The RNA adenosine deaminases (ADAR) efficiently catalyze this reaction in duplex RNA. Previous studies have shown that the m⁶A methylation significantly hinders the deamination activity of ADAR proteins. Less than 2% activity with slower kinetics was observed with m⁶A as the substrate (**FIG. 6A**). ADAR can be used to convert all A in isolated RNA to inosines while keeping m⁶A mostly intact. Subsequent amplification and high-throughput sequencing will read m⁶A as A while unmodified A is read as G (**FIG. 6B**). In some embodiments, *Drosophila* ADAR (dADAR) is used. dADAR exhibits non-specific RNA editing activity on long double-stranded RNA (dsRNA).

[00125] The proposed use of ADAR for Deam-m⁶A-seq of m⁶A is not straightforward. Several major challenges must be resolved: 1. ADAR only works on duplex RNA. We must generate anti-sense RNA and form duplexes with sense RNA in order for the deamination reaction to occur. 2. ADAR-catalyzed deamination of duplex RNA can hardly reach 30% conversion because the deamination reaction converts A in the duplex RNA to I, which progressively weakens the duplex stability until dissociation of the two RNA strands leading to termination of the reaction. 3. The need for a control to correct various factors that may affect the deamination reaction. The intrinsically present RNA editing product should not influence the *in vitro* deamination (A has already been converted to I). However, an accurate assignment of the methylation fraction at each modification site could be affected if the same site is also intrinsically deaminated to some extent. In addition, the ADAR enzyme may exhibit certain sequence and/or structure biases that need to be corrected. Therefore, a control sample with minimum methylation is required in order to correct these factors.

[00126] Several strategies exist with regard to generating duplex RNA. The third challenge can be addressed with a demodification strategy, also detailed above in the “controls” section. The first and second challenges are most critical to overcome.

[00127] Challenge 1, Generating double-stranded RNA: Because ADAR only works on duplex RNA one solution is to generate a transcriptome-wide anti-sense RNA strand pool in order to form dsRNA for the application of Deam-m⁶A-seq. To do this, one can use cDNA library to prepare anti-sense RNA fragments and then anneal to the transcripts. Well-established procedures to clone mammalian or other cDNA into plasmids can be used. The anti-sense RNAs can be transcribed *in vitro* using T7 RNA polymerase and are annealed to the methylated and the demethylated transcriptomes; the resulting dsRNAs can serve as the substrates for the dADAR-mediated deamination.

[00128] Alternatively, the 3' primer-free Phi6 RNA replicase can be utilized to generate dsRNA using purified transcripts as templates. Phi6 RNA replicase is a primer-independent viral RNA replicase. It can generate dsRNA directly from ssRNA templates without primers. This approach serves as an alternative to produce dsRNA for dADAR-mediated deamination.

[00129] Challenge 2, Inefficient deamination: To improve the deamination efficiency of ADAR on the sense strand of dsRNA, Applicants devised a strategy to inhibit adenosine deamination of the anti-sense strand of dsRNA. Applicants employed N⁶-methyladenosine

triphosphate (m^6 ATP or me-ATP) in the *in vitro* transcription reaction to generate the complementary strand, replacing all A with m^6 A on the anti-sense RNA strand in order to prevent the deamination reaction and increase the stability of dsRNA. Applicants cloned recombinant dADAR into an insect cell expression system with a FLAG tag on the N terminus and a polyhistidine tag on the C terminus. The protein was expressed and purified to high purity via anti-FLAG affinity resin and heparin column. A target RNA probe was generated through transcription and purified as a model RNA (FIG. 7). The complementary strand was generated with unmodified A or with m^6 A replacing all A by using m^6 ATP during the transcription; Applicants have confirmed that m^6 ATP can be efficiently recognized by various polymerases (including Phi6 RNA replicase) in order to generate the anti-sense strand with m^6 A replacing A. The two strands were annealed. Deaminations of both dsRNA using recombinant dADAR were tested. The treated product was reverse transcribed, amplified, and then inserted into a vector through TA-cloning. The A-to-G mutation ratio on the target strand was counted through colony picking and Sanger sequencing. As expected, with A on the complementary strand, ~20-30% deamination was observed for each of the seven A on the target strand. A similar deamination ratio for each A on the complementary strand was also observed. When all A was replaced with m^6 A on the complementary strand, almost no deamination of the complementary strand and close to 50-60% deamination for each A on the target strand was observed (FIG. 7). The deamination ratio of the target strand doubled with m^6 A replacing unmodified A on the complementary strand.

[00130] Challenge 3, quantification controls: In order to generate a control sample, Applicants further propose a demethylation approach to provide control transcripts with minimum m^6 A methylation (FIG. 8A). Cellular mRNA can be isolated and subjected to ALKBH5-catalyzed m^6 A demethylation. ALKBH5 is one of the two known m^6 A RNA demethylases identified. Recombinant ALKBH5 is highly active and can remove most m^6 A (90%) from mammalian mRNA *in vitro* as shown in FIG. 8B. For a quantification control, the following steps can be performed: i) Separate the purified polyA-tailed RNA into two portions. One portion will be subjected to the ALKBH5-catalyzed m^6 A demethylation to remove most m^6 A on RNA. ii) Then, both mRNA samples (after forming duplex RNA) will be subjected to the dADAR-mediated deamination, RT-PCR amplification, and high-throughput sequencing. iii) Because m^6 A is resistant to deamination it will be read as A in the sample without ALKBH5 treatment. In the ALKBH5-treated control m^6 A is converted to A, which is deaminated to inosine in the deamination step and will be read as G. A

comparison of the two parallel sequencing data will accurately reveal m⁶A sites at base resolution and eliminate potential RNA editing at the modification site (unmodified A-to-I) and potential biases of the deamination step.

[00131] Applicants tested whether the A-to-G mutation ratio changes with and without demethylation reaction as proposed. A- and m⁶A-containing target strands were synthesized and mixed together in 1:1 ratio. The ssRNA substrate was split into two portions, a control portion and a portion subjected to ALKBH5-mediated demethylation (**FIG. 8A**). The dsRNA was deaminated by dADAR and then converted to DNA for Sanger sequencing. The results revealed significant differences between samples with and without demethylation treatment (**FIG. 8B** and **FIG. 8C**). The A-to-G transition occurred on partially methylated target strand, which represents the ratio of deamination. In the absence of demethylation, the deamination efficiency for the target strand ranged around 5-15% for the 1:1 mixture of A:m⁶A. With demethylation treatment, the deamination efficiency went up to around 20-35% for the target strand. The comparison of the two results can lead to accurate assignments of the m⁶A sites as well as correction of other factors that may affect the dADAR-mediated deamination.

[00132] Applicants have applied Deam-m⁶A-seq to isolated mRNA from HeLa cells by using Phi6 RNA replicase to generate duplex RNA (with m⁶A on the opposite anti-sense strand). A shallow sequencing was performed with promising results. In MALAT1 lncRNA as an example, we observed 20-60% A-to-G transition mutations at most A sites.

[00133] One main concern is that the ADAR-mediated deamination of unmodified A is not stoichiometric. But with m⁶A in the complementary strand to extend deamination of the target strand and a new buffer system that stabilizes duplex and promotes dADAR activity, the deamination efficiency can be improved up to ~75%. To provide quantitative information of the modification percentage at each site, spike-in controls that contain different percentages of m⁶A/A can be used. Generating a calibration curve will enable more precise measurements of the methylation fractions at each sites. The use of spike-in controls and generation of a calibration curve provided more accurate estimates of the modification fractions.

Adenosine deamination in RNA-DNA duplex

[00134] Applicants have recently discovered a new buffer system (Example 2) that stabilizes duplex and significantly promotes deamination of unmodified A in duplex RNA (with m⁶A on the complementary strand) by dADAR to ~75%. Under the same conditions dADAR is active on RNA-DNA duplex substrates. We observed ~25% A-to-G mutations for unmodified A in RNA of an RNA-DNA model substrate after treating with dADAR (**FIG. 9A**). Since both duplex RNA and RNA-DNA duplex adopt the similar A-form conformation, this result may not be surprising; the observation nevertheless opens a possibility to further increase the deamination efficiency for A (**FIG. 9B**): 1. Through regular RNase H-minus RT, RNA-DNA hybrid duplex can readily be generated from a pool of isolated mRNA. dADAR does not work on the DNA strand. Therefore, its activity will be confined to the RNA strand. The dADAR-mediated deamination leads to progressive weakening of the duplex until the two strands separate. 2. The mixture can then be treated with DNase to digest all DNA, purify RNA, and perform another round of RT to generate another pool of RNA-DNA hybrid duplexes that form perfectly complementary strands, now with the deaminated RNA product from step 1. 3. The dADAR-mediated deamination will be performed, and then the procedure goes back to step 1 for iterative rounds of duplex generation and RNA deamination. 4. After several rounds of RT-deamination-DNA digestion-RT, the deamination efficiency of unmodified A could in principle reach stoichiometric. Library will be constructed for subsequent sequencing.

[00135] With the RNA-DNA hybrid duplex as the substrate, Applicants can selectively digest DNA after the strand separation (because of deamination) and perform reverse transcription to generate the new DNA stands complementary to the deaminated and undeaminated RNAs for another round of deamination. In principle, iterative rounds of this procedure will allow for much higher deamination efficiency. An ALKBH5-treated control sample with minimum m⁶A will be processed in parallel. Comparison of these two samples could accurately reveal m⁶A with base-resolution accuracy. Potential deamination of m⁶A can be monitored in order to ensure minimum interference. This approach offers one of the best current solutions for m⁶A sequencing at base resolution.

Example 2: Deam-m⁶A-seq

[00136] In RNA technologies, RNA processing and modification are important features, closely related to its biological functions. Particularly, N⁶-methyladenosine (m⁶A) is a widely present modification found within eukaryotic messenger RNA and various nuclear non-

coding RNAs. Recent discoveries have revealed that methylation of adenosine in mRNA is a dynamic and reversible process. m⁶A formation in the nucleus is catalyzed by a complex containing methyltransferase like 3 (METTL3), methyltransferase like 14 (METTL14), and Wilms' tumor 1-associating protein (WTAP). Two human AlkB family proteins, fat mass and obesity-associated protein (FTO) and ALKBH5, serve as RNA demethylases to remove m⁶A in mammalian polyA⁺-tailed RNA. The "reader" protein YTHDF2, an m⁶A specific binding protein which is shown to interact with thousands of mRNA targets, mediates its substrates to a methylation-dependent mRNA decay, demonstrating a significant role of methylation in mRNA metabolism. The latest work on METTL3 knockout embryonic stem cells also indicates the critical role of m⁶A in cell differentiation and its regulatory relationship with microRNA.

[00137] Currently, transcriptome-wide m⁶A detection is based on antibody-specific enrichment, followed by high-throughput sequencing (m⁶A-seq, or MeRIP-seq). Even a photo-crosslinking-assisted strategy has been developed to significantly improve the resolution of m⁶A-seq (PA-m⁶A-seq), the lack of direct conversion tool to differentiate methylated and unmethylated adenosine still hinders the single-nucleoside resolution detection and quantitative analyses of methylation across the entire transcriptome, which is of great importance to further investigate the biological significance of the methylation modification.

[00138] Described herein is an enzyme-based approach, ADAR-mediated adenosine deamination sequencing (Deam-m⁶A-seq), to map m⁶A in a transcriptome-wide manner at single-nucleoside resolution.

[00139] Design and Innovation: *Drosophila* Adenosine Deaminase that Acting on RNA (dADAR) was chosen as the tool enzyme to catalyze deamination process (**FIG. 6**).

[00140] The following describes exemplary method steps useful in the methods and compositions of the disclosure. Certain embodiments may comprise the reagents and methods described below.

I. Step 1: dADAR substrate preparation:

A. Formation of dsRNA by employing Phi6 RNA replicase and *N*⁶-methyl-ATP to replace normal ATP

[00141] Phi6 RNA replicase is a high-efficient RNA replicase from virus, which is able to generate complementary anti-sense RNA strand without the assistance of primer. Phi6 RNA replicase recognizes free 3' hydroxyl group of RNA molecule to initiate the synthesis, typically producing the full-length dsRNA for the RNA/DNA template.

[00142] To avoid the nonsense deamination occurring on complementary RNA strand which destabilizes dsRNA and also consumes the catalytic turnover capability of dADAR, Applicants proposed to incorporate m⁶A instead of A in complementary strand by utilizing *N*⁶-methyl-ATP, which was not tested before. Surprisingly, the incorporation efficiency of *N*⁶-methyl-ATP by Phi6 RNA replicase is similar to that of normal ATP, ensuring the productivity of m⁶A-incorporated dsRNA for further deamination treatment (**FIG. 10A**).

B. Formation of RNA-DNA hybrid as dADAR substrate.

[00143] dADAR does not recognize RNA-DNA hybrid in typical buffer system. dATP and GTP- containing reaction buffers were tested to investigate whether the deamination activity could be improved. It was eventually found that dADAR could react with RNA-DNA hybrid with GTP in the reaction buffer (**FIG. 10B**).

II. Step 2: deamination control setup

A. Demethylated control group

[00144] The deamination activity of dADAR on substrate is barely affected by sequences of substrate. However, the tiny uneven deamination reactivity on adenosine in different contexts has great negative effect on the power of differentiating methylated adenosine from unmethylated. Thus, setting up an effective control group for further data analyses is extremely significant.

[00145] In theory, comparing deamination results of methylated and unmethylated transcripts is the best way to 1) rule out any context biases on unmethylated adenosine and 2) amplify the deamination conversion effect on methylated adenosine. Applicants considered the use of m⁶A demethylases to get rid of m⁶A modification transcriptome-wide. Applicants tested both FTO and ALKBH5, finding that ALKBH5 affords the better efficiency of

removing methyl group of adenosine (**FIG. 8B**). As the flowchart shows, fragmented transcriptome is split into two parallel experimental group and after the treatment of ALKBH5, one is original and the other is demethylated control group, both of which are applied to substrate preparation step. The strategy indeed shows its efficiency to enhance A-to-G transition ratio on methylated sites on our model study (**FIG. 8C**).

B. Relationship between deamination efficiency and methylation level demonstrated by calibration curve

[00146] Even we have already optimized the entire deamination reaction from substrate to reaction condition, it is still unlikely to convert all unmethylated adenosine to inosine in deaminase treatment. Considering the relatively low level of methylation, a cutoff/criteria is required for confidently detecting m⁶A and quantifying methylation on each site. Therefore, a relationship between deamination efficiency and methylation level on each adenosine is necessary for quality control and data analyses.

[00147] T7 and T3 RNA polymerases are compatible with several modified nucleoside triphosphate and are able to generate modified nucleoside containing RNA fragments. *We found that N⁶-methyl-ATP can be used to form the RNA strand.* By using this strategy, we can prepare a set of samples with different methylation levels, with which a calibration curve, demonstrating the relationship between deamination efficiency (indicated by A-to-G transition ratio in high-throughput sequencing) and methylation level, can be prepared.

C. Step 3: deamination condition optimization

[00148] The best buffer system for deamination reaction of dADAR (on dsRNA and RNA-DNA hybrid) is shown below:

1X Reaction buffer system	
<i>Component</i>	<i>Final Concentration</i>
Tris-HCl (pH 7.9)	20 mM
KCl	100 mM
ZnCl ₂	20 μM
DTT	1 mM
MgCl ₂	1 μM
GTP	4 mM

[00149] The reactivity under two different concentrations of GTP in the buffer system were tested. With 4 mM GTP in buffer, the reactivity enhanced significantly (**FIG. 9A**).

III. Exemplary procedure for effective deamination of RNA in RNA-DNA hybrid duplex

[00150] The observation that dADAR works effectively on RNA-DNA duplex in the presence of GTP opens a possibility to further increase the deamination efficiency for A (FIG. 9B):

[00151] Through regular RNase H-minus RT Applicants can readily generate RNA-DNA hybrid duplex from a pool of isolated mRNA. dADAR does not work on the DNA strand. Therefore, its activity will be confined to the RNA strand. The dADAR-mediated deamination leads to progressive weakening of the duplex until the two strands separate. Applicants will then treat the mixture with DNase to digest all DNA, purify RNA, and perform another round of RT to generate another pool of RNA-DNA hybrid duplexes that form perfectly complementary strands, now with the deaminated RNA product from step 1. The dADAR-mediated deamination will be performed, and then the procedure goes back to step 1 for iterative rounds of duplex generation and RNA deamination. After several rounds of RT-deamination-DNA digestion-RT, the deamination efficiency of unmodified A could in principle reach stoichiometric. Library will be constructed for subsequent sequencing.

[00152] With the RNA-DNA hybrid duplex as the substrate, Applicants can selectively digest DNA after the strand separation (because of deamination) and perform reverse transcription to generate the new DNA stands complementary to the deaminated and un-deaminated RNAs for another round of deamination. In principle, iterative rounds of this procedure will allow us to achieve much higher deamination efficiency. An ALKBH5-treated control sample with minimum m⁶A will be processed in parallel. Comparison of these two samples could accurately reveal m⁶A with base-resolution accuracy. Applicants can monitor potential deamination of m⁶A in order to ensure minimum interference. This approach offers one of the best current solutions for m⁶A sequencing at base resolution.

Example 3: RNA Immobilization and iterative Deam-seq

[00153] The deamination process converts unmethylated adenosine to inosine and generates inosine-uracil/thymine mismatch in dsRNA/RNA-DNA hybrid, which significantly destabilizes the duplex structure. However, the presence of the duplex structure is critical for achieving high transition ratio through effective deamination.

[00154] To overcome this drawback, Applicants propose to iteratively perform deamination reaction on target strand to convert as many unmethylated adenosines to inosine as possible. In dsRNA Deam-seq, the introduction of the anti-sense complementary strand is critical for the deamination reaction; however, the addition of the complementary strand also
5 interferences on iterative deamination reaction since after several rounds of formation of dsRNA, the original target strand, which is the “real” transcriptome, is diluted by artificially synthesized strand.

[00155] The best approach to differentiate the original target strand from artificially synthesized complementary strand is to selectively label the original one and immobilize it on
10 a solid support. Therefore, we have designed the RNA immobilization and iterative Deam-seq strategy (FIG. 11).

[00156] RNA fragment is first attached with a 5'-phosphothioate group by using T4 polynucleotide kinase and ATP- γ S. Then the labeled RNA fragment reacts with a solid support with iodoacetamide (or other functional groups that react with phosphothioate) that is
15 commercially available. The immobilize RNA serves as the template for the formation of dsRNA/RNA-DNA duplex and is applied to deamination reaction system.

[00157] After the conversion of as many adenosines as possible to inosine during deamination, the artificial anti-sense strand no longer stably interacts with target strand, which can be denatured and washed out. Then, a second-round of the formation of
20 dsRNA/RNA-DNA duplex and subsequent deamination is followed (FIG. 11). By using this strategy, iterative deamination can be achieved. Not only does the deamination efficiency increase, but also the target strand is the only strand to be sequenced at the end of the process.

[00158] Besides reacting ATP- γ S with iodoacetamide, other 5' end labeling and immobilization strategies can be applied. For instance, after 5'-phosphothioate transfer,
25 labeled RNA/DNA can also react with maleimide derivatives (for example, maleimide biotin) and then be immobilized on streptavidin beads.

[00159] The high reactivity of phosphothioate transfer and high yield of solid phase capture may also enable our strategy to enrich cell free oligonucleotides (RNA or DNA) for clinical diagnostics. Current enrichment approaches are generally based on direct extraction and
30 absorptions through electrostatic or hydrophobic interaction, which either is less efficient or hardly to be further treated for further experiments. Our approach anchors oligonucleotides

at 5' terminal, which has little effect on most biochemical treatment; also, the solid phase separation can afford iterative flow-through and clean-up steps, leading to high efficiency and low background noises.

EXAMPLE 2: PHI6 RNA REPLICASE EXPRESSION AND PURIFICATION

5 [00160] The Phi6 RNA replicase gene was synthesized at GeneArt (Thermo Fisher). The gene was directly cloned into pMCSG19 vector by using Gibson assembly method. The construct was verified by Sanger sequencing then transformed to PRK1037 competent cell.

10 [00161] For protein expression and purification, the transformant was grown at 37 °C overnight as a starter culture. In the next day it was used to inoculate LB media and grown at 37 °C to an absorbance at 600 nm of 0.8. Then, the media culture was cooled down to 16 °C and induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside at 16 °C for 18 hrs. The bacterial cells were pelleted by centrifugation and homogenized in lysis buffer containing 20 mM Tris-HCl pH 8.0, 200 mM NaCl and 1 mM phenylmethanesulfonyl-fluoride (PMSF) by
15 a cell homogenizer. The supernatant was subjected to Ni-NTA columns for affinity purification. The protein was eluted in 20 mM Tris-HCl, pH 8.0, 200 mM NaCl and 500 mM imidazole, then directly subjected to Heparin column (GE Healthcare). Elution of the bound protein was performed with a 150 mM to 1 M NaCl gradient buffered with 50 mM Tris-HCl, pH 8.0 and 1 mM EDTA. Fractions containing Phi6 RNA replicase was further purified by
20 Source Q column (GE Healthcare) with a 100 mM to 1 M NaCl gradient. The fraction was pooled together and concentrated for storage.

EXAMPLE 3: AN ALTERNATIVE BIOTINYLATION LABELING REACTION

[00162] Besides maleimide sulfur reaction, an alternative approach was also developed. The reaction between the phosphothioate group at the 5' terminal of RNA and
25 methanethiosulfonate biotin (MTSEA-biotin) labels the RNA molecule with biotin via the formation of disulfide bond, which can be cleaved by DTT treatment.

[00163] The advantage of this methanethiosulfonate disulfide formation strategy is: 1) the high activity of MTSEA-biotin makes the labeling more efficient under milder condition (incubated at room temperature for 20 min); 2) the DTT cleavage step provides high recovery

yield of selective pulldown RNA molecule from the streptavidin beads, ensuring the application of Deam-seq to limited amount samples.

EXAMPLE 4: DATA ANALYSIS PIPELINE

[00164] Regarding the fact that Deam-seq original reads contain multiple specific mutations which is very similar to bisulfite sequencing, the inventors chose to adapt Bismark, a widely used bisulfite sequencing analysis tool, to the Deam-seq analysis by performing A-to-G transition instead of its original C-to-T transition. The modified Bismark script is able to uniquely map the raw data back to human transcriptome and report both the transition sites and transition frequency of each site as analysis output, which could be used for further statistical test.

[00165] With the adapted Bismark tool, the pipeline to analyze Deam-seq and translate the raw data into frequency is described as following:

[00166] Step 1: use a homemade script to convert the raw data `.fastq` file to its reverse complementary sequence `.fastq.rc`. The Illumina TruSeq stranded mRNA library preparation kit specifically labels the transcriptome to effectively distinguish the first-strand cDNA from the second strand, keeping the stranded information in raw data. Given the great likelihood that Deam-seq libraries contain both “sense” (the transcriptome) and “anti-sense” (the artificial “reverse complement-ome”) reads, it is of great necessity to process all reads to make sure that the reads are in their original directions as how they are annotated in genome.

[00167] Step 2: apply the adapted Bismark tool to map the converted raw data `.fastq.rc` file back to reference genome. In this step, both the raw reads from Step 1 and the reference genome are first transformed to replace all A sites with G sites, then two parallel alignment instances between original and converted versions of raw data and reference enable one to precisely and uniquely map the reads; meanwhile, conversion status of each A site is also recorded based on the parallel alignment, which is further reported as the A-to-G transition frequency.

[00168] Step 3: employ Bedtools intersect to extract the transcriptome oriented A-to-G transition frequency for further statistical analysis. Even the reverse complementary strand in dsRNA could be deaminated in the treatment, only the strand with the same direction as the transcriptome is the target for m⁶A analysis. Using the transcriptome based reference,

Bedtools intersect tool efficiently extracts the “on-transcript-strand” part and reports in a separate file recording the conversion status of each A sites covered by raw data. An example report is shown in **Table 1**.

5 **Table 1. Transcriptome direction A-to-G report “on-transcript-strand”**

Categories of A sites on-transcript-strand	Counts
A->A only Events ^a	8,576,879
A->A only Sites ^b	2,217,080
A->G only Events ^c	9,234,159
A->G only Sites ^d	2,110,312
A&G both A->A Events ^e	25,384,512
A&G both A->G Events ^f	28,141,722
A&G both Sites ^g	2,702,153

Notes:

^a A->A only Events: on A sites which do not have A-to-G mutation detected, the covered A reads counted as “events”.

^b A->A only Sites: the A sites which do not have A-to-G mutation detected.

10 ^c A->G only Events: on A sites which have all A read as G, the covered G reads counted as “events”.

^d A->G only Sites: the A sites which have all A read as G.

^e A&G both A->A Events: on A sites which have both A and G detected, the covered A reads counted as “events”.

15 ^f A&G both A->G Events: on A sites which have both A and G detected, the covered G reads counted as “events”.

^g A&G both Sites: the A sites which have both A and G detected.

[00169] All of the methods disclosed and claimed herein can be made and executed without
 20 undue experimentation in light of the present disclosure. While the compositions and
 methods of this invention have been described in terms of preferred embodiments, it will be
 apparent to those of skill in the art that variations may be applied to the methods and in the
 steps or in the sequence of steps of the method described herein without departing from the
 concept, spirit and scope of the invention. More specifically, it will be apparent that certain
 25 agents which are both chemically and physiologically related may be substituted for the
 agents described herein while the same or similar results would be achieved. All such similar

substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. All publications described herein are specifically incorporated by reference for all purposes.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

- 5 1. Cantara WA, Crain PF, Rozenski J, McCloskey JA, Harris KA, Zhang X, Vendeix FAP, Fabris D, Agris PF. The RNA modification database, RNAMDB: 2011 update. *Nucleic Acids Res.* 2011;39 (Database issue):D195-D201. PMC3013656.
2. Machnicka MA, Milanowska K, Osman Oglou O, Purta E, Kurkowska M, Olchowik A, Januszewski W, Kalinowski S, Dunin-Horkawicz S, Rother KM, Helm M, Bujnicki JM, 10 Grosjean H. MODOMICS: a database of RNA modification pathways--2013 update. *Nucleic Acids Res.* 2013; D262-7. PMC3531130.
3. He C. Grand challenge commentary: RNA epigenetics? *Nat Chem Biol.* 2010;6(12):863-5. PMC pending.
4. Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, Yi C, Lindahl T, Pan T, Yang Y, He 15 C. *N*⁶-Methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nat Chem Biol.* 2011;7(12):885-7. PMC3218240.
5. Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L, Jia G, Yu M, Lu Z, Deng X, Dai Q, Chen W, He C. A METTL3-METTL14 complex mediates mammalian nuclear RNA *N*⁶-adenosine methylation. *Nat Chem Biol.* 2014;10(2):93-5. PMC3911877.
- 20 6. Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, Fu Y, Parisien M, Dai Q, Jia G, Ren B, Pan T, He C. *N*⁶-methyladenosine-dependent regulation of messenger RNA stability. *Nature.* 2014;505(7481):117-20. PMC3877715.
7. Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang C-M, Li CJ, Vågbø CB, Shi Y, Wang W-L, Song S-H, Lu Z, Bosmans Ralph PG, Dai Q, Hao Y-J, Yang X, Zhao W-M, Tong W- 25 M, Wang X-J, Bogdan F, Furu K, Fu Y, Jia G, Zhao X, Liu J, Krokan HE, Klungland A, Yang Y-G, He C. ALKBH5 Is a Mammalian RNA Demethylase that Impacts RNA Metabolism and Mouse Fertility. *Mol Cell.* 2013;49(1):18-29. PMC3646334.
8. Fu Y, Dominissini D, Rechavi G, He C. Gene expression regulation mediated through reversible m⁶A RNA methylation. *Nat Rev Genet.* 2014;15(5):293-306. PMC pending.

9. Carlile TM, Rojas-Duran MF, Zinshteyn B, Shin H, Bartoli KM, Gilbert WV. Pseudouridine profiling reveals regulated mRNA pseudouridylation in yeast and human cells. *Nature*. 2014; 515(7525):143-6. PMC4224642.
10. Schwartz S, Bernstein Douglas A, Mumbach Maxwell R, Jovanovic M, Herbst
5 Rebecca H, León-Ricardo Brian X, Engreitz Jesse M, Guttman M, Satija R, Lander Eric S, Fink G, Regev A. Transcriptome-wide Mapping Reveals Widespread Dynamic-Regulated Pseudouridylation of ncRNA and mRNA. *Cell*. 2014;159(1):148-62. PMC4180118.
11. Dominissini D, Moshitch-Moshkovitz S, Schwartz S, Salmon-Divon M, Ungar L, Osenberg S, Cesarkas K, Jacob-Hirsch J, Amariglio N, Kupiec M, Sorek R, Rechavi G.
10 Topology of the human and mouse m⁶A RNA methylomes revealed by m⁶A-seq. *Nature*. 2012;485(7397):201-6. PMC pending.
12. Meyer K, Saletore Y, Zumbo P, Elemento O, Mason C, Jaffrey S. Comprehensive Analysis of mRNA Methylation Reveals Enrichment in 3' UTRs and near Stop Codons. *Cell*. 2012;149(7):1635-46. PMC3383396.
13. Batista PJ, Molinie B, Wang J, Qu K, Zhang J, Li L, Bouley DM, Lujan E, Haddad B, Daneshvar K, Carter AC, Flynn RA, Zhou C, Lim K-S, Dedon P, Wernig M, Mullen AC, Xing Y, Giallourakis CC, Chang HY. m⁶A RNA Modification Controls Cell Fate Transition in Mammalian Embryonic Stem Cells. *Cell Stem Cell*. 2014;15(6):707-19. PMC4278749.
14. Chen T, Hao Y-J, Zhang Y, Li M-M, Wang M, Han W, Wu Y, Lv Y, Hao J, Wang L,
20 Li A, Yang Y, Jin K-X, Zhao X, Li Y, Ping X-L, Lai W-Y, Wu L-G, Jiang G, Wang H-L, Sang L, Wang X-J, Yang Y-G, Zhou Q. m⁶A RNA Methylation Is Regulated by MicroRNAs and Promotes Reprogramming to Pluripotency. *Cell Stem Cell*. 2015;16(3):289-301. PMC pending.
15. Geula S, Moshitch-Moshkovitz S, Dominissini D, Mansour AA, Kol N, Salmon-
25 Divon M, Hershkovitz V, Peer E, Mor N, Manor YS, Ben-Haim MS, Eyal E, Yunger S, Pinto Y, Jaitin DA, Viukov S, Rais Y, Krupalnik V, Chomsky E, Zerbib M, Maza I, Rechavi Y, Massarwa R, Hanna S, Amit I, Levanon EY, Amariglio N, Stern-Ginossar N, Novershtern N, Rechavi G, Hanna JH. m⁶A mRNA methylation facilitates resolution of naïve pluripotency toward differentiation. *Science*. 2015;347(6225):1002-6. PMC pending.
16. Ping X-L, Sun B-F, Wang L, Xiao W, Yang X, Wang W-J, Adhikari S, Shi Y, Lv Y, Chen Y-S, Zhao X, Li A, Yang Y, Dahal U, Lou X-M, Liu X, Huang J, Yuan W-P, Zhu X-F, Cheng T, Zhao Y-L, Wang X, Danielsen JMR, Liu F, Yang Y-G. Mammalian WTAP is a

regulatory subunit of the RNA *N*⁶-methyladenosine methyltransferase. *Cell Res.* 2014;24(2):177-89. PMC3915904.

17. Schwartz S, Agarwala Sudeep D, Mumbach Maxwell R, Jovanovic M, Mertins P, Shishkin A, Tabach Y, Mikkelsen Tarjei S, Satija R, Ruvkun G, Carr Steven A, Lander Eric S, Fink Gerald R, Regev A. High-Resolution Mapping Reveals a Conserved, Widespread, Dynamic mRNA Methylation Program in Yeast Meiosis. *Cell.* 2013;155(6):1409-21. PMC3956118.
18. Wang Y, Li Y, Toth JJ, Petroski MD, Zhang Z, Zhao JC. *N*⁶-methyladenosine modification destabilizes developmental regulators in embryonic stem cells. *Nat Cell Biol.* 2014;16(2):191-8. PMC pending.
19. Liu N, Dai Q, Zheng G, He C, Parisien M, Pan T. *N*⁶-methyladenosine-dependent RNA structural switches regulate RNA-protein interactions. *Nature.* 2015;518(7540):560-4. PMC4355918.
20. Spitale RC, Flynn RA, Zhang QC, Crisalli P, Lee B, Jung J-W, Kuchelmeister HY, Batista PJ, Torre EA, Kool ET, Chang HY. Structural imprints in vivo decode RNA regulatory mechanisms. *Nature.* 2015;519(7544):486-90. PMC4376618.
21. Bokar JA. The biosynthesis and functional roles of methylated nucleosides in eukaryotic mRNA. *Fine-tuning of RNA functions by modification and editing*; Springer; 2005. p. 141-77. PMC pending.
22. Clancy MJ, Shambaugh ME, Timpte CS, Bokar JA. Induction of sporulation in *Saccharomyces cerevisiae* leads to the formation of *N*⁶-methyladenosine in mRNA: a potential mechanism for the activity of the IME4 gene. *Nucleic Acids Res.* 2002;30(20):4509-18. PMC137137.
23. Hongay CF, Orr-Weaver TL. *Drosophila* Inducer of MEiosis 4 (IME4) is required for Notch signaling during oogenesis. *Proc Natl Acad Sci U.S.A.* 2011;108(36):14855-60. PMC3169142.
24. Zhong S, Li H, Bodi Z, Button J, Vespa L, Herzog M, Fray RG. MTA is an Arabidopsis messenger RNA adenosine methylase and interacts with a homolog of a sex-specific splicing factor. *The Plant Cell Online.* 2008;20(5):1278-88. PMC2438467.
25. Yi C, Pan T. Cellular Dynamics of RNA Modification. *Acc Chem Res.* 2011;44(12):1380-8. PMC pending.

26. Phizicky EM, Hopper AK. tRNA processing, modification, and subcellular dynamics: past, present, and future. *RNA*. 2015;21(4):483-5. PMC4371247.
27. Kiss-László Z, Henry Y, Bachellerie J-P, Caizergues-Ferrer M, Kiss T. Site-Specific Ribose Methylation of Preribosomal RNA: A Novel Function for Small Nucleolar RNAs. *Cell*. 1996;85(7):1077-88. PMC pending.
28. Squires JE, Patel HR, Nousch M, Sibbritt T, Humphreys DT, Parker BJ, Suter CM, Preiss T. Widespread occurrence of 5-methylcytosine in human coding and non-coding RNA. *Nucleic Acids Res*. 2012;40(11):5023-33. PMC3367185.
29. Umeda N, Suzuki T, Yukawa M, Ohya Y, Shindo H, Watanabe K, Suzuki T. Mitochondria-specific RNA-modifying Enzymes Responsible for the Biosynthesis of the Wobble Base in Mitochondrial tRNAs: Implications for the molecular pathogenesis of human mitochondria diseases. *J Biol Chem*. 2005;280(2):1613-24. PMC pending.
30. Torres AG, Batlle E, Ribas de Pouplana L. Role of tRNA modifications in human diseases. *Trends Mol Med*. 20(6):306-14. PMC pending.
31. Desrosiers R, Friderici K, Rottman F. Identification of Methylated Nucleosides in Messenger RNA from Novikoff Hepatoma Cells. *Proc Natl Acad Sci U.S.A*. 1974;71(10):3971-5. PMC434308.
32. Desrosiers RC, Friderici KH, Rottman FM. Characterization of Novikoff hepatoma mRNA methylation and heterogeneity in the methylated 5' terminus. *Biochemistry*. 1975;14(20):4367-74. PMC pending.
33. Charette M, Gray MW. Pseudouridine in RNA: What, Where, How, and Why. *IUBMB Life*. 2000;49(5):341-51. PMC pending.
34. Dubin DT, Stollar V, Hsueh C-C, Timko K, Guild GM. Sindbis virus messenger RNA: the 5'-termini and methylated residues of 26 and 42 S RNA. *Virology*. 1977;77(2):457-70. PMC pending.
35. Horowitz S, Horowitz A, Nilsen TW, Munns TW, Rottman FM. Mapping of N6-methyladenosine residues in bovine prolactin mRNA. *Proc Natl Acad Sci U.S.A*. 1984;81(18):5667-71. PMC pending.
36. Rottman F, Shatkin AJ, Perry RP. Sequences containing methylated nucleotides at the 5' termini of messenger RNAs: Possible implications for processing. *Cell*. 3(3):197-9. PMC pending.

37. Yu YT, Shu MD, Steitz JA. A new method for detecting sites of 2'-O-methylation in RNA molecules. *RNA*. 1997;3(3):324-31. PMC1369484.
38. Jia G, Fu Y, He C. Reversible RNA adenosine methylation in biological regulation. *Trends Genet*. 2013;29(2):108-15. PMC3558665.
- 5 39. Meyer KD, Jaffrey SR. The dynamic epitranscriptome: *N*⁶-methyladenosine and gene expression control. *Nat Rev Mol Cell Biol*. 2014;15(5):313-26. PMC4393108.
40. Pan T. *N*⁶-methyl-adenosine modification in messenger and long non-coding RNA. *Trends Biochem Sci*. 2013;38(4):204-9. PMC3608796.
41. Bokar JA, Shambaugh ME, Polayes D, Matera AG, Rottman FM. Purification and
10 cDNA cloning of the AdoMet-binding subunit of the human mRNA (*N*⁶-adenosine)-methyltransferase. *RNA*. 1997;3(11):1233-47. PMC1369564.
42. Bokar JA, Rath-Shambaugh ME, Ludwiczak R, Narayan P, Rottman F. Characterization and partial purification of mRNA *N*⁶-adenosine methyltransferase from HeLa cell nuclei. Internal mRNA methylation requires a multisubunit complex. *J Biol Chem*.
15 1994;269(26):17697-704. PMC pending.
43. Bodi Z, Zhong S, Mehra S, Song J, Li H, Graham N, May S, Fray RG. Adenosine methylation in Arabidopsis mRNA is associated with the 3' end and reduced levels cause developmental defects. *Front Plant Sci*. 2012;3:48. PMC3355605.
44. Fustin J-M, Doi M, Yamaguchi Y, Hida H, Nishimura S, Yoshida M, Isagawa T,
20 Morioka Masaki S, Kakeya H, Manabe I, Okamura H. RNA-Methylation-Dependent RNA Processing Controls the Speed of the Circadian Clock. *Cell*. 2013;155(4):793-806. PMC pending.
45. Alarcon CR, Lee H, Goodarzi H, Halberg N, Tavazoie SF. *N*⁶-methyladenosine marks primary microRNAs for processing. *Nature*. 2015;519(7544):482-5. PMC pending.
- 25 46. Boissel S, Reish O, Proulx K, Kawagoe-Takaki H, Sedgwick B, Yeo GSH, Meyre D, Golzio C, Molinari F, Kadhon N, Etchevers HC, Saudek V, Farooqi IS, Froguel P, Lindahl T, O'Rahilly S, Munnich A, Colleaux L. Loss-of-Function Mutation in the Dioxygenase-Encoding FTO Gene Causes Severe Growth Retardation and Multiple Malformations. *Am J Hum Genet*. 2009;85(1):106-11. PMC2706958.
- 30 47. Dina C, Meyre D, Gallina S, Durand E, Korner A, Jacobson P, Carlsson LMS, Kiess W, Vatin V, Lecoecur C, Delplanque J, Vaillant E, Pattou F, Ruiz J, Weill J, Levy-Marchal C,

Horber F, Potoczna N, Hercberg S, Le Stunff C, Bougneres P, Kovacs P, Marre M, Balkau B, Cauchi S, Chevre J-C, Froguel P. Variation in *FTO* contributes to childhood obesity and severe adult obesity. *Nat Genet.* 2007;39(6):724-6. PMC pending.

48. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM,
5 Perry JRB, Elliott KS, Lango H, Rayner NW, Shields B, Harries LW, Barrett JC, Ellard S, Groves CJ, Knight B, Patch A-M, Ness AR, Ebrahim S, Lawlor DA, Ring SM, Ben-Shlomo Y, Jarvelin M-R, Sovio U, Bennett AJ, Melzer D, Ferrucci L, Loos RJF, Barroso I, Wareham NJ, Karpe F, Owen KR, Cardon LR, Walker M, Hitman GA, Palmer CNA, Doney ASF, Morris AD, Smith GD, Consortium TWTCC, Hattersley AT, McCarthy MI. A Common
10 Variant in the *FTO* Gene Is Associated with Body Mass Index and Predisposes to Childhood and Adult Obesity. *Science.* 2007;316(5826):889-94. PMC pending.
49. Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ, Li Y, Duren WL, Erdos MR, Stringham HM, Chines PS, Jackson AU, Prokunina-Olsson L, Ding C-J, Swift AJ, Narisu N, Hu T, Pruim R, Xiao R, Li X-Y, Conneely KN, Riebow NL, Sprau AG, Tong M, White PP,
15 Hetrick KN, Barnhart MW, Bark CW, Goldstein JL, Watkins L, Xiang F, Saramies J, Buchanan TA, Watanabe RM, Valle TT, Kinnunen L, Abecasis GR, Pugh EW, Doheny KF, Bergman RN, Tuomilehto J, Collins FS, Boehnke M. A Genome-Wide Association Study of Type 2 Diabetes in Finns Detects Multiple Susceptibility Variants. *Science.* 2007;316(5829):1341-5. PMC pending.
- 20 50. Scuteri A, Sanna S, Chen W-M, Uda M, Albai G, Strait J, Najjar S, Nagaraja R, Orrù M, Usala G, Dei M, Lai S, Maschio A, Busonero F, Mulas A, Ehret GB, Fink AA, Weder AB, Cooper RS, Galan P, Chakravarti A, Schlessinger D, Cao A, Lakatta E, Abecasis GR. Genome-Wide Association Scan Shows Genetic Variants in the *FTO* Gene Are Associated with Obesity-Related Traits. *PLoS Genet.* 2007;3(7):e115. PMC pending.
- 25 51. Church C, Moir L, McMurray F, Girard C, Banks GT, Teboul L, Wells S, Bruning JC, Nolan PM, Ashcroft FM, Cox RD. Overexpression of *Fto* leads to increased food intake and results in obesity. *Nat Genet.* 2010;42(12):1086-92. PMC3018646.
52. Fischer J, Koch L, Emmerling C, Vierkotten J, Peters T, Bruning JC, Ruther U. Inactivation of the *Fto* gene protects from obesity. *Nature.* 2009;458(7240):894-8. PMC
30 pending.

53. Ng H-H, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, Tempst P, Reinberg D, Bird A. MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat Genet.* 1999;23(1):58-61. PMC pending.
54. Obata Y, Furusawa Y, Endo TA, Sharif J, Takahashi D, Atarashi K, Nakayama M, Onawa S, Fujimura Y, Takahashi M, Ikawa T, Otsubo T, Kawamura YI, Dohi T, Tajima S, Masumoto H, Ohara O, Honda K, Hori S, Ohno H, Koseki H, Hase K. The epigenetic regulator Uhrf1 facilitates the proliferation and maturation of colonic regulatory T cells. *Nat Immunol.* 2014;15(6):571-9. PMC pending.
55. Sharif J, Muto M, Takebayashi S-i, Suetake I, Iwamatsu A, Endo TA, Shinga J, Mizutani-Koseki Y, Toyoda T, Okamura K, Tajima S, Mitsuya K, Okano M, Koseki H. The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. *Nature.* 2007;450(7171):908-12. PMC pending.
56. Xu C, Wang X, Liu K, Roundtree IA, Tempel W, Li Y, Lu Z, He C, Min J. Structural basis for selective binding of m6A RNA by the YTHDC1 YTH domain. *Nat Chem Biol.* 2014;10(11):927-9. PMC pending.
57. Liu N, Parisien M, Dai Q, Zheng G, He C, Pan T. Probing N6-methyladenosine RNA modification status at single nucleotide resolution in mRNA and long noncoding RNA. *Rna.* 2013;19(12):1848-56. PMC3884656
58. Canaani D, Kahana C, Lavi S, Groner Y. Identification and mapping of N⁶-methyladenosine containing sequences in Simian Virus 40 RNA. *Nucleic Acids Res.* 1979;6(8):2879-99. PMC327900.
59. Dimock K, Stoltzfus CM. Sequence specificity of internal methylation in B77 avian sarcoma virus RNA subunits. *Biochemistry-US.* 1977;16(3):471-8. PMC pending.
60. Harper JE, Miceli SM, Roberts RJ, Manley JL. Sequence specificity of the human mRNA N6-adenosine methylase *in vitro*. *Nucleic Acids Res.* 1990;18(19):5735-41. PMC pending.
61. Kane SE, Beemon K. Precise Localization of m6A in Rous Sarcoma Virus RNA Reveals Clustering of Methylation Sites: Implications for RNA Processing. *Mol Cell Biol.* 1985;5(9):2298-306. PMC pending.

62. Schibler U, Kelley DE, Perry RP. Comparison of methylated sequences in messenger RNA and heterogeneous nuclear RNA from mouse L cells. *J Mol Biol.* 1977;115(4):695-714. PMC pending.
63. Wei C-M, Moss B. Nucleotide Sequences at the *N*⁶-Methyladenosine Sites of HeLa
5 Cell messenger Ribonucleic Acid. *Biochemistry-US.* 1977;16(8):1672-6. PMC pending.
64. Ganot P, Bortolin M-L, Kiss T. Site-Specific Pseudouridine Formation in Preribosomal RNA Is Guided by Small Nucleolar RNAs. *Cell.* 1997;89(5):799-809. PMC pending.
65. Decatur WA, Fournier MJ. RNA-guided Nucleotide Modification of Ribosomal and
10 Other RNAs. *J Biol Chem.* 2003;278(2):695-8. PMC pending.
66. Barbosa E, Moss B. mRNA(nucleoside-2'-)-methyltransferase from vaccinia virus. Purification and physical properties. *J Biol Chem.* 1978;253(21):7692-7. PMC pending.
67. McMahon M, Contreras A, Ruggero D. Small RNAs with big implications: new insights into H/ACA snoRNA function and their role in human disease. Wiley
15 *Interdisciplinary Reviews: RNA.* 2015;6(2):173-89. PMC4390053.
68. Khoddami V, Cairns BR. Identification of direct targets and modified bases of RNA cytosine methyltransferases. *Nat Biotech.* 2013;31(5):458-64. PMC3791587.
69. Hussain S, Sajini Abdulrahim A, Blanco S, Dietmann S, Lombard P, Sugimoto Y, Paramor M, Gleeson Joseph G, Odom Duncan T, Ule J, Frye M. NSun2-Mediated Cytosine-5
20 Methylation of Vault Noncoding RNA Determines Its Processing into Regulatory Small RNAs. *Cell Reports.* 2013;4(2):255-61. PMC3730056.
70. Arnez JG, Steitz TA. Crystal structure of unmodified tRNA^{Gln} complexed with glutaminyl-tRNA synthetase and ATP suggests a possible role for pseudo-uridines in stabilization of RNA structure. *Biochemistry.* 1994;33(24):7560-7. PMC pending.
71. Newby MI, Greenbaum NL. Sculpting of the spliceosomal branch site recognition motif by a conserved pseudouridine. *Nat Struct Mol Biol.* 2002;9(12):958-65. PMC pending.
72. Davis DR. Stabilization of RNA stacking by pseudouridine. *Nucleic Acids Res.* 1995;23(24):5020-6.

73. Newby MI, Greenbaum NL. A conserved pseudouridine modification in eukaryotic U2 snRNA induces a change in branch-site architecture. *RNA*. 2001;7(06):833-45. PMC pending.
74. Newby MI, Greenbaum NL. Investigation of Overhauser effects between
5 pseudouridine and water protons in RNA helices. *Proc Natl Acad Sci U.S.A.* 2002;99(20):12697-702. PMC pending.
75. Wu G, Xiao M, Yang C, Yu YT. U2 snRNA is inducibly pseudouridylated at novel sites by Pus7p and snR81 RNP. *The EMBO journal*. 2011;30(1):79-89. PMC3020122.
76. Meier UT. Pseudouridylation goes regulatory. *The EMBO journal*. 2011;30(1):3-4.
10 PMC3020123
77. Karikó K, Muramatsu H, Keller JM, Weissman D. Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin. *Molecular Therapy*. 2012. 20(5):948-53. PMC3345990.
78. Jambhekar A, DeRisi JL. *Cis*-acting determinants of asymmetric, cytoplasmic RNA
15 transport. *RNA*. 2007;13(5):625-42. PMC pending.
79. Kudla G, Murray AW, Tollervey D, Plotkin JB. Coding-sequence determinants of gene expression in *Escherichia coli*. *Science*. 2009;324(5924):255-8. PMC3902468.
80. Somogyi P, Jenner A, Brierley I, Inglis S. Ribosomal pausing during translation of an RNA pseudoknot. *Mol Cell Biol*. 1993;13(11):6931-40. PMC pending.
- 20 81. Shah P, Ding Y, Niemczyk M, Kudla G, Plotkin JB. Rate-limiting steps in yeast protein translation. *Cell*. 2013;153(7):1589-601. PMC3694300.
82. Tan X, Lu ZJ, Gao G, Xu Q, Hu L, Fellmann C, Li MZ, Qu H, Lowe SW, Hannon GJ. Tiling genomes of pathogenic viruses identifies potent antiviral shRNAs and reveals a role for secondary structure in shRNA efficacy. *Proc Natl Acad Sci U.S.A.* 2012;109(3):869-
25 74. PMC3271875.
83. Karijolich J, Yu Y-T. Converting nonsense codons into sense codons by targeted pseudouridylation. *Nature*. 2011;474(7351):395-8. PMC3381908.
84. Fernández IS, Ng CL, Kelley AC, Wu G, Yu Y-T, Ramakrishnan V. Unusual base pairing during the decoding of a stop codon by the ribosome. *Nature*. 2013;500(7460):107-
30 10. PMC3732562.

85. Lee SH, Kim I, Chung BC. Increased urinary level of oxidized nucleosides in patients with mild-to-moderate Alzheimer's disease. *Clinical Biochemistry*. 2007;40(13):936-8. PMC pending.
86. Grozdanov PN, Fernandez-Fuentes N, Fiser A, Meier UT. Pathogenic NAP57
5 mutations decrease ribonucleoprotein assembly in dyskeratosis congenita. *Hum Mol Genet*. 2009;18(23):4546-51. PMC2773269.
87. Heiss NS, Knight SW, Vulliamy TJ, Klauck SM, Wiemann S, Mason PJ, Poustka A, Dokal I. X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. *Nat Genet*. 1998;19(1):32-8. PMC pending.
- 10 88. Schaefer M, Pollex T, Hanna K, Tuorto F, Meusburger M, Helm M, Lyko F. RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. *Genes Dev*. 2010;24(15):1590-5. PMC2912555.
89. Tuorto F, Liebers R, Musch T, Schaefer M, Hofmann S, Kellner S, Frye M, Helm M, Stoecklin G, Lyko F. RNA cytosine methylation by Dnmt2 and NSun2 promotes tRNA
15 stability and protein synthesis. *Nat Struct Mol Biol*. 2012;19(9):900-5. PMC pending.
90. Kiani J, Grandjean V, Liebers R, Tuorto F, Ghanbarian H, Lyko F, Cuzin F, Rassoulzadegan M. RNA-Mediated Epigenetic Heredity Requires the Cytosine Methyltransferase Dnmt2. *PLoS Genet*. 2013;9(5):e1003498. PMC3662642.
91. Metodiev MD, Spåhr H, Loguercio Polosa P, Meharg C, Becker C, Altmueller J,
20 Habermann B, Larsson N-G, Ruzzenente B. NSUN4 Is a Dual Function Mitochondrial Protein Required for Both Methylation of 12S rRNA and Coordination of Mitochondrial Assembly. *PLoS Genet*. 2014;10(2):e1004110. PMC3916286.
92. Cámara Y, Asin-Cayuela J, Park Chan B, Metodiev Metodi D, Shi Y, Ruzzenente B, Kukat C, Habermann B, Wibom R, Hultenby K, Franz T, Erdjument-Bromage H, Tempst P,
25 Hallberg BM, Gustafsson Claes M, Larsson N-G. MTERF4 Regulates Translation by Targeting the Methyltransferase NSUN4 to the Mammalian Mitochondrial Ribosome. *Cell Metab*. 2011;13(5):527-39. PMC pending.
93. Okamoto M, Hirata S, Sato S, Koga S, Fujii M, Qi G, Ogawa I, Takata T, Shimamoto F, Tatsuka M. Frequent increased gene copy number and high protein expression of tRNA
30 (cytosine-5-)-methyltransferase (NSUN2) in human cancers. *DNA Cell Biol*. 2012;31(5):660-71. PMC pending.

94. Frye M, Watt FM. The RNA methyltransferase Misu (NSun2) mediates Myc-induced proliferation and is upregulated in tumors. *Curr Biol.* 2006;16(10):971-81. PMC pending.
95. Khosronezhad N, Colagar AH, Jorsarayi SGA. T26248G-transversion mutation in exon7 of the putative methyltransferase Nsun7 gene causes a change in protein folding associated with reduced sperm motility in asthenospermic men. *Reprod Fertil Dev.* 2015;27(3):471-80. PMC pending.
96. Hussain S, Tuorto F, Menon S, Blanco S, Cox C, Flores JV, Watt S, Kudo NR, Lyko F, Frye M. The Mouse Cytosine-5 RNA Methyltransferase NSun2 Is a Component of the Chromatoid Body and Required for Testis Differentiation. *Mol Cell Biol.* 2013;33(8):1561-70. PMC3624257.
97. Harris T, Marquez B, Suarez S, Schimenti J. Sperm Motility Defects and Infertility in Male Mice with a Mutation in Nsun7, a Member of the Sun Domain-Containing Family of Putative RNA Methyltransferases. *Biol Reprod.* 2007;77(2):376-82. PMC pending.
98. Khan MA, Rafiq MA, Noor A, Hussain S, Flores JV, Rupp V, Vincent AK, Malli R, Ali G, Khan FS. Mutation in NSUN2, which encodes an RNA methyltransferase, causes autosomal-recessive intellectual disability. *Am J Hum Genet.* 2012;90(5):856-63. PMC3376419.
99. Begley U, Dyavaiah M, Patil A, Rooney JP, DiRenzo D, Young CM, Conklin DS, Zitomer RS, Begley TJ. Trm9-catalyzed tRNA modifications link translation to the DNA damage response. *Mol Cell.* 2007;28(5):860-70. PMC2211415.
100. Chan CT, Pang YLJ, Deng W, Babu IR, Dyavaiah M, Begley TJ, Dedon PC. Reprogramming of tRNA modifications controls the oxidative stress response by codon-biased translation of proteins. *Nature Commun.* 2012;3:937. PMC3535174.
101. Kaiser S, Rimbach K, Eigenbrod T, Dalpke AH, Helm M. A modified dinucleotide motif specifies tRNA recognition by TLR7. *RNA.* 2014;20(9):1351-5. PMC4138318.
102. Fu D, Brophy JAN, Chan CTY, Atmore KA, Begley U, Paules RS, Dedon PC, Begley TJ, Samson LD. Human AlkB Homolog ABH8 Is a tRNA Methyltransferase Required for Wobble Uridine Modification and DNA Damage Survival. *Mol Cell Biol.* 2010;30(10):2449-59. PMC2863699.
103. Saikia M, Fu Y, Pavon-Eternod M, He C, Pan T. Genome-wide analysis of N¹-methyl-adenosine modification in human tRNAs. *RNA.* 2010;16(7):1317-27. PMC2885681.

104. Anderson J, Phan L, Hinnebusch AG. The Gcd10p/Gcd14p complex is the essential two-subunit tRNA (1-methyladenosine) methyltransferase of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U.S.A.* 2000;97(10):5173-8. PMC pending.
105. Anderson J, Phan L, Cuesta R, Carlson BA, Pak M, Asano K, Björk GR, Tamame M,
5 Hinnebusch AG. The essential Gcd10p–Gcd14p nuclear complex is required for 1-methyladenosine modification and maturation of initiator methionyl-tRNA. *Genes Dev.* 1998;12(23):3650-62. PMC pending.
106. Li J, Yang Z, Yu B, Liu J, Chen X. Methylation Protects miRNAs and siRNAs from a 3'-End Uridylation Activity in *Arabidopsis*. *Curr Biol.* 2005;15(16):1501-7. PMC pending.
107. Bakin A, Ofengand J. Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: analysis by the application of a new sequencing technique. *Biochemistry.* 1993;32(37):9754-62. PMC pending.
108. Kiss-László Z, Henry Y, Kiss T. Sequence and structural elements of methylation guide snoRNAs essential for site-specific ribose methylation of pre-rRNA. *The EMBO Journal.* 1998;17(3):797-807. PMC pending.
15
109. Luo G-Z, MacQueen A, Zheng G, Duan H, Dore LC, Lu Z, Liu J, Chen K, Jia G, Bergelson J, He C. Unique features of the m⁶A methylome in *Arabidopsis thaliana*. *Nat Commun.* 2014;5:5630. PMC4248235.
110. Chen K, Lu Z, Wang X, Fu Y, Luo G-Z, Liu N, Han D, Dominissini D, Dai Q, Pan T,
20 He C. High-Resolution N6-Methyladenosine (m6A) Map Using Photo-Crosslinking-Assisted m6A Sequencing. *Angew Chem Int Ed.* 2015;54(5):1587-90. PMC4396828.
111. Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH, Ecker JR. Highly Integrated Single-Base Resolution Maps of the Epigenome in *Arabidopsis*. *Cell.* 2008;133(3):523-36. PMC2723732.
112. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo Q-M, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature.* 2009;462(7271):315-22. PMC2857523.
25
113. Yu M, Hon Gary C, Szulwach Keith E, Song C-X, Zhang L, Kim A, Li X, Dai Q,
30 Shen Y, Park B, Min J-H, Jin P, Ren B, He C. Base-Resolution Analysis of 5-

Hydroxymethylcytosine in the Mammalian Genome. *Cell*. 2012;149(6):1368-80.

PMC3589129.

114. Véliz EA, Easterwood LM, Beal PA. Substrate Analogues for an RNA-Editing Adenosine Deaminase: Mechanistic Investigation and Inhibitor Design. *Journal of the American Chemical Society*. 2003;125(36):10867-76. PMC pending.

115. Song C-X, Szulwach Keith E, Dai Q, Fu Y, Mao S-Q, Lin L, Street C, Li Y, Poidevin M, Wu H, Gao J, Liu P, Li L, Xu G-L, Jin P, He C. Genome-wide Profiling of 5-Formylcytosine Reveals Its Roles in Epigenetic Priming. *Cell*. 2013;153(3):678-91.

PMC3657391.

116. Lu X, Song C-X, Szulwach K, Wang Z, Weidenbacher P, Jin P, He C. Chemical Modification-Assisted Bisulfite Sequencing (CAB-Seq) for 5-Carboxylcytosine Detection in DNA. *J Am Chem Soc*. 2013;135(25):9315-7. PMC3727251.

117. Fu Y, Luo G-Z, Chen K, Deng X, Yu M, Han D, Hao Z, Liu J, Lu X, Doré Louis C, Weng X, Ji Q, Mets L, He C. *N*⁶-Methyldeoxyadenosine Marks Active Transcription Start Sites in *Chlamydomonas*. *Cell*. 2015; 161(4):879-92. PMC4427561.

118. Zhang G, Huang H, Liu D, Cheng Y, Liu X, Zhang W, Yin R, Zhang D, Zhang P, Liu J, Li C, Liu B, Luo Y, Zhu Y, Zhang N, He S, He C, Wang H, Chen D. *N*⁶-Methyladenine DNA Modification in *Drosophila*. *Cell*. 2015;161(4):893-906. PMC pending.

119. Greer Eric L, Blanco Mario A, Gu L, Sendinc E, Liu J, Aristizábal-Corrales D, Hsu C-H, Aravind L, He C, Shi Y. DNA Methylation on *N*⁶-Adenine in *C. elegans*. *Cell*. 2015;161(4):868-78. PMC4427530.

120. Keegan LP, Gallo A, O'Connell MA. The many roles of an RNA editor. *Nat Rev Genet*. 2001;2(11):869-78. PMC pending.

121. Wulff B-E, Sakurai M, Nishikura K. Elucidating the inosinome: global approaches to adenosine-to-inosine RNA editing. *Nat Rev Genet*. 2011;12(2):81-5. PMC3075016.

122. Gubler U, Hoffman BJ. A simple and very efficient method for generating cDNA libraries. *Gene*. 1983;25(2-3):263-9. PMC3688977.

123. Makeyev EV, Bamford DH. Replicase activity of purified recombinant protein P2 of double-stranded RNA bacteriophage ϕ 6. *The EMBO Journal*. 2000;19(1):124-33.

PMC1171784.

124. Chen I, Dorr BM, Liu DR. A general strategy for the evolution of bond-forming enzymes using yeast display. *Proc Natl Acad Sci U.S.A.* 2011;108(28):11399-404. PMC3136257.
125. Dorr BM, Ham HO, An C, Chaikof EL, Liu DR. Reprogramming the specificity of sortase enzymes. *Proc Natl Acad Sci U.S.A.* 2014;111(37):13343-8. PMC4169943.
126. Pepper LR, Cho YK, Boder ET, Shusta EV. A decade of yeast surface display technology: where are we now? *Comb Chem High Throughput Screen.* 2008;11(2):127-34. PMC2681324.
127. Yin J, Straight PD, McLoughlin SM, Zhou Z, Lin AJ, Golan DE, Kelleher NL, Kolter R, Walsh CT. Genetically encoded short peptide tag for versatile protein labeling by Sfp phosphopantetheinyl transferase. *Proc Natl Acad Sci U.S.A.* 2005;102(44):15815-20. PMC1276090.
128. Puthenveetil S, Liu DS, White KA, Thompson S, Ting AY. Yeast display evolution of a kinetically efficient 13-amino acid substrate for lipoic acid ligase. *J Am Chem Soc.* 2009;131(45):16430-8. PMC2799336.
129. Macbeth MR, Schubert HL, Vandemark AP, Lingam AT, Hill CP, Bass BL. Inositol hexakisphosphate is bound in the ADAR2 core and required for RNA editing. *Science.* 2005;309(5740):1534-9. PMC1850959.
130. Savva Y, Rieder L, Reenan R. The ADAR protein family. *Genome Biol.* 2012;13(12):252. PMC3580408.
131. Krueger F, Kreck B, Franke A, Andrews SR. DNA methylome analysis using short bisulfite sequencing data. *Nat Meth.* 2012;9(2):145-51. PMC pending.
132. Randau L, Stanley BJ, Kohlway A, Mechta S, Xiong Y, Söll D. A Cytidine Deaminase Edits C to U in Transfer RNAs in Archaea. *Science.* 2009;324(5927):657-9. PMC2857566.
133. Conticello S. The AID/APOBEC family of nucleic acid mutators. *Genome Biol.* 2008;9(6):229. PMC2481415.
134. Prochnow C, Bransteitter R, Klein MG, Goodman MF, Chen XS. The APOBEC-2 crystal structure and functional implications for the deaminase AID. *Nature.* 2007;445(7126):447-51. PMC pending.

135. Motorin Y, Muller S, Behm-Ansmant I, Branlant C. Identification of modified residues in RNAs by reverse transcription-based methods. *Methods Enzymol.* 2007;425:21-53. PMC pending.
136. Tijerina P, Mohr S, Russell R. DMS footprinting of structured RNAs and RNA-protein complexes. *Nat Protoc.* 2007;2(10):2608-23. PMC2701642.
137. Pang YL, Abo R, Levine SS, Dedon PC. Diverse cell stresses induce unique patterns of tRNA up- and down-regulation: tRNA-seq for quantifying changes in tRNA copy number. *Nucleic Acids Res.* 2014;42(22):e170. PMC4267671.
138. Mohr S, Ghanem E, Smith W, Sheeter D, Qin Y, King O, Polioudakis D, Iyer VR, Hunicke-Smith S, Swamy S, Kuersten S, Lambowitz AM. Thermostable group II intron reverse transcriptase fusion proteins and their use in cDNA synthesis and next-generation RNA sequencing. *RNA.* 2013;19(7):958-70. PMC3683930.
139. Trewick SC, Henshaw TF, Hausinger RP, Lindahl T, Sedgwick B. Oxidative demethylation by *Escherichia coli* AlkB directly reverts DNA base damage. *Nature.* 2002;419(6903):174-8. PMC pending.
140. Aas PA, Otterlei M, Falnes PO, Vagbo CB, Skorpen F, Akbari M, Sundheim O, Bjoras M, Slupphaug G, Seeberg E, Krokan HE. Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. *Nature.* 2003;421(6925):859-63. PMC pending.
141. Holland PJ, Hollis T. Structural and mutational analysis of *Escherichia coli* AlkB provides insight into substrate specificity and DNA damage searching. *PLoS ONE.* 2010;5(1):e8680. PMC2800194.
142. Yang CG, Yi C, Duguid EM, Sullivan CT, Jian X, Rice PA, He C. Crystal structures of DNA/RNA repair enzymes AlkB and ABH2 bound to dsDNA. *Nature.* 2008;452(7190):961-5. PMC2587245.
143. Wang R, Zheng W, Yu H, Deng H, Luo M. Labeling substrates of protein arginine methyltransferase with engineered enzymes and matched S-adenosyl-L-methionine analogues. *J Am Chem Soc.* 133(20):7648-51. PMC3104021.
144. Calabretta A, Leumann CJ. Base pairing and miscoding properties of 1,N⁶-ethenoadenine- and 3,N⁴-ethenocytosine-containing RNA oligonucleotides. *Biochemistry.* 2013;52(11):1990-7. PMC pending.

145. Luo M. Current chemical biology approaches to interrogate protein methyltransferases. *ACS Chem Biol.* 2012;7(3):443-63. PMC3306480.
146. Bakin A, Ofengand J. Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: analysis by the application
5 of a new sequencing technique. *Biochemistry.* 1993;32(37):9754-62. PMC pending.
147. Bakin A, Ofengand J. Mapping of the 13 pseudouridine residues in *Saccharomyces cerevisiae* small subunit ribosomal RNA to nucleotide resolution. *Nucleic Acids Res.* 1995;23(16):3290-4. PMC307190.
148. Bakin AV, Ofengand J. Mapping of pseudouridine residues in RNA to nucleotide
10 resolution. *Methods Mol Biol.* 1998;77:297-309. PMC pending.
149. Xu Y, Liu L, Lopez-Estrano C, Michaeli S. Expression studies on clustered trypanosomatid box C/D small nucleolar RNAs. *J Biol Chem.* 2001;276(17):14289-98. PMC pending.
150. Mag M, Luking S, Engels JW. Synthesis and selective cleavage of an
15 oligodeoxynucleotide containing a bridged internucleotide 5'-phosphorothioate linkage. *Nucleic Acids Res.* 1991;19(7):1437-41. PMC333898.
151. Satterlee JS, Basanta-Sanchez M, Blanco S, Li JB, Meyer K, Pollock J, Sadri-Vakili G, Rybak-Wolf A. Novel RNA modifications in the nervous system: form and function. *J Neurosci.* 2014;34(46):15170-7. PMC4402329.
- 20 152. Chen C, Tuck S, Bystrom AS. Defects in tRNA modification associated with neurological and developmental dysfunctions in *Caenorhabditis elegans* elongator mutants. *PLoS Genet.* 2009;5(7):e1000561. PMC2702823.
153. Honda K, Smith MA, Zhu X, Baus D, Merrick WC, Tartakoff AM, Hattier T, Harris PL, Siedlak SL, Fujioka H, Liu Q, Moreira PI, Miller FP, Nunomura A, Shimohama S, Perry
25 G. Ribosomal RNA in Alzheimer disease is oxidized by bound redox-active iron. *J Biol Chem.* 2005;280(22):20978-86. PMC pending.
154. LaSalle JM, Powell WT, Yasui DH. Epigenetic layers and players underlying neurodevelopment. *Trends Neurosci.* 2013;36(8):460-70. PMC3735843.
155. Bonaguidi MA, Wheeler MA, Shapiro JS, Stadel RP, Sun GJ, Ming GL, Song H. *In vivo* clonal analysis reveals self-renewing and multipotent adult neural stem cell
30 characteristics. *Cell.* 2011;145(7):1142-55. PMC3124562.

156. Christian KM, Song H, Ming GL. Functions and dysfunctions of adult hippocampal neurogenesis. *Annu Rev Neurosci.* 2014;37:243-62. PMC pending.
157. Guo JU, Ma DK, Mo H, Ball MP, Jang MH, Bonaguidi MA, Balazer JA, Eaves HL, Xie B, Ford E, Zhang K, Ming GL, Gao Y, Song H. Neuronal activity modifies the DNA methylation landscape in the adult brain. *Nat Neurosci.* 2011;14(10):1345-51. PMC3183401.
- 5 158. Guo JU, Su Y, Zhong C, Ming GL, Song H. Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell.* 2011;145(3):423-34. PMC3088758.
159. Chen E, Sharma MR, Shi X, Agrawal RK, Joseph S. Fragile X mental retardation protein regulates translation by binding directly to the ribosome. *Mol Cell.* 54(3):407-17. PMC4019695.
- 10 160. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, Fraser CE, Stone EF, Chen C, Fak JJ, Chi SW, Licatalosi DD, Richter JD, Darnell RB. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell.* 2011;146(2):247-61. PMC3232425.
- 15 161. Maxwell EK, Campbell JD, Spira A, Baxeianis AD. SubmiRine: assessing variants in microRNA targets using clinical genomic data sets. *Nucleic Acids Res.* 2015;43(8):3886-98. PMC pending.
162. Song J, Zhong C, Bonaguidi MA, Sun GJ, Hsu D, Gu Y, Meletis K, Huang ZJ, Ge S, Enikolopov G, Deisseroth K, Luscher B, Christian KM, Ming GL, Song H. Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. *Nature.* 2012;489(7414):150-4. PMC3438284.
- 20 163. Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H. GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature.* 2006;439(7076):589-93. PMC1420640.
- 25 164. Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y, Liu XB, Yang CH, Jordan JD, Ma DK, Liu CY, Ganesan S, Cheng HJ, Ming GL, Lu B, Song H. Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. *Cell.* 2007;130(6):1146-58. PMC2002573.
- 30 165. Song J, Sun J, Moss J, Wen Z, Sun GJ, Hsu D, Zhong C, Davoudi H, Christian KM, Toni N, Ming GL, Song H. Parvalbumin interneurons mediate neuronal circuitry-

neurogenesis coupling in the adult hippocampus. *Nat Neurosci.* 2013;16(12):1728-30. PMC4096812.

166. Ge S, Yang CH, Hsu KS, Ming GL, Song H. A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. *Neuron.* 2007;54(4):559-66.

5 PMC2040308.

167. Kim JY, Duan X, Liu CY, Jang MH, Guo JU, Pow-anpongkul N, Kang E, Song H, Ming GL. DISC1 regulates new neuron development in the adult brain via modulation of AKT-mTOR signaling through KIAA1212. *Neuron.* 2009;63(6):761-73. PMC3075620.

168. Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell
10 RA, Lu B, Ming GL, Song H. Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. *Science.* 2009;323(5917):1074-7. PMC2726986.

169. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C. Selective chemical labeling reveals the genome-wide distribution of 5-

15 hydroxymethylcytosine. *Nat Biotechnol.* 2011;29(1):68-72. PMC3107705.

170. Yu H, Su Y, Shin J, Zhong C, Guo JU, Weng YL, Gao F, Geschwind DH, Coppola G, Ming GL, Song H. Tet3 regulates synaptic transmission and homeostatic plasticity via DNA oxidation and repair. *Nat Neurosci.* 2015. advance online publication. PMC pending.

CLAIMS

1. A method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising contacting the target RNA with an adenosine deaminase enzyme to generate a target RNA with deaminated adenosines and sequencing the target RNA with deaminated adenosines; wherein any modified adenosine in the target RNA is read as an adenosine in the sequencing of the target RNA with deaminated adenosines.
2. The method of claim 1, wherein the adenosine deaminase enzyme is ADAR (adenosine deaminase, RNA-specific).
3. The method of claim 1 or 2, wherein the method further comprises: contacting target RNA with a demethylating enzyme; contacting the demethylated target RNA with the adenosine deaminase enzyme to produce control RNA; and sequencing the control RNA; wherein any modified adenosine in the target RNA is read as a guanosine in the sequencing of the control RNA with deaminated adenosines.
4. The method of claim 3 further comprising comparing the sequence of the target RNA with deaminated adenosines to the sequence of the demethylated target RNA .
5. The method of claim 3 or 4, wherein the demethylating enzyme is an *N*⁶-methyladenosine-specific demethylating enzyme.
6. The method of any one of claims 3-6, wherein the demethylating enzyme is ALKBH5.
7. The method of any one of claims 1-6, wherein the target and/or demethylated target RNA is in a duplex with a complementary strand of RNA or DNA.
8. The method of claim 7, wherein the complementary strand is DNA.
9. The method of any one of claims 7-8, wherein the complementary strand comprises modified adenosine.
10. The method of any one of claims 1-9, wherein adenosine deaminase enzyme is from *Drosophila*.
11. The method of any one of claims 1-10, wherein the target RNA is mRNA, lncRNA, pri-microRNA, pre-piRNA, rRNA, tRNA, snoRNA, or snRNA.
12. The method of claim 11, wherein the target RNA is mRNA or lncRNA.

13. The method of any one of claims 1-12, wherein the method further comprises isolating RNA.
14. The method of any one of claims 1-13, wherein the method further comprises generating a nucleic acid strand that is complementary with the target and/or demethylated target RNA and hybridizing the complementary nucleic acid strand with the target RNA and/or demethylated target RNA.
15. The method of claim 14, wherein generating the nucleic acid strand that is complementary with the target and/or demethylated target RNA comprises synthesis of a nucleic acid strand complementary to the target and/or demethylated target RNA.
16. The method of claim 15, wherein the synthesis of the nucleic acid strand comprises a synthesis reaction composition comprising adenosine triphosphate, wherein all of the adenosine triphosphate is modified adenosine triphosphate.
17. The method of any one of claims 1-16, wherein the modified adenosine is N^6 -methyladenosine.
18. The method of any one of claims 1-16, wherein the modified adenosine is N^1 -methyladenosine.
19. The method of any one of claims 15-18, wherein the complementary nucleic acid strand is RNA.
20. The method of claim 15, wherein the complementary nucleic acid strand is DNA.
21. The method of any one of claims 14-19, wherein the method further comprises contacting the target and/or demethylated target RNA with RNA replicase to synthesize a complementary RNA strand.
22. The method of claim 21, wherein the RNA replicase is Phi6.
23. The method of any one of claims 1-21, wherein the target RNA strand is immobilized on a solid support.
24. The method of any one of claims 1-23, wherein the target RNA strand is labeled.
25. The method of claim 24, wherein the label is a phosphothioate group.
26. The method of claim 25, wherein the target RNA is immobilized by reaction of the phosphothioate group with a thiol-reactive group.

27. The method of claim 26, wherein the thiol-reactive group is iodoacetamide, maleimide, or methanethiosulfonate.
28. The method of any one of claims 23-26, wherein the target RNA is fragmented prior to immobilization on the solid support.
29. The method of claim 28, wherein the RNA is fragmented into RNA molecules 50-300 nucleotides in length.
30. The method of claim 28, wherein the average RNA molecule fragment is 100 nucleotides.
31. The method of any one of claim 23 to 30, wherein the method comprises contacting the target and/or demethylated target RNA with RNA replicase to synthesize a complementary RNA strand that forms a duplex with the target RNA or demethylated target RNA.
32. The method of claim 31 wherein the method comprises denaturing of the RNA duplex to create mobilized complementary RNA.
33. The method of claim 32, wherein the method further comprises removal of the mobilized complementary RNA.
34. The method of any one of claims 23 to 33, wherein the method further comprises mobilizing the target RNA by separating it from the solid support.
35. The method of any one of claims 23 to 34, wherein the method comprises the steps of a) immobilizing the target RNA, b) contacting the target and/or demethylated target RNA with RNA replicase to synthesize a complementary RNA strand that forms a duplex with the target RNA or demethylated target RNA, c) contacting the target RNA with an adenosine deaminase enzyme to generate a target RNA with deaminated adenosines, d) denaturing of the RNA duplex to create mobilized complementary RNA; e) removal of the mobilized complementary RNA; and f) sequencing of the target RNA.
36. The method of claim 35, wherein the method further comprises repeating steps b-e one or more times prior to step f.
37. The method of claim 36, wherein the steps are repeated at least four more times.

38. The method of any one of claims 1-38, wherein sequencing of the target RNA comprises construction of a library of nucleic acid molecules comprising the target RNA sequence.
39. The method of claim 15, wherein the method comprises synthesis of a complementary nucleic acid from a cDNA library.
40. The method of claim 39, wherein the complementary nucleic acid strand is RNA.
41. The method of any one of claims 14-15 or 20, wherein the method further comprises contacting the target and/or demethylated target RNA with reverse transcriptase to synthesize a complementary DNA strand.
42. The method of any one of claims 1-41, wherein the target RNA is 10-1000 nucleic acids in length.
43. The method of any one of claims 1-42, wherein the sequence of the target RNA is known.
44. The method of claim 43, wherein the method further comprises comparing the known sequence of the target RNA with the sequence of the target RNA with deaminated adenosines.
45. The method of any one of claims 1-44, wherein contacting the target and/or demethylated target RNA with the adenosine deaminase enzyme is done in the presence of GTP.
46. The method of claim 45, wherein the GTP is in a concentration of 0.5-5 mM.
47. The method of any one of claims 8-15, 20, or 39-46, wherein the method comprises contacting the RNA-DNA duplex with a DNA digesting agent prior to sequencing the RNA.
48. The method of claim 47, wherein the DNA digesting agent is a DNase.
49. The method of claim 47 or 48, wherein the method further comprises the steps of: a) generating a DNA strand that is complementary to the target and/or demethylated target RNA and hybridizing the complementary DNA strand with the target and/or demethylated target RNA to generate an RNA-DNA duplex; b) contacting the RNA-DNA duplex with the adenosine deaminase enzyme; and c) contacting the RNA-DNA duplex with a DNA digesting agent; wherein all the steps are done prior to target RNA sequencing.

50. The method of claim 49, wherein the method further comprises repeating steps a, b, and c one or more times.
51. The method of claim 50, wherein the steps are repeated at least two more times.
52. The method of claim 51, wherein the steps are repeated at least four more times.
53. The method of any one of claims 50-52, wherein the method further comprises step of strand separation after step b).
54. The method of any one of claims 50-53, wherein the method further comprises purification of the target and/or demethylated target RNA after step c) and prior to target RNA sequencing.
55. The method of any one of claims 1-54, wherein the method further comprises: providing a quantification RNA control comprising a known percentage of modified adenosine; contacting the quantification control RNA with the adenosine deaminase enzyme; and sequencing the deaminated quantification control RNA
56. The method of claim 55, wherein 0, 25, 50, 75, or 100% of the adenosine in the quantification RNA control is modified.
57. The method of claim 55, wherein 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% of the adenosine in the quantification RNA control is modified.
58. A kit comprising: an adenosine deaminase enzyme and instructions for detecting modified adenosines in target RNA.
59. The kit of claim 58 further comprising a control nucleic acid.
60. The kit of claim 59, wherein the control nucleic acid is a non-naturally occurring nucleic acid or non-widely present nucleic acid.
61. The kit of claim 59, wherein the control nucleic acid comprises a non-naturally occurring sequence.
62. The kit of claim 59, wherein the control nucleic acid comprises RNA or DNA comprising modified adenosines.
63. The kit of claim 62, wherein the percentage of adenosines that are modified in the control nucleic acid is known.

64. The kit of any one of claims 59-63, wherein the control nucleic acid comprises a duplex with a complementary nucleic acid strand.
65. The kit of claim 64, wherein the complementary nucleic acid strand is DNA or RNA with modified adenosines.
66. The kit of claim 64, wherein the complementary nucleic acid strand is RNA with modified adenosines.
67. The kit of any one of claims 58-66 further comprising an adenosine demethylase.
68. The kit of claim 67, wherein the adenosine demethylase is specific for *N*⁶-methyladenosine.
69. The kit of claim 67, wherein the adenosine demethylase is specific for *N*⁶-methyladenosine.
70. The kit of any one of claims 58-69, wherein the kit further comprises an adenosine deaminase enzyme reaction composition comprising GTP.
71. The kit of any one of claims 58-70, further comprising a DNase.
72. The kit of any one of claims 58-71, further comprising a reverse transcriptase.
73. A method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising contacting a double-stranded nucleic acid molecule comprising the target RNA with ADAR to generate a target RNA with deaminated adenosines and sequencing the target RNA with deaminated adenosines; wherein the modified adenosine is detected when the sequence of the target RNA with deaminated adenosines is adenosine.
74. A method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising:
- a) providing a target RNA;
 - b) generating a DNA strand that is complementary with the target RNA and hybridizing the complementary DNA strand with the target RNA to generate a RNA-DNA duplex comprising the target RNA;
 - c) contacting the RNA-DNA duplex with ADAR to generate target RNA with deaminated adenosines; and
 - d) contacting the RNA-DNA duplex with a DNA digesting agent; and

e) sequencing the target RNA with the deaminated adenosines.

75. A method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising:

a) providing a target RNA;

b) generating a DNA strand that is complementary with the target RNA and hybridizing the complementary DNA strand with the target RNA to generate a RNA-DNA duplex comprising the target RNA;

c) contacting the RNA-DNA duplex with ADAR to generate target RNA with deaminated adenosines; and

d) contacting the RNA-DNA duplex with a DNA digesting agent;

e) repeating steps b, c, and d one or more times; and

f) sequencing the target RNA with the deaminated adenosines.

76. A method for detecting modified adenosine in a target ribonucleic acid (RNA) comprising:

a) providing a target RNA;

b) generating an RNA strand with modified adenosine that is complementary with the target RNA to generate an RNA-RNA duplex comprising the target RNA;

c) contacting the RNA-RNA duplex with ADAR to generate target RNA with deaminated adenosines; and

d) sequencing the target RNA with the deaminated adenosines.

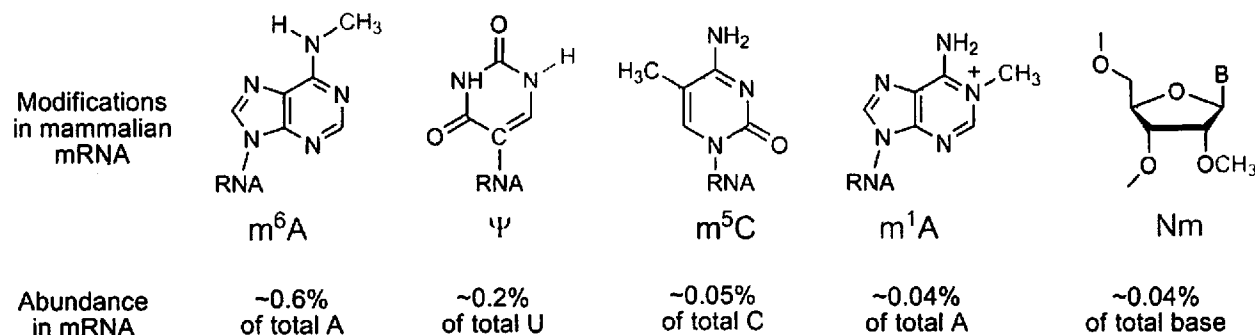


FIG. 1

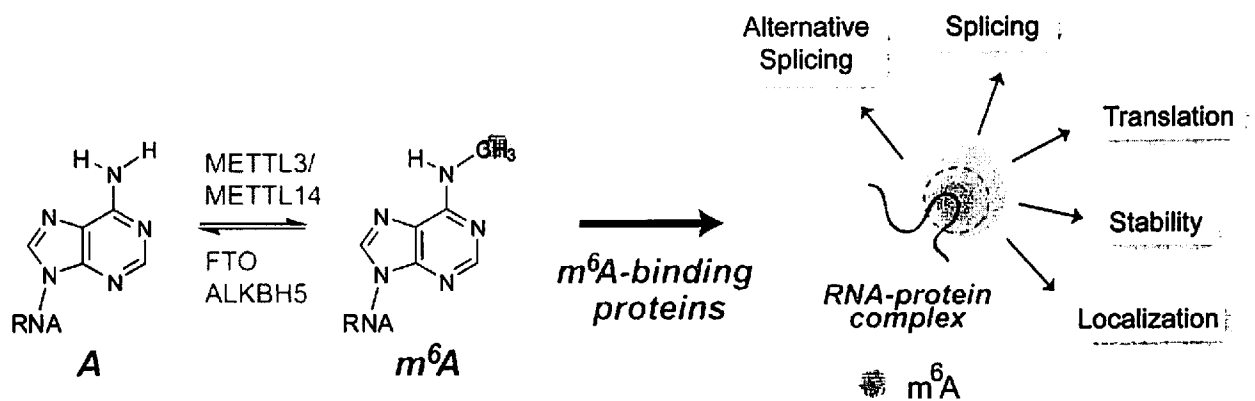


FIG. 2

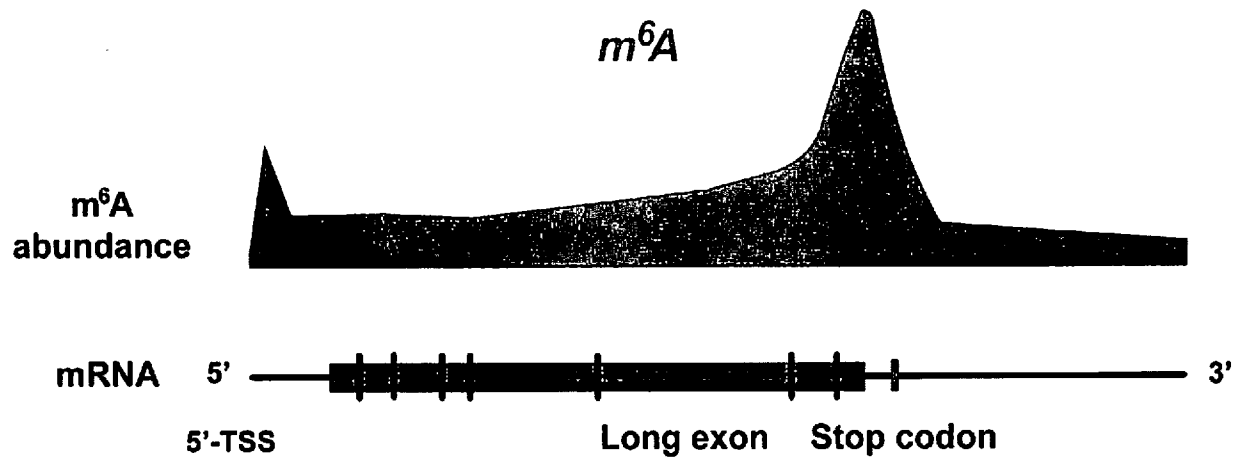


FIG. 3

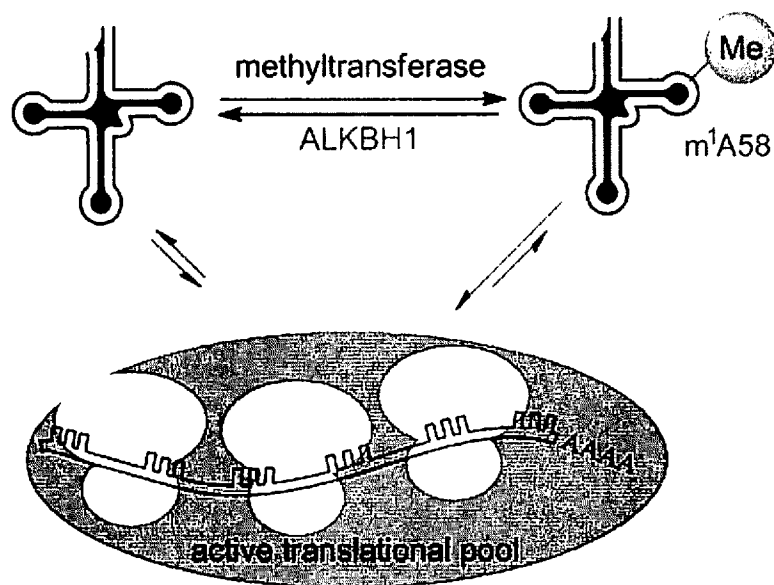


FIG. 4

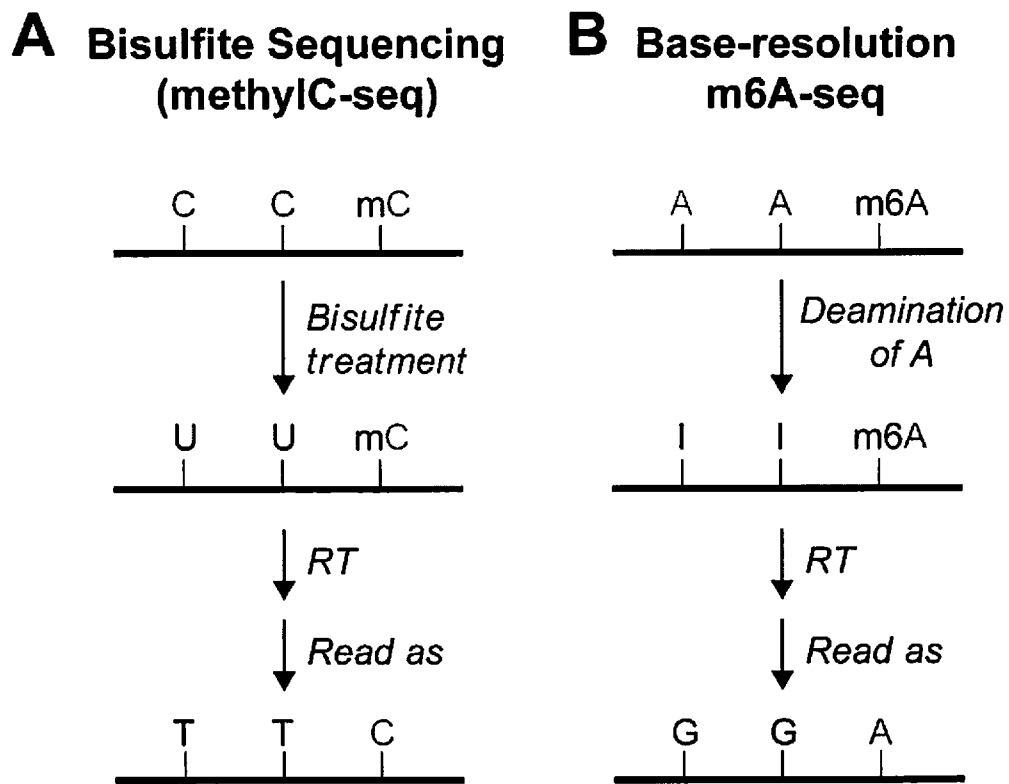


FIG. 5A-B

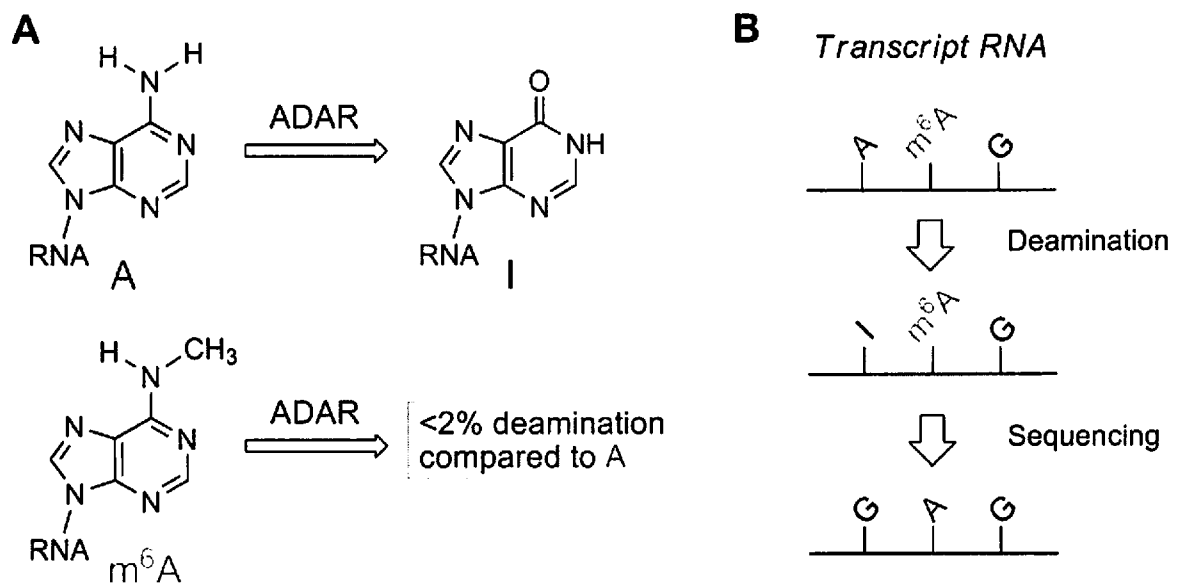


FIG. 6A-B

5'---CCG UUCG U UC GCUU UCG---3
3'---GGCUUXXGCUXUXGUUCGXXUXGC---5'

X = m⁶A: Deamination ratio on target strand ~50-60%

X = A: Deamination ratio on target strand ~20-30%

FIG. 7

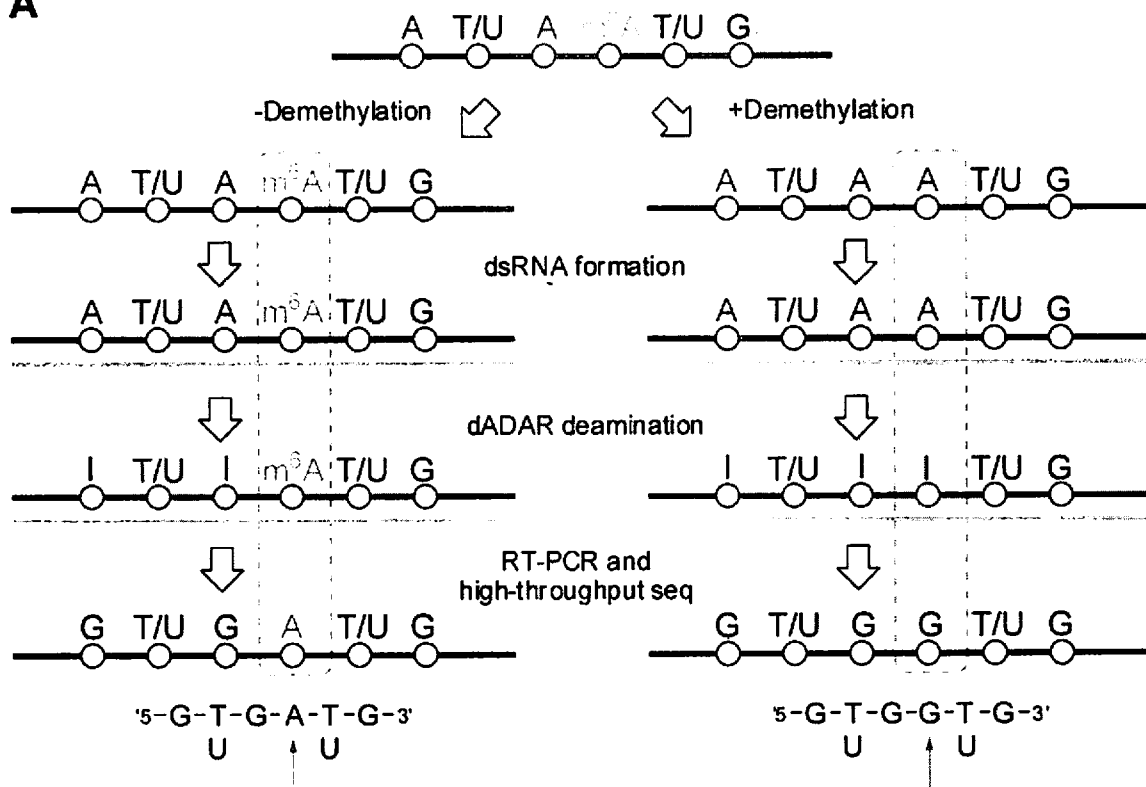
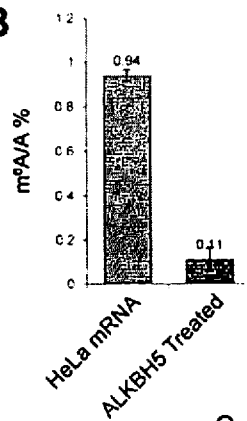
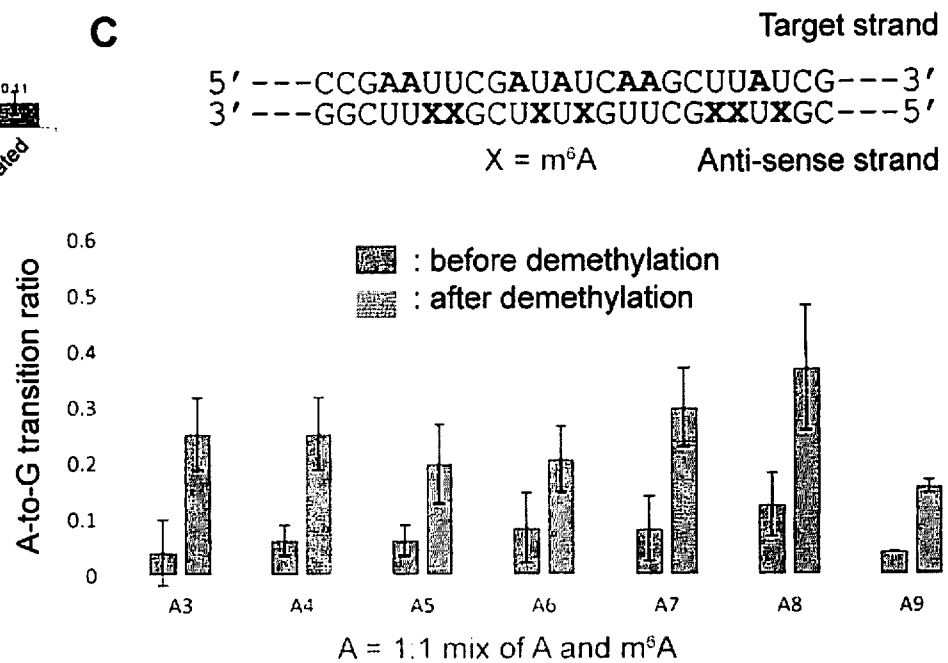
A**B****C**

FIG. 8A-C

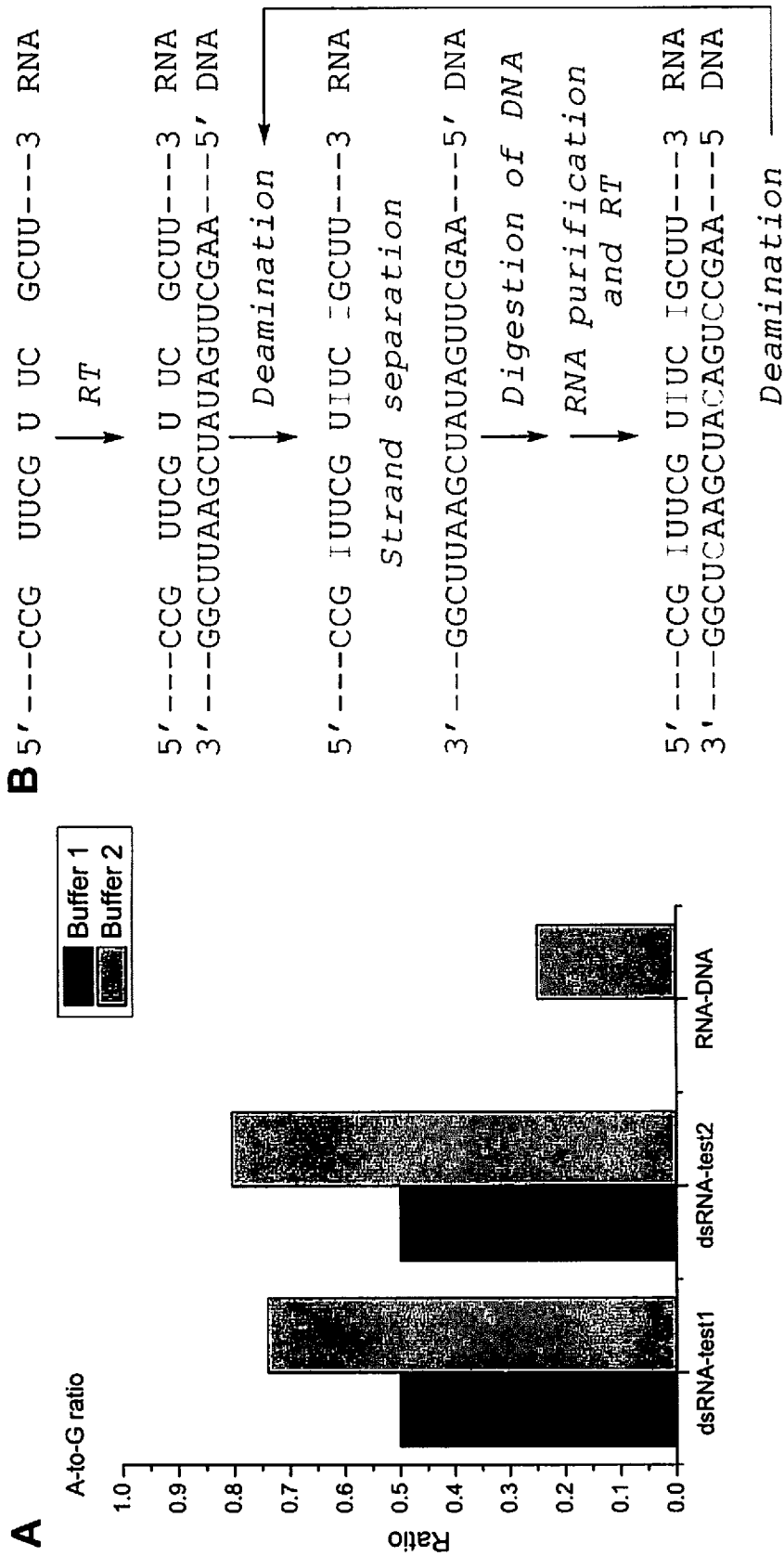


FIG. 9A-B



FIG. 10A-B

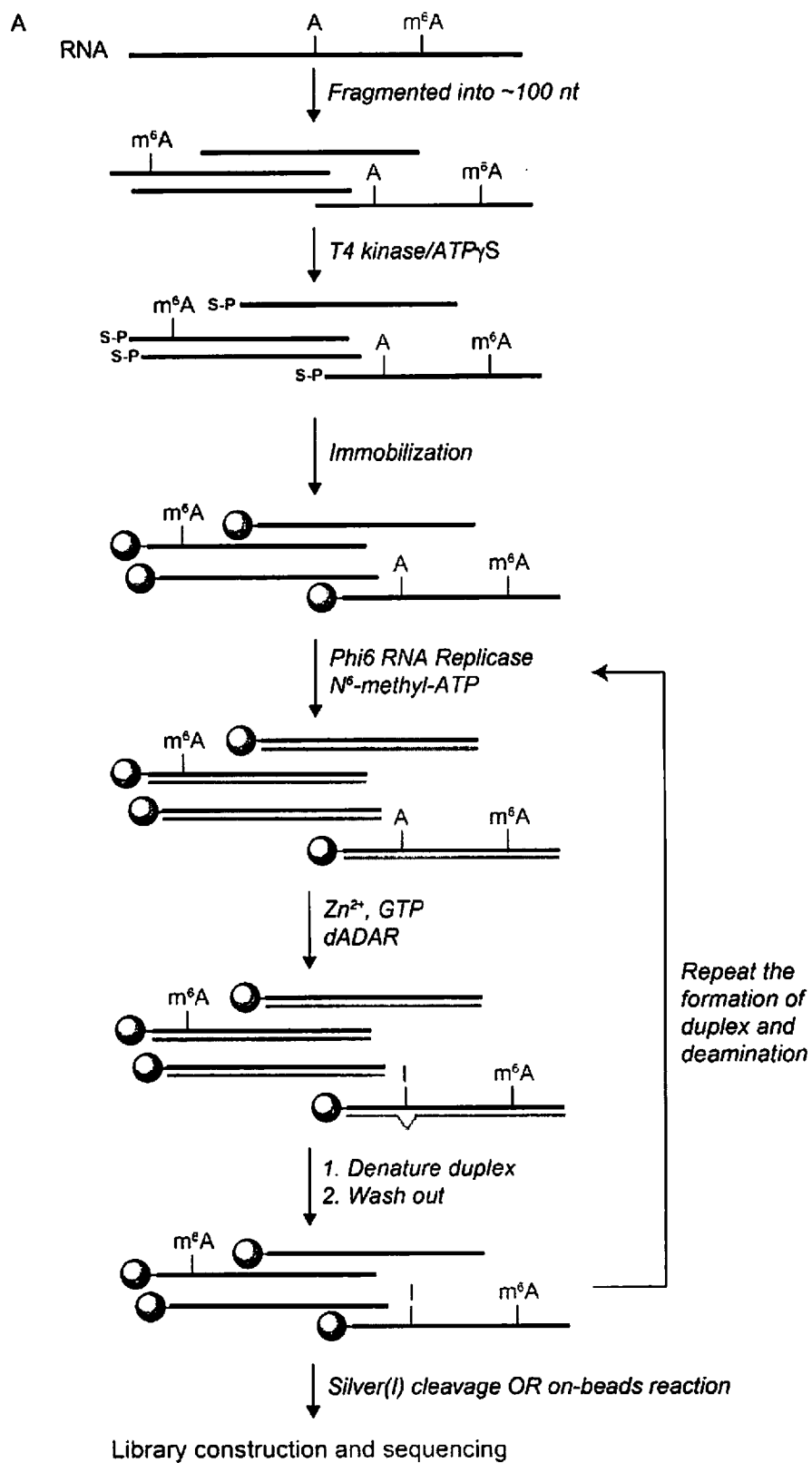


FIG. 11A

9 / 11

B

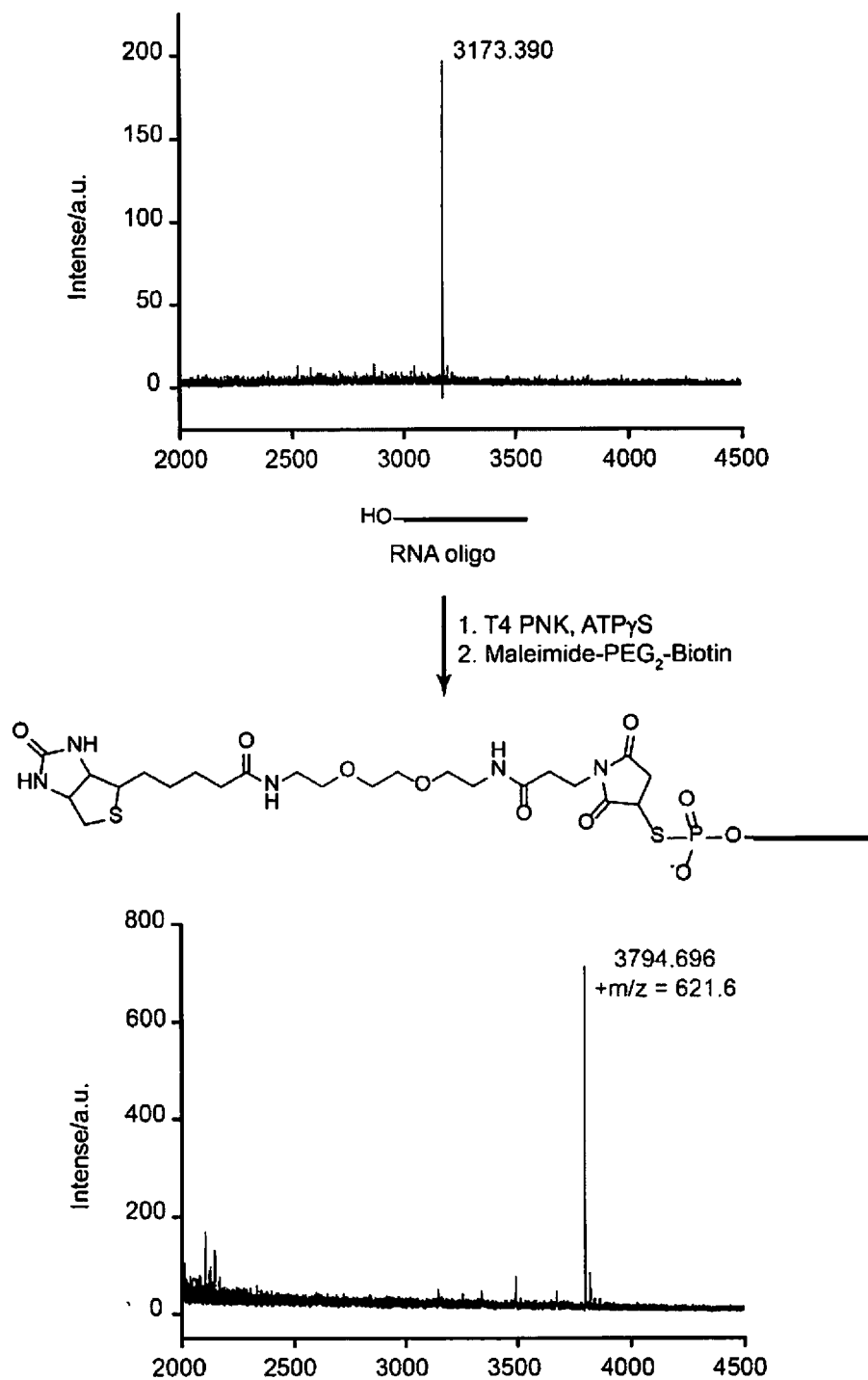


FIG. 11B

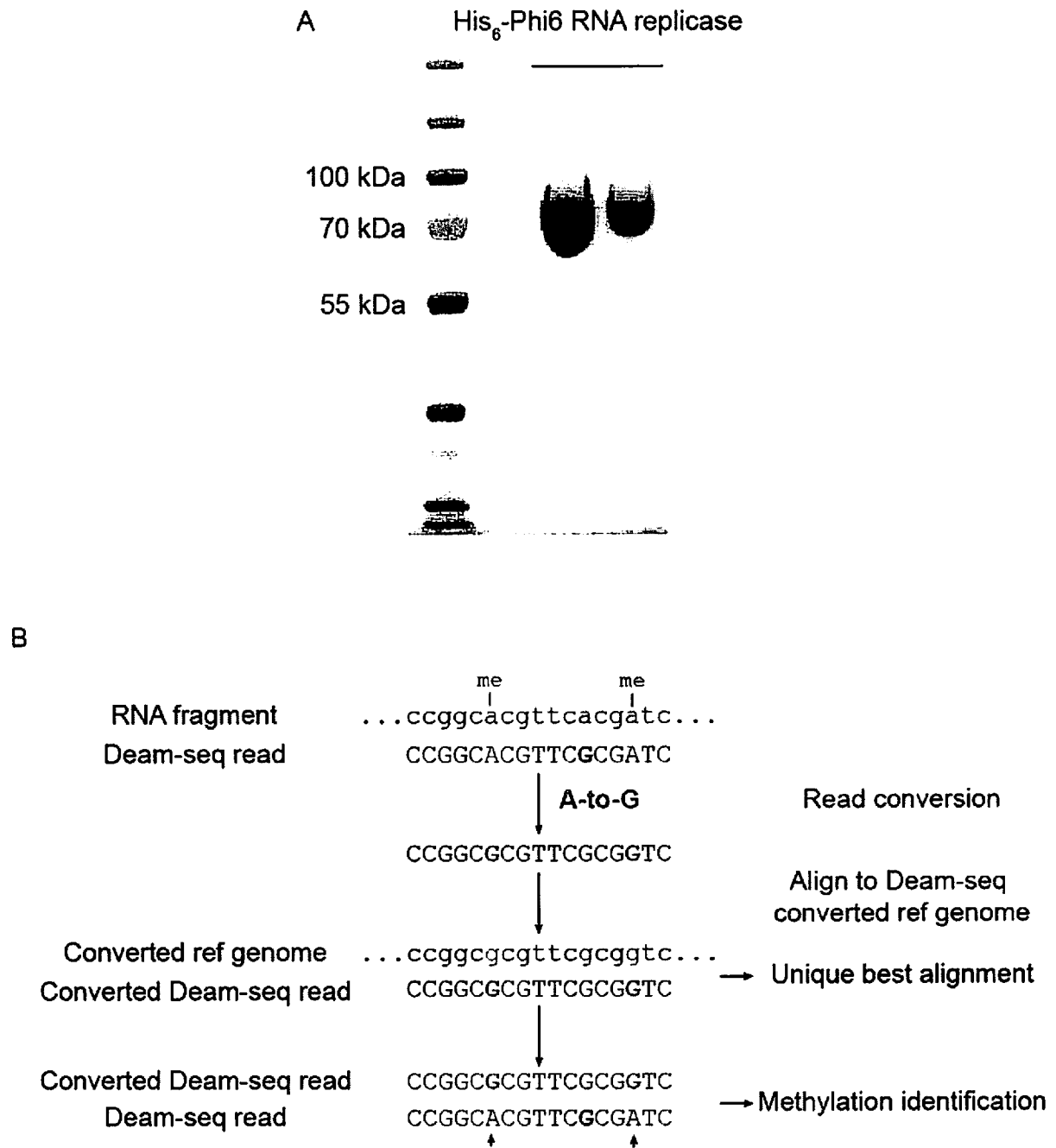


FIG. 12A-B

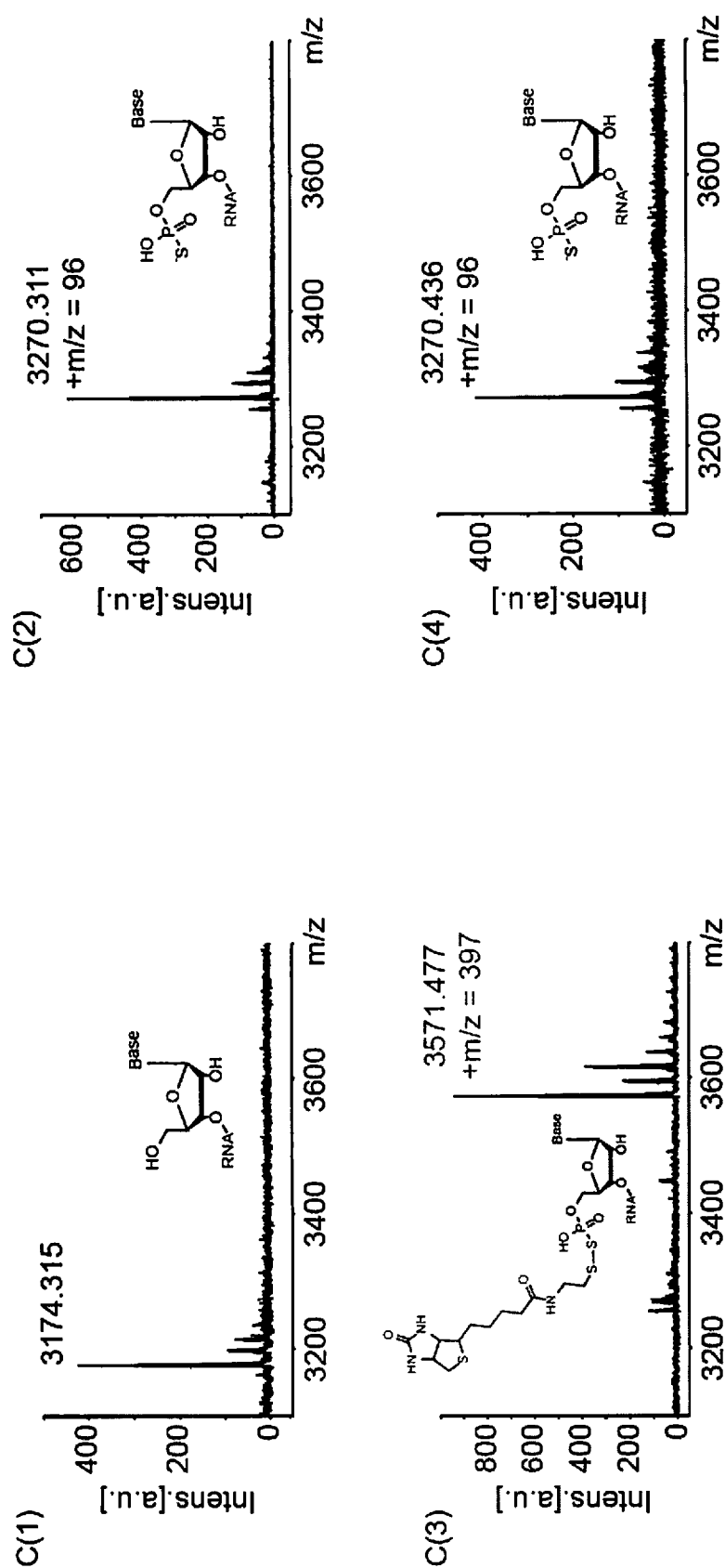


FIG. 12C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/49407

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/48; C12N 9/78 (2016.01)

CPC - A61K 38/48; C12N 9/78; C12Y 305/04004; A61K 38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 38/48; C12N 9/78 (2016.01)

CPC - A61K38/48; C12N9/78; C12Y305/04004; A61K38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 536/23.1, 536/24.3, 536/24.33Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
pubWEST; PatBase; Google Scholar

search terms - Adenosine deaminase, deamina\$, dsRAD, DRADA, ADA2, ADAR, ADAR1, ADAR2 sequencing, sequenced, RNA-specific, RNA specific, RNA-RNA, RNA-DNA, messenger, double stranded, duplex, deaminated Adenosine, deaminated Aden

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.**
X ----- Y	MIZRAHI et al. "Nucleoside analog studies indicate mechanistic differences between RNA-editing adenosine deaminases." Nucleic acids research 40.19 (2012): 9825-9835. Full document, especially p. 9827-9830 and Fig. 3.	1, 2, 73, 76 ----- 3, 4, 74, 75
Y	ZHENG et al. "Sprouts of RNA epigenetics: the discovery of mammalian RNA demethylases." RNA biology 10.6 (2013): 915-918. Full document, especially p. 916.	3, 4
Y	VUKOVIC et al. "Substrate recognition and specificity of double-stranded RNA binding proteins." Biochemistry 53.21 (2014): 3457-3466. Full document, especially abstract and p. 3457.	74, 75
A	DAWSON et al. "Structure and sequence determinants required for the RNA editing of ADAR2 substrates." Journal of Biological Chemistry 279.6 (2004): 4941-4951. Full document, especially p. 4942 - p. 4943.	1



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 December 2016

Date of mailing of the international search report

30 DEC 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/49407

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-57, 67-72
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-4 and 73-76, directed to a method for detecting modified adenosine in a target ribonucleic acid (RNA).
Group II, claims 58-66, directed to a kit.

The inventions listed as Groups I and II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

--continued on first extra sheet attached hereto--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-4, 73-76

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

--continuation of Box No III: Observations where unity of invention is lacking--

Special technical features

Group I has the special technical feature of a method comprising contacting a target RNA with an adenosine deaminase enzyme and sequencing the target RNA with deaminated adenosines; wherein any modified adenosine in the target RNA is read as an adenosine in the sequencing of the target RNA with deaminated adenosines, that is not required by Group II.

Group II has the special technical feature of a kit comprising an enzyme and instructions, that is not required by Group I.

Common technical features:

Groups I-II share the common technical feature of adenosine deaminase enzyme for detecting modified adenosines in target RNA. However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is anticipated by the article by Dawson et al., "Structure and sequence determinants required for the RNA editing of ADAR2 substrates." (Journal of Biological Chemistry 279.6, 2004: 4941-4951), (hereinafter Dawson).

Dawson teaches adenosine deaminase enzyme for detecting modified adenosines in target RNA (p. 4942, last para . p. 4943, para 1 'In Vitro Editing/Deamination assays. Editing assays were performed under single turnover conditions at 37 C with 7.5 nm FLAG-rADAR2b protein and 0.3 nm in vitro transcribed RNA as substrate in a buffer...To analyze the editing of ADAR2 at site -1, first-strand cDNA was synthesized with an oligonucleotide primer complementary to the unique 3'-extension of the ADAR2 RNA substrate that was not present in competitor RNAs. First-strand cDNA was amplified by PCR and the extent of editing was quantified by primer-extension analysis as described').

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-II inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.

NOTE, continuation of number 4 above: claims 5-57 and 67-72 are held unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).