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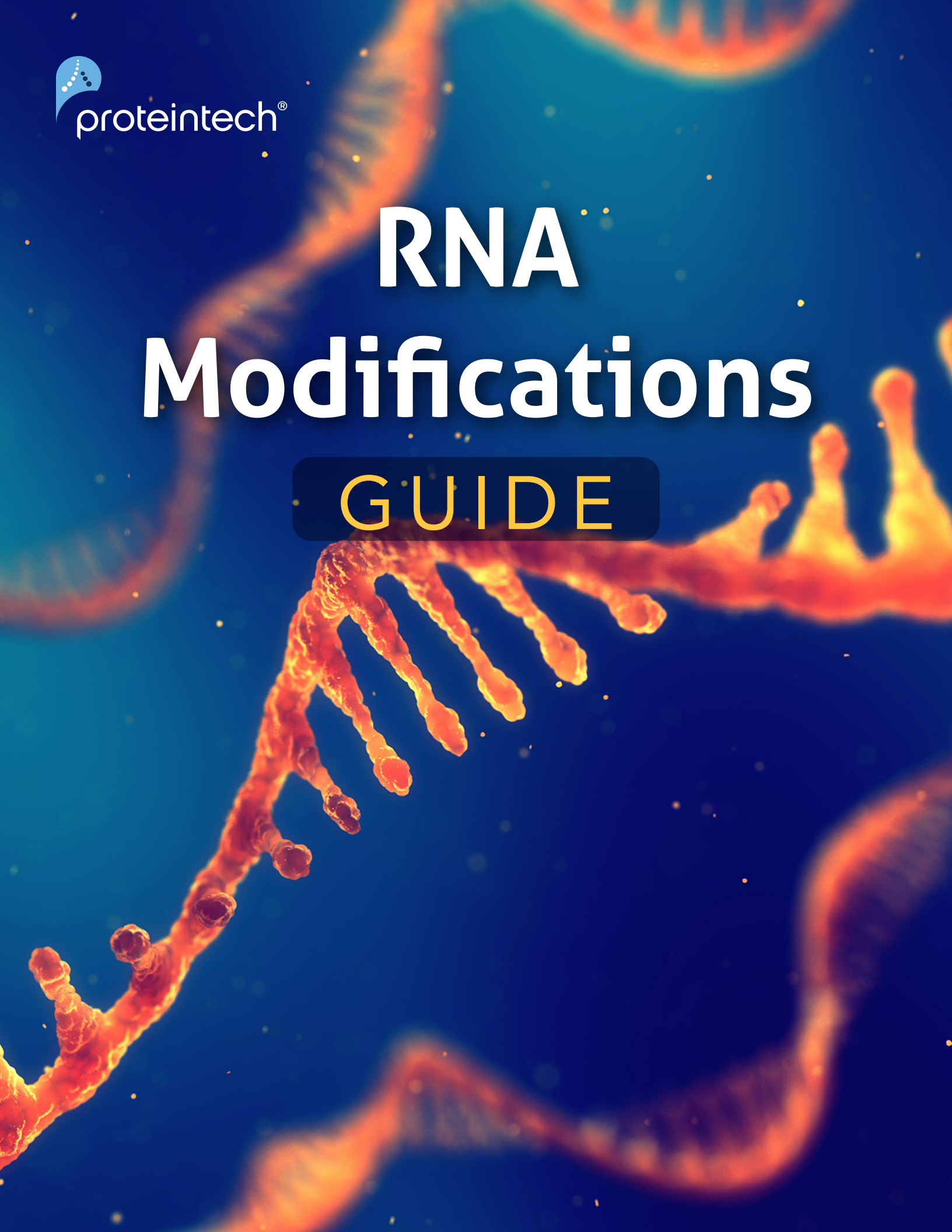
RNA Modifications

GUIDE



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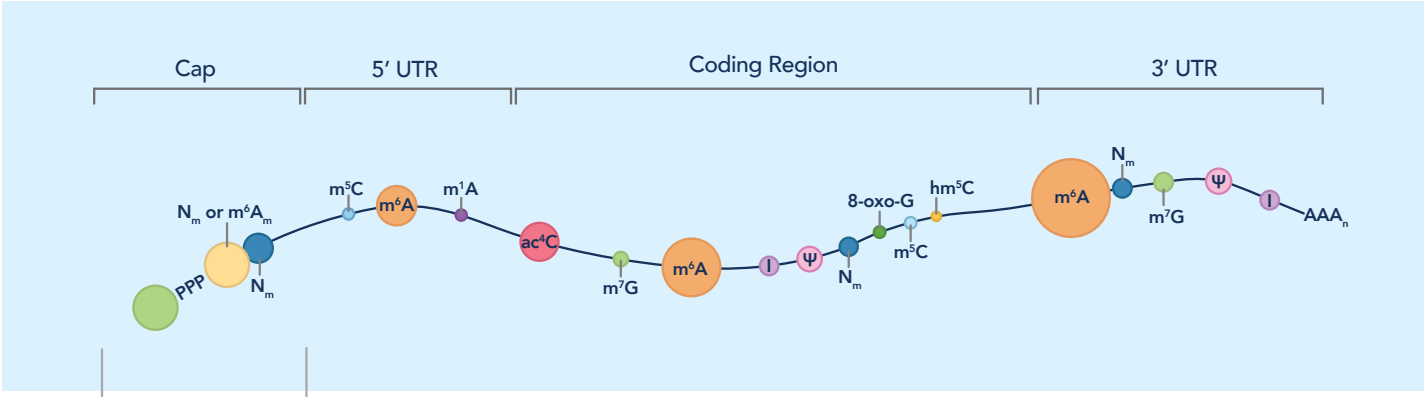
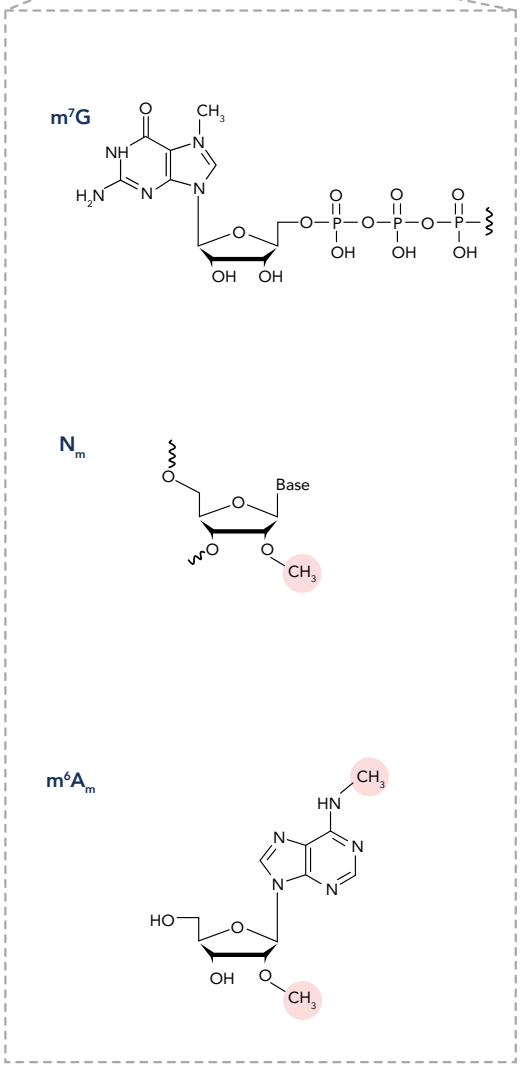
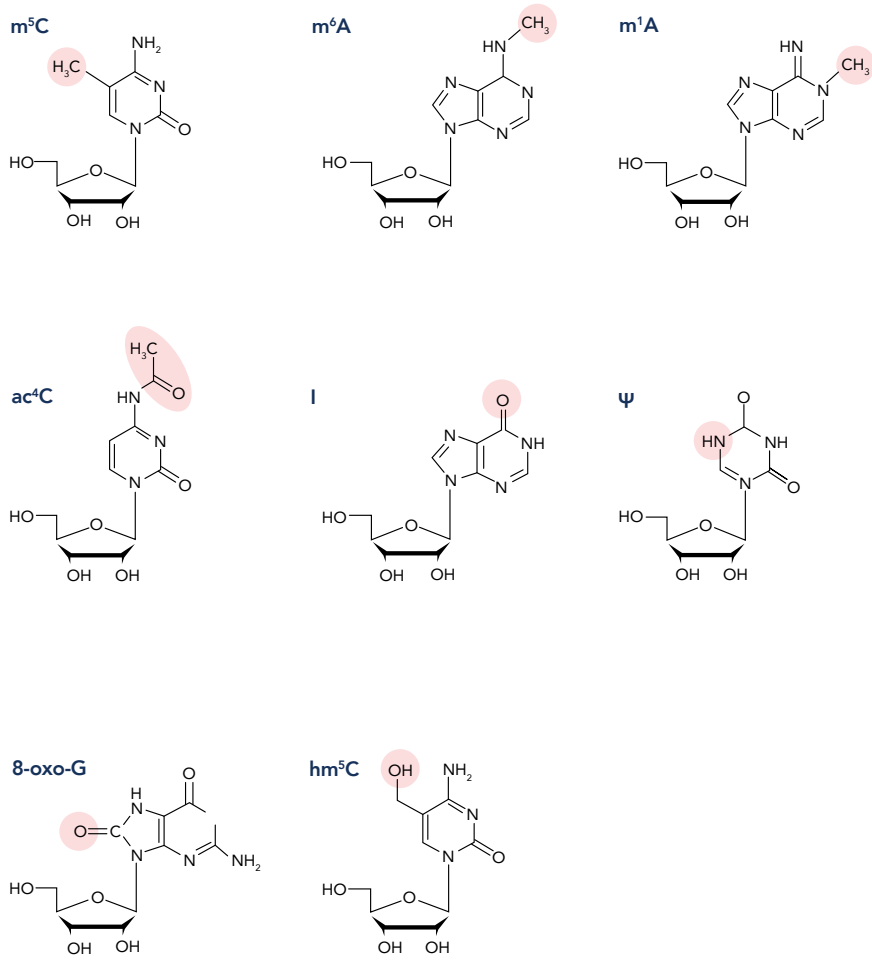
INTRODUCTION

RNA molecules can undergo posttranscriptional modifications involving the addition, removal, or changes in chemical groups within the nucleotides. These alterations can regulate various biological functions, including RNA structure, stability, and translation efficiency. The study of RNA modifications has gained attention in recent years due to their emerging roles in gene expression regulation and their association with various diseases, such as cancer. Over 170 different RNA modifications have been identified and characterized on various types of RNA, including mRNA, tRNA, rRNA, and non-coding RNAs. With the advancement of techniques for the detection of RNA modifications, the number of known modifications continues to rise.

Types of RNA MODIFICATIONS







▲ Examples of modifications observed on mRNA. Adapted from Zaccara et al., 2019

RNA modifications and functions (selective list)

Modification	Full Name	RNA Showing Modification	Main Functional Roles
m^6A	N6-methyladenosine	mRNA, tRNA, rRNA, snRNA, lncRNA, snoRNA, circRNA	RNA stability, splicing, export, translation efficiency, processing
m^7G	7-methylguanosine	mRNA, tRNA, rRNA, snRNA, snoRNA	5′ terminal cap, RNA structure, stability
m^5C	5-methylcytidine	mRNA, tRNA, rRNA, lncRNA, snoRNA	RNA structure, stability, translation efficiency, localization
m^1A	1-methyladenosine	mRNA, tRNA, rRNA, lncRNA, snoRNA	RNA structure, stability, translation efficiency
ψ	Pseudouridine	mRNA, tRNA, rRNA, snRNA, snoRNA	RNA structure, stability, translation efficiency
N_m	2′-O-methylation	mRNA, tRNA, rRNA, snRNA	RNA structure, stability, translation efficiency
m^6A_M	2′-O-methyladenosine	mRNA	RNA stability, translation, cap structure
ac^4C	N4-acetylcytidine	mRNA, tRNA, rRNA	RNA structure, stability, translation efficiency, codon recognition
8-oxo-G	8-Oxoguanine	mRNA, tRNA, rRNA, lncRNAs, snoRNAs	RNA stability, structure, translation efficiency
I	Inosine	mRNA, tRNA, lncRNA, snRNA	Codon recognition, splicing, stability
hm^5C	5-hydroxymethylcytidine	mRNA, tRNA, rRNA	Translation efficiency, RNA structure
m^2, 2G	N2,2′-O-dimethylguanosine	tRNA	Unknown
m^4C	N4-methylcytosine	mRNA, tRNA, rRNA, lncRNAs, snoRNAs	RNA structure, stability, translation efficiency
m^2A	N2-methyladenosine	mRNA, tRNA, rRNA, lncRNA, snRNA	RNA stability, structure, translation efficiency, splicing
m^6, 6A	N6-6-dimethyladenosine	tRNA	RNA structure, stability, translation efficiency
m^8A	8-methyladenosine	tRNA	RNA structure, stability, translation efficiency
m^1G	N1-methylguanosine	mRNA, tRNA, rRNA, lncRNA, snRNA	RNA stability, structure, translation efficiency

Finding highly sensitive antibodies to study RNA modifications, such as RNA methylation, can be a challenge. Proteintech offers the most expansive portfolio on the market to study RNA methylation.

View our full portfolio of products for RNA methylation

Common Applications for
STUDYING RNA MODIFICATIONS

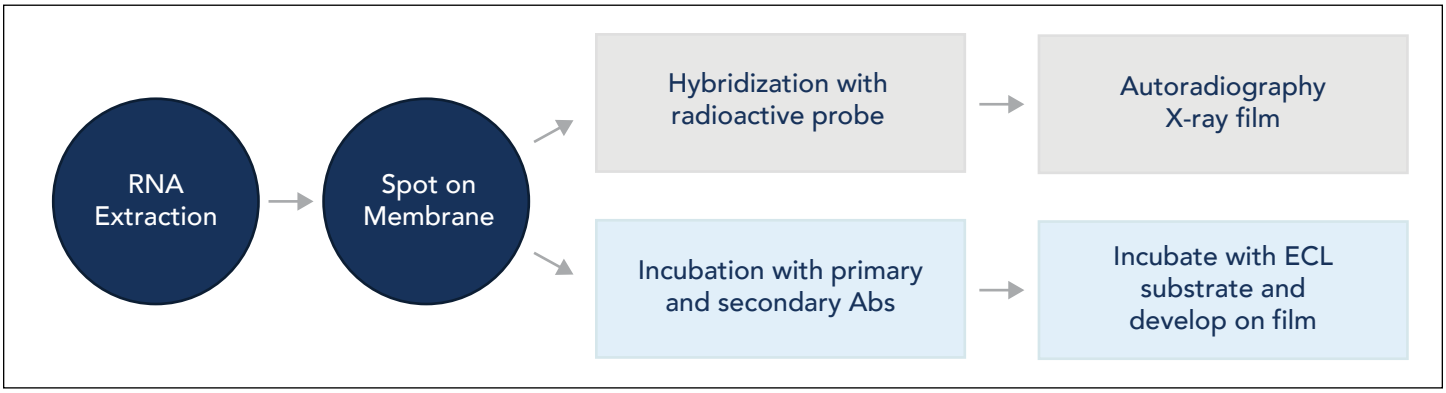
Dot Blot

What is an RNA dot blot?

An RNA dot blot is a technique that allows for the detection and semi-quantification of RNA molecules in a sample. The technique can also be used to detect RNA modifications, including RNA methylation.

How does an RNA dot blot work?

The protocol for RNA dot blotting is similar to northern blotting except RNA samples are not separated by electrophoresis. Instead, extracted RNA is directly spotted onto a nitrocellulose (NC) or nylon membrane followed by hybridization with a radioactive probe to detect the target RNA sequences. Alternatively, traditional blotting techniques using primary and secondary antibody incubation can also be used to detect post-transcriptional modifications to RNA sequences, such as RNA methylation.



▲ Simplified examples of RNA dot blot workflows

Why is dot blotting a good technique to observe RNA methylation?

RNA methylation is a post-transcriptional modification that adds a methyl group (-CH3) to specific RNA nucleotides. These modifications play critical roles in many biological processes, including RNA metabolism and gene expression, development and differentiation, and disease. N6-methyladenosine (m6A) is the most common RNA modification in eukaryotes. Other types of RNA methylation include N1-methyladenosine (m1A), 5-methylcytosine (m5C), and 7-methylguanosine (m7G).

By using antibodies designed to recognize methylated RNA molecules, dot blotting can be a powerful tool for analyzing RNA methylation for several reasons:

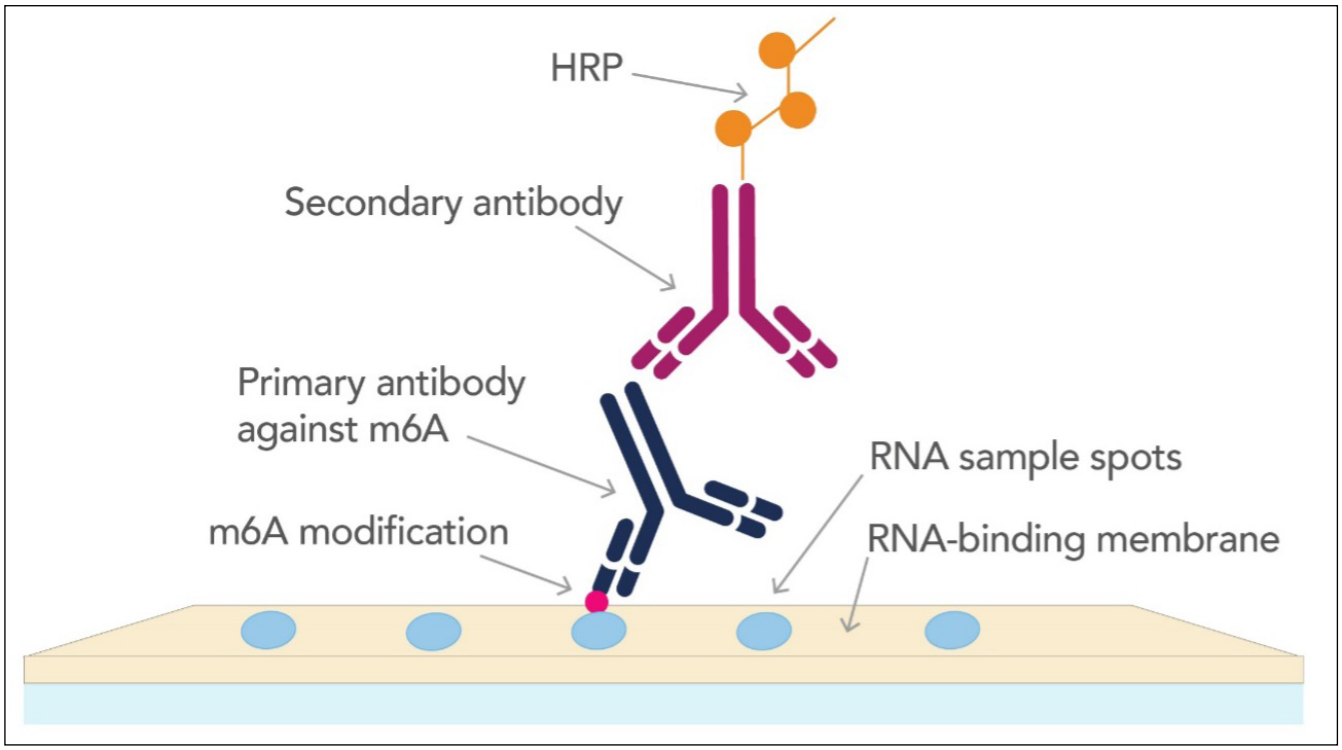
Sensitivity: Dot blotting is highly sensitive and can detect RNA methylation at very low levels. Since RNA methylation is often low in RNA extracts, highly sensitive techniques are required to detect it.

Simplicity: Dot blotting is a relatively simple technique that requires minimal sample preparation and equipment. It does not require RNA sequencing or other advanced techniques, making it a cost-effective and efficient option for detecting RNA methylation.

Specificity: Dot blotting can be highly specific for detecting RNA methylation if a specific antibody or probe is used. This can be important for distinguishing RNA methylation from other post-transcriptional modifications or products from RNA degradation.

High-throughput: Dot blotting can be easily adapted for high-throughput screening of a large number of samples. This makes it a useful technique for large-scale studies of RNA methylation in various biological contexts.

It is important to note that dot blotting is a semi-quantitative technique, and quantitative measurements of RNA modifications may require other techniques such as RNA sequencing or mass spectrometry.



▲ Graphical representation of identifying m6A modification by dot blot

Protocol for RNA dot blot using antibodies for RNA methylation

1. Extract RNA from samples with extraction reagent (or kit) and formulate a proper final concentration between 0.05-2 μg/μL. Dilute with PBS if the concentration is too high. Avoid any contamination with RNase.

2. Directly dot the extracted RNA onto the nitrocellulose (NC) membrane. Mark the dot position and front side of the membrane. The appropriate amount of RNA for each dot is between 0.2–2 μg. Serial dilutions are recommended to determine the optimal amount. Adjust each dot to the same volume by dilution with PBS if needed.

3. Dry the membrane for 5–15 minutes at room temperature, and block with 1% BSA (in PBST) solution for one hour. If methylene blue staining is applied as a loading control, the dried membrane can be directly stained without blocking. Blocking will interfere with methylene blue staining.

4. Briefly wash with PBS, PBST, or TBST once. Dilute the primary antibody with 1% BSA (in PBST) solution. The following dilutions are recommended for Proteintech's m6A, m5C, and m7G antibodies:

   m6A Monoclonal Antibody (68055-1-Ig): 1:2000

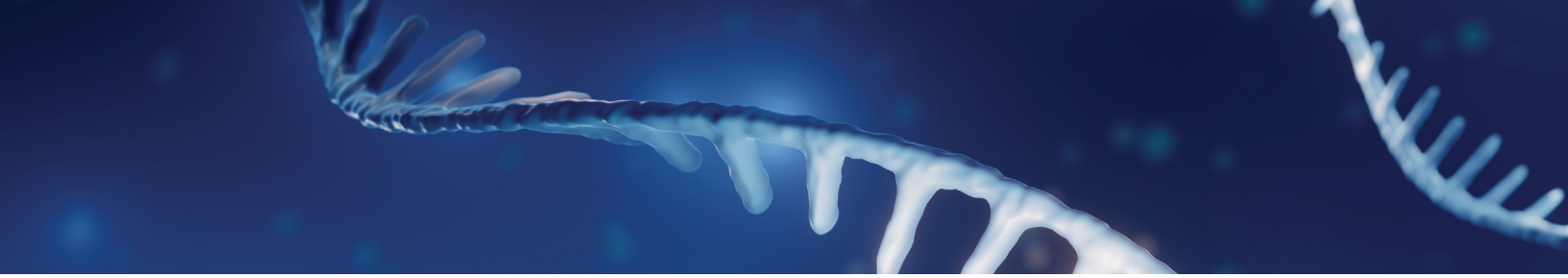
   m5C Monoclonal Antibody (68301-1-Ig): 1:5000

   m7G Monoclonal Antibody (68302-1-Ig): 1:5000

   Add the diluted primary antibody solution to the membrane and incubate at room temperature for 1.5 hours. Ensure that the primary antibody solution completely covers the membrane. Avoid using milk for dilution.

5. Wash with PBST 4 times for 5 minutes each time. Discard the washing buffer, add the secondary antibody diluted in 1% BSA, and incubate at room temperature for 1.5 hours or at 37°C for 1 hour. Ensure that the secondary antibody solution completely covers the membrane. Avoid using milk for dilution.

6. Wash in PBST 4 times for 5 minutes each time. Completely discard the washing buffer and add ECL substrate for the luminescent development.



RNA IMMUNOPRECIPITATION (RIP)/RIP-SEQ

*Written by **Sripriya Raja**, Graduate Student at University of Pittsburgh*

What is RIP/RIP-seq?

RIP/RIP-seq, or RNA immunoprecipitation/RNA immunoprecipitation sequencing, is a high-throughput experimental technique used to study the interaction between RNA and RNA-binding proteins. It allows for the identification of specific RNA-bound proteins within cells or tissues.

Why is it beneficial to study RNA modifications?

Studying RNA modifications is essential for unraveling the complexity of RNA biology, understanding gene expression regulation, deciphering disease mechanisms, and exploring diagnostic and therapeutic applications. It provides a deeper level of understanding of RNA molecules and their roles in cellular processes and diseases.

The dysregulation of RNA modifications has been implicated in various diseases, including cancer, neurological disorders, and metabolic disorders. Understanding RNA modifications opens opportunities for developing targeted therapeutic approaches. By manipulating the enzymes responsible for adding or erasing specific RNA modifications, it may be possible to modulate gene expression, alter RNA structure, or correct aberrant RNA modifications in disease states. Additionally, modulating RNA modifications pharmacologically may offer new avenues for therapeutic intervention. Understanding the landscape of RNA modifications and their functional consequences can guide the development of RNA-targeted therapies and precision medicine approaches.

RIP/RIP-seq provides key insights into the binding targets of RNA, and the mechanistic roles of RNA binding proteins in RNA biology, gene expression regulation, and disease. RIP-seq allows researchers to gain insights into the RNA targets and regulatory roles of RBPs in various cellular processes, such as RNA splicing, stability, transport, and translation. By comparing RIP-seq data from different experimental conditions or cell types, researchers can investigate changes in RBP binding patterns and uncover potential regulatory mechanisms underlying gene expression. It has contributed to our understanding of RNA-protein interactions and has been particularly valuable in uncovering the roles of RBPs in various biological processes and diseases.

How does RIP/RIP-seq work?

RIP is a method that involves the immunoprecipitation of RBPs along with their associated RNA molecules. The principle behind this technique is to crosslink RBPs to RNA using a chemical crosslinker, typically formaldehyde, which "freezes" the interactions between RNA and RBPs at a particular moment in time. The cell or tissue lysate is then subjected to immunoprecipitation using antibodies specific to the RBP of interest. The immunoprecipitated RNA-protein complexes are then purified and the crosslinks are reversed to release the RNA molecules. Finally, the enriched RNA is analyzed to identify the bound transcripts. RIP-seq combines the RIP technique with high-throughput sequencing technologies, allowing for the genome-wide identification and characterization of RNA molecules bound to RBPs. After the immunoprecipitation step, the purified RNA is converted into a library of cDNA fragments, which are then sequenced using next-generation sequencing platforms. The resulting sequence data provides information about the RNA sequences that were bound by the RBPs, allowing researchers to identify the target transcripts.

Overview of the RIP/RIP-seq workflow

1. **Cross-linking**: The first step in RIP-seq involves cross-linking the RBPs to the RNA molecules within the cell. This is typically done by treating the cells with a chemical cross-linker such as formaldehyde. The cross-linking helps to preserve the RBP-RNA interactions and prevent their dissociation during subsequent processing steps.

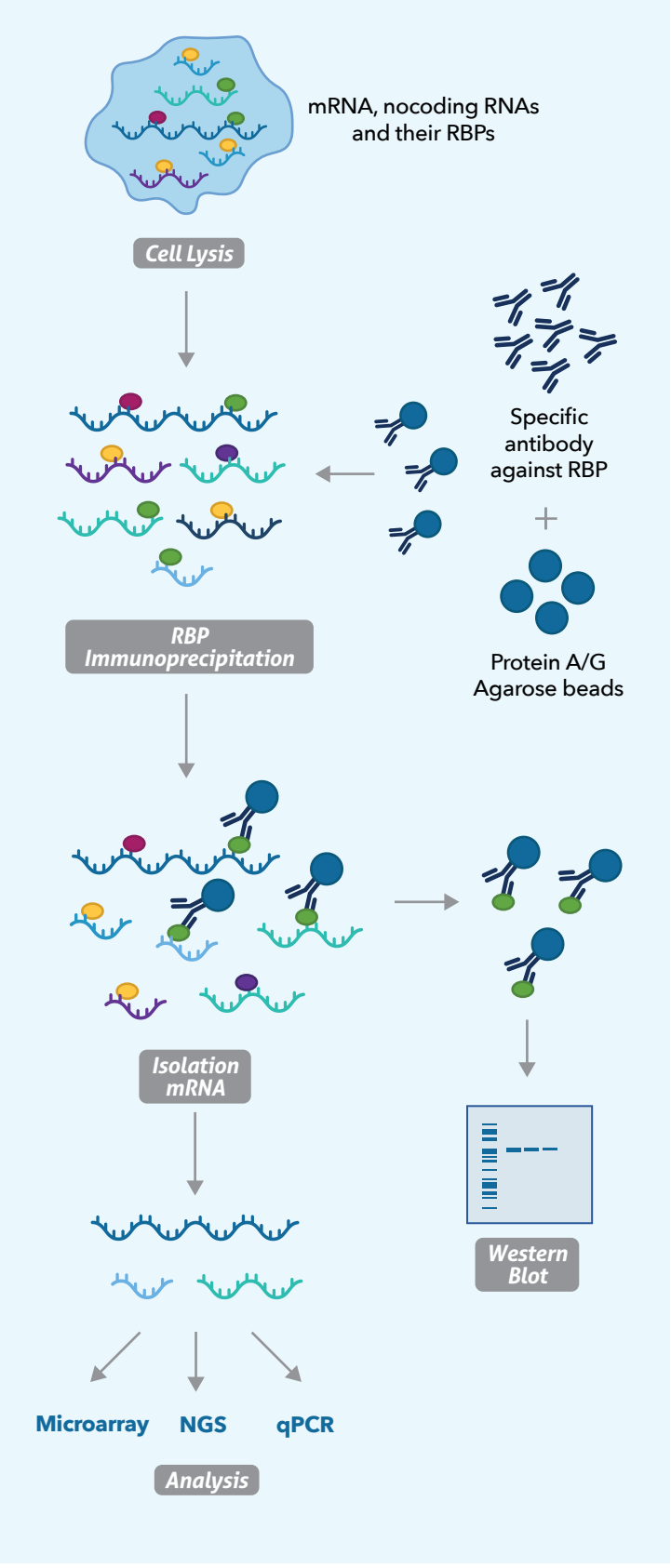
2. **Cell lysis and immunoprecipitation**: The cross-linked cells are then lysed to release the cellular contents. Antibodies specific to the target RBP are added to the lysate, and these antibodies selectively bind to the RBPs of interest. The RBP-RNA complexes are then immunoprecipitated using techniques like protein A/G beads, which bind to the antibody-RBP complexes.

3. **RNA fragmentation**: The immunoprecipitated RNA-protein complexes are treated with RNase to digest the unbound RNA molecules, leaving behind the RBP-bound RNA. The RBP is then removed from the complexes, leaving only the RNA molecules associated with the protein of interest.

4. **RNA purification**: The RNA is purified from the remaining protein fragments, contaminants, and cross-links. This step involves various enzymatic treatments and column purification to obtain high-quality RNA.

5. **Library preparation and sequencing**: The purified RNA is converted into a sequencing library by adding adapters and amplifying the RNA fragments. The library is then subjected to high-throughput sequencing using platforms like next-generation sequencing (NGS) technologies.

6. **Data analysis**: The resulting sequencing reads are aligned to a reference genome or transcriptome to identify the regions of the RNA molecules that were bound by the RBPs. The data analysis involves identifying enriched regions (peaks) that represent the binding sites of the RBPs, as well as downstream analysis to understand the functional implications of RBP-RNA interactions.



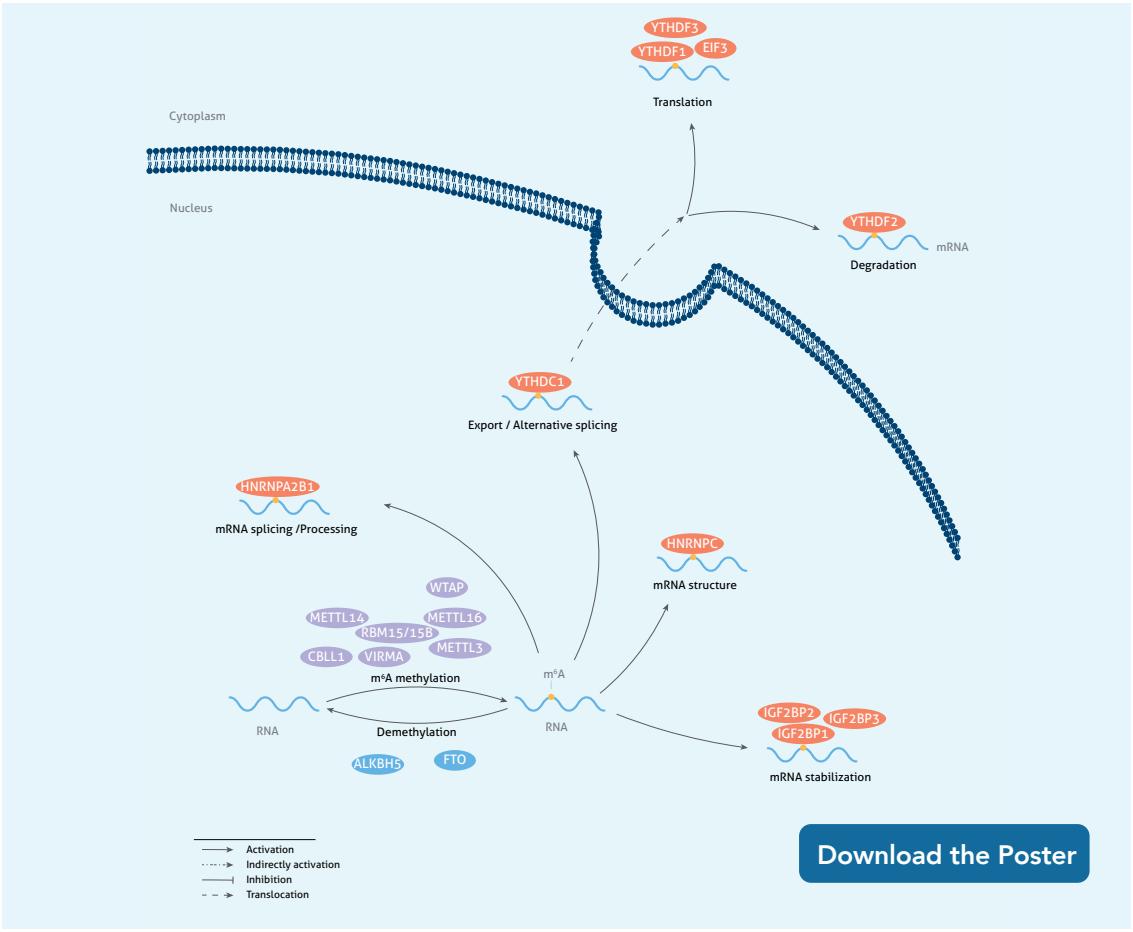
▲ RIP/RIPseq workflow

RNA METHYLATION

RNA methylation is a type of RNA modification involving the addition of a methyl group to specific nucleotide bases. Similar to other modifications, RNA methylation is determined by the dynamic interplay between regulators called "writers" (methyltransferases), "readers" (binding proteins), and "erasers" (demethylases). m6A methylation is the most common modification observed in eukaryotic RNA. Proteintech offers the most complete portfolio to study RNA methylation.

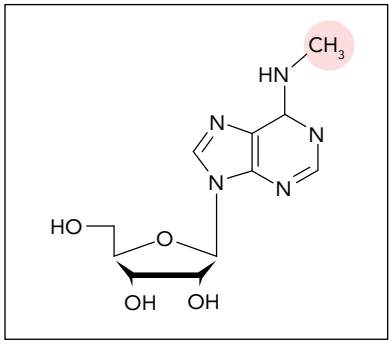
View our full portfolio of products for RNA methylation

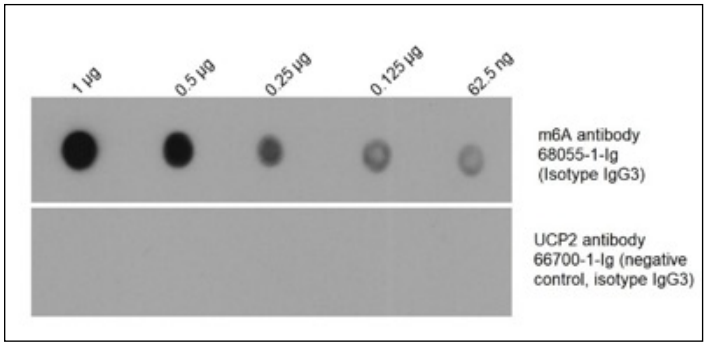
N6-Methyladenosine (m^{6}A)



▲ m^{6}A regulators

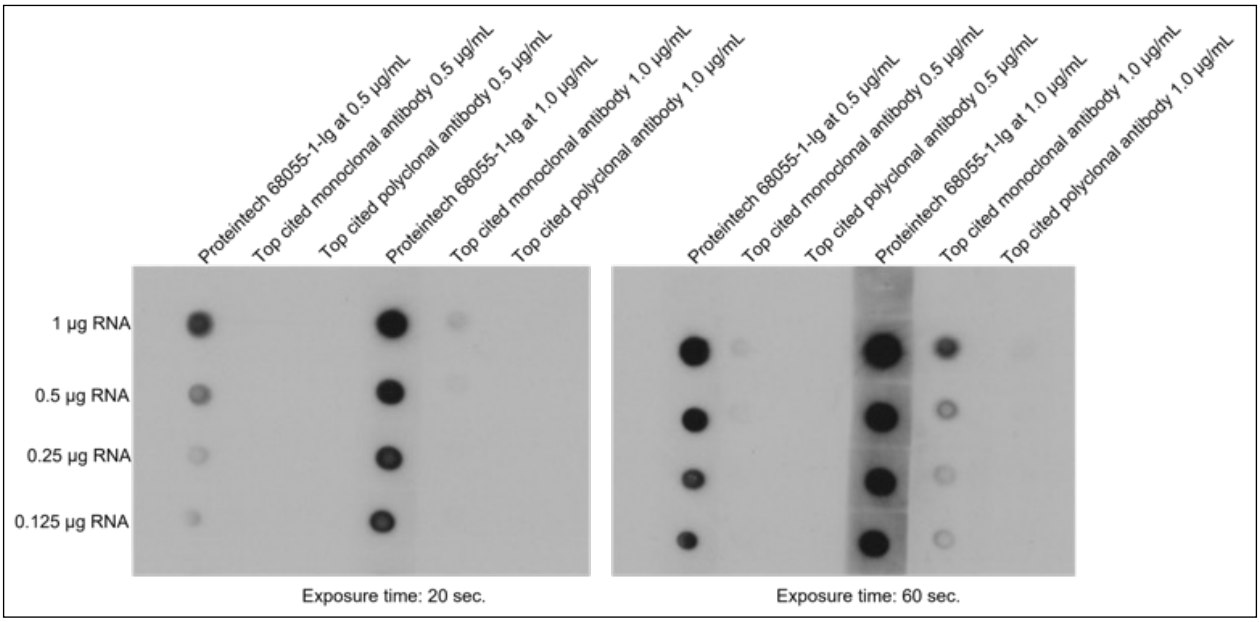
m^{6}A Monoclonal Antibody (68055-1-Ig)





◀ Total RNA was isolated from HEK-293 cells and dotted on an NC membrane at different amounts. The membrane was blotted with **m6A antibody (68055-1-Ig, 1:2000)** followed by incubation with HRP-goat anti-mouse secondary antibody. A parallel dot blot was performed using an antibody with the same isotype **(UCP2 antibody 66700-1-Ig)** at the same dose.

Proteintech's m⁶A antibody is significantly more sensitive than antibodies from the leading competitor!



▲Total RNA was extracted from HeLa cell line and dotted to NC membrane at different doses followed by blocking and blotting with m6A antibodies at 0.5μg/mL and 1.0μg/mL.

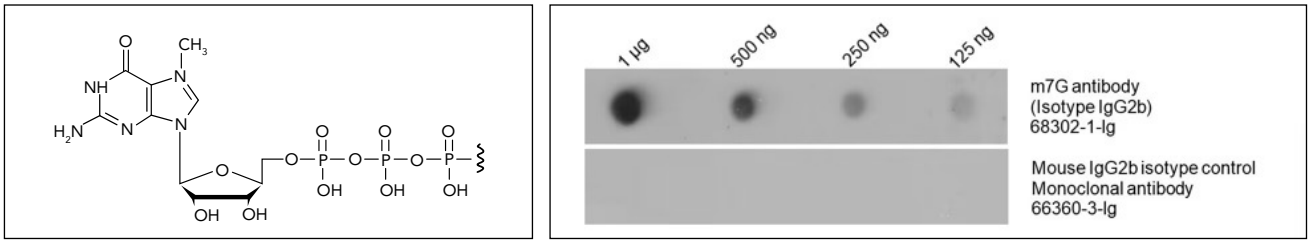
m⁶A Regulators

Writers (Methyltransferases)	Erasers (Demethylases)	Readers (Binding proteins)
METTL3	FTO	YTHDC1
METTL14	ALKBH5	YTHDC2
METTL16		YTHDF1
WTAP		YTHDF2
KIAA1429		YTHDF3

**Explore products for m⁶A regulators by clicking on each target*

7-methylguanosine (m^{7}G)

m^{7}G Monoclonal Antibody (68302-1-Ig)



▲Total RNA was isolated from HeLa cell line and dotted on an NC membrane at different amounts. The membrane was blotted with m7G antibody (68302-1-Ig, 1:5000) followed by incubation of HRP-goat anti-mouse secondary antibody. A parallel dot blot was performed using Mouse IgG2b isotype control Monoclonal antibody 66360-3-Ig at the same dose.

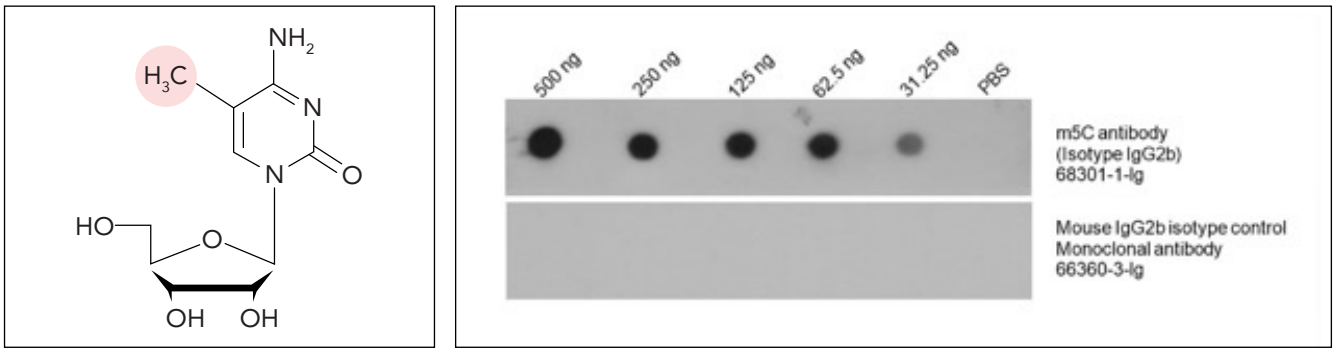
m^{7}G Regulators

Writers (Methyltransferases)	Erasers (Demethylases)
RNMT	NUDT16
METTL1	

*Explore products for m^{7}G regulators by clicking on each target

5-methylcytidine (m^{5}C)

m^{5}C Monoclonal Antibody (68301-1-Ig)



▲Total DNA was isolated from HeLa cell line and dotted on an NC membrane at different amounts. The membrane was blotted with m5C antibody (68301-1-Ig, 1:5000) followed by incubation of HRP-goat anti-mouse secondary antibody. A parallel dot blot was performed using Mouse IgG2b isotype control Monoclonal antibody 66360-3-Ig at the same dose.

m^{5}C Regulators

Writers (Methyltransferases)	Erasers (Demethylases)	Readers (Binding proteins)
DNMT1	TET2	ALYREF
DNMT2	ALKBH1	YBX1
NSUN2		

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