RayBio® combined 4-RNA modification

Dot Blot Kit

Catalog #: DB-4-RNA

User Manual
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RayBiotech
Empowering your proteomics

ISO 13485 Certified

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Table of Contents

Please read the entire manual carefully before starting your experiment

I. Introduction

RNA modifications are dynamic and reversible chemical modifications on substrate RNA that are regulated by specific modifying enzymes. They play important roles in the regulation of many biological processes in various diseases, including cancer, neurological disorders, cardiovascular diseases, metabolic diseases, genetic and developmental diseases, as well as immune disorders.

Advanced sequencing technologies have increasingly highlighted the significance of RNA modifications in human diseases which caught increasing attention in scientific research. Recent studies have mapped out the locations and abundance of key RNA modifications, such as N6-methyladenosine (m6A), 5-methylcytosine (m5C), N1-methyladenosine (m1A), and N7-methylguanosine (m7G), primarily through antibody immunoprecipitation or chemical treatments coupled with next-generation sequencing. It is well known that through the “writing (Methyltransferase) - erasing (Demethyltransferase) -reading (Binding protein)” mechanisms, RNA modifications regulate the stability, translation, and localization of pivotal disease-related mRNAs to manipulate disease development. Further research into RNA modifications, their roles, inhibitors, and activators, is essential for disease diagnosis, treatment, and prognosis.

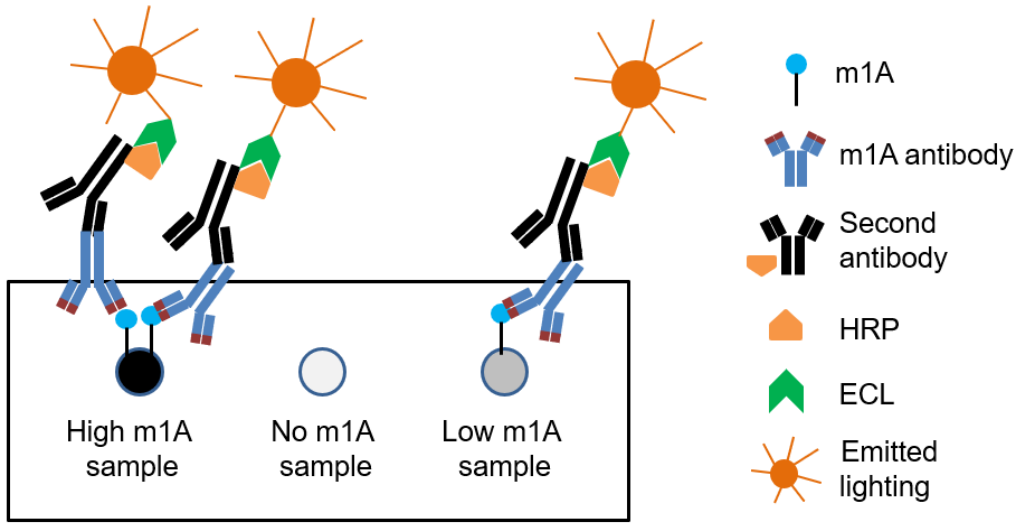
Here, we introduce a combined m1A, m7G, m6A, and m5C dot blot kit, which offers a reliable, simple, and rapid method of quantifying the 4 most common global RNA modification levels in RNA, urine, serum, plasma, or other nucleic samples collected from humans, rats, and mice.

II. General Description

The RayBio® combined 4-RNA modifications dot blot Kit is an *in vitro* semi-quantitative assay for detecting m1A, m7G, m6A and m5C. The RNA-containing sample is directly spotted onto a nitrocellulose (NC) or nylon membrane followed by incubation with their individual primary and secondary antibodies. Compared with traditional blotting techniques, our optimized dot blot is a powerful tool for analyzing the 4 most common global RNA modifications. This kit is highly sensitive and specific**,** requires minimal sample preparation, no specialty equipment, and can be completed within 3-4 hours, making it a cost-effective and efficient option. Moreover, dot blotting presents a convenient method for conducting high-throughput screening in large-scale studies involving a substantial volume of clinical and experimental samples. 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.

In this assay, the sample containing denatured RNA is directly spotted onto an NC membrane. Then, the RNA sample is crosslinked to the membrane using UV light, blocked for 1 hour, and incubated with the 4 individual RNA modification antibody for another hour, respectively. After a wash step, any bound antibody then interacts with an HRP-secondary antibody for 1 hour, which catalyzes a color development reaction with the ECL substrate.

III. How It Works



Graphical representation of m1A quantification by bot blot assay for example

IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 12 months from the date of shipment. For extended storage, it is recommended to store it at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

Component	**Size / Description**	**Storage / Stability After Preparation**
m1A Antibody (1000X)	1 vial (10 µl)	1 month at 4°C
m7G Antibody (1000X)	1 vial (10 µl)	1 month at 4°C
m6A Antibody (1000X)	1 vial (10 µl)	1 month at 4°C
m5C Antibody (1000X)	1 vial (10 µl)	1 month at 4°C
Anti-Rabbit IgG-HRP Antibody (1000X)	1 vial (20 µl)	1 month at 4°C
Detection Buffer C	1 bottle (10 ml)	1 month at 4°C
Detection Buffer D	1 bottle (10 ml)	1 month at 4°C
Wash Buffer (20X)	1 bottle (30 ml)	1 month at 4°C
Blocking Buffer (4X)	1 bottle (30 ml)	1 month at 4°C
MB Staining Buffer	1 bottle (30 ml)	Room temperature
NC Membrane	4 sheets (6x8cm)	Room temperature
Plastic Sheet	8 sheets (9x11cm)	Room temperature
Incubation Dish	1 dish with four chambers	Room temperature

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. RNase-free tubes
2. Heat blocker
3. 10 cm plastic petri dish
4. UV cross-linker
5. Chemiluminescent Imaging system

VII. Assay Procedure

1. Sample preparation:

I. RNA Samples: Extract RNA from cell or tissue samples with TRIZOL reagent (or kit) and formulate a final concentration between 100-2000 ng/μL. Dissolve purified RNA/DNA in nuclease-free water at 100-1000 ng/dot with 0.5-2 μl. For optimal quantification, the input RNA amount should be 300 ng/dot in 1 μl.

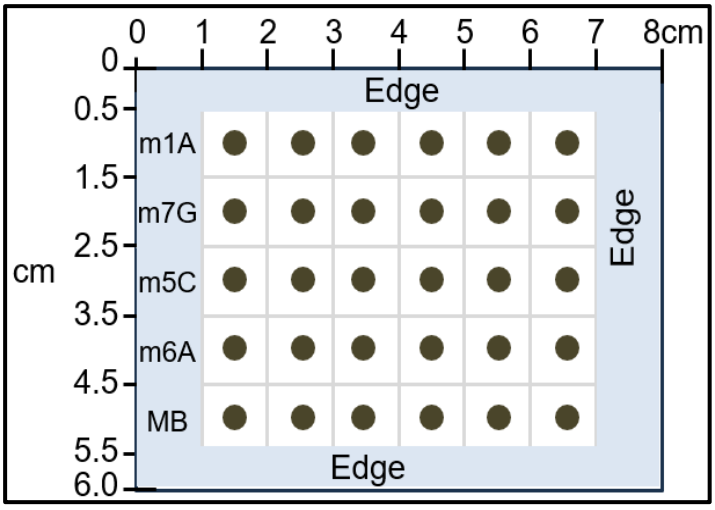
II. Plasma: Avoid hemolyzed and lipemic blood samples. Collect blood with EDTA, heparin, or citrate and centrifuge at 5000 g for 10 minutes at room temperature. Remove the plasma layer and avoid disturbing the white buffy layer. Dilute plasma sample with nuclease-free water at 1:3~1:9 for dot blot assay.

III. Serum: Avoid hemolyzed and lipemic blood samples. Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 5000 x g for 10 minutes at room temperature. Remove the yellow serum supernatant without disturbing the white buffy layer. Dilute serum sample with nuclease-free water at 1:3~1:9 for dot blot assay.

2. Incubate the diluted samples at 95°C in a heat block for 5 minutes to remove any RNA secondary structure.

3. Chill the tubes on ice for 5 minutes immediately after denaturation to prevent the re-formation of secondary structures of RNA.

4. Cut the NC membrane to an appropriate size. Mark the grid on the membrane lightly with a pencil to guide sample loading. The right image is an example of sample loading.



5. Mix the sample by pipetting it up and down. Using a narrow-mouth pipette tip, dispense 1 µL of the sample pre spot onto the membrane. The spots are necessary for running the m1A, m7G, m5C and m6A dot blots, as well as the methylene blue (MB)-staining which serves as an internal control.

NOTE: Avoid touching the membrane with the pipette tip. Let the pipetted RNA droplets diffuse onto the membrane via surface tension. Change tips after each loading, even between the same sample.

6. Air dry at room temperature for 5 minutes.

7. Using the UV crosslinker set to the Autocross link mode (1,200 microjoules [x100]; 25-50 sec), crosslink RNA to the membrane twice.

8. Wash the membrane in 10 ml of wash buffer in a clean washing tray, which does not need to be RNase-free, for 5 minutes at room temperature with gentle shaking to wash off the unbound RNA.

9. Cut off the MB line of the spotted sample from the membrane to use for MB staining. Transfer the membrane to a petri dish containing 10 mL MB staining buffer for 10 minutes with gentle shaking. Wash the membrane with Water three times, and for 5 minutes each time, until the background is clean. Image the MB-stained membrane with your imaging system.

10. Incubate the rest of the membrane with 1x blocking buffer on a rotating shaker for 1h at room temperature.

11. Decant the blocking solution and rinse the membrane with 1x Washing buffer.

12. Cut off the membrane for m1A, m7G, m5C and m6A dot blot assay, and incubate each membrane with their corresponding antibody (1:1000 dilution) in four chambers of the incubation dish for 1 hour at room temperature or overnight at 4 °C with gentle shaking.

13. Discard the primary antibody and wash with 1x Washing buffer for 5 – 10 minutes on a rotating shaker. Repeat this wash step 2 additional times.

14. After washing, incubate the membrane with the diluted 1x Anti-Rabbit IgG-HRP antibody for 1 hour at room temperature with gentle shaking.

15. Discard the secondary antibody and wash with 1x Washing buffer 3 times, each time 5 - 10 minutes on a rotating shaker.

16. Using a pipette, mix equal volumes (1:1) of Detection Buffer C and Detection Buffer D in a single clean tube.

17. Place all 4 membranes on top of the provided plastic sheet and gently pipette 500 µl of the Detection Buffer mixture onto the membranes. Incubate for 2 minutes at room temperature. Place another plastic sheet on top of the membranes to "sandwich" the membrane. Then, gently roll the flexible plastic sheet across the surface to the opposite end to smooth out any air bubbles.

NOTE: Exposure should ideally start within 5 minutes after finishing Step 17 and be completed within 10-15 minutes as chemiluminescence signals will fade over time.

18. Transfer the sandwiched membranes to the chemiluminescence imaging system such as a CCD camera (recommended) and expose.

NOTE: Optimal exposure times will vary so performing multiple exposure times is strongly recommended.

19. To obtain 4-RNA modification levels from the chemiluminescent images, Image J software (or similar image processing and analysis software) can be used.

VIII. Typical Results

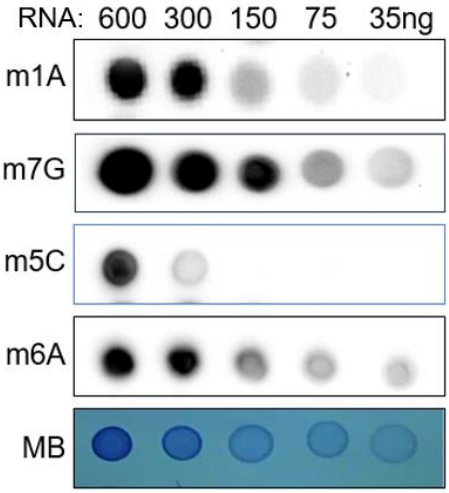


Figure 1. Representative image of 4-RNA methylation-specific dot blot assay of RNA samples. Total RNA was isolated from the human lung cancer cell line of A431 and quantified by nanodrop. The dot blot assay followed this protocol.

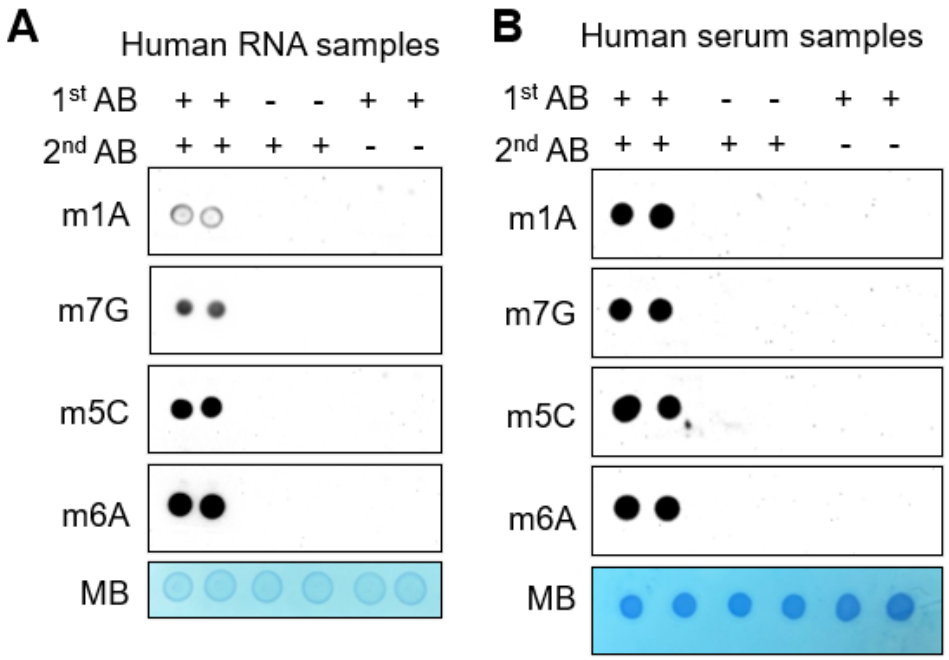


Figure 2. Representative images of the negative control for 4-RNA methylation by 1st and 2nd Antibody. **A**, total RNA was isolated from the human lung cancer cell line of A431 and A549 and then 300ng of total RNA was used for this assay. **B**, two human plasma samples were collected from two normal health controls. The plasma samples were diluted into 4-fold with water and 1 µl samples were loaded.

IX. Troubleshooting Guide

PROBLEM	CAUSE	RECOMMENDATION
No signals (not even the positive controls spots)	Chemiluminescent imager is not working properly	Contact image manufacturer
	Too Short Exposure	Ensure sufficient incubation time and change sample incubation step to overnight
	Degradation of components due to improper storage	Store entire kit at ≤ - 20°C. Do not use the kit after the expiration date. See storage guidelines.
	Improper preparation or dilution of the antibodies	Centrifuge vial briefly before use, mix well, and do not dilute more than 1000-fold
	Waiting too long before exposing	The entire detection process should be completed in 10-15 minutes
	Low sample protein levels	Decrease sample dilution, concentrate samples, or load more protein initially
Uneven signals and/or background	Bubbles present on or below membrane	Don't rock/rotate the tray too vigorously or pipette the sample or reagent with excessive force
	Insufficient sample or reagent volume	Load enough sample and reagent to completely cover the membrane
	Insufficient mixing of reagents	Gently mix all reagents before loading onto the membrane, especially the HRP-second antibody
	Rocking/Rotating on an uneven surface while incubating	Rock/rotate on a flat surface or the sample or reagent can "pool" to one side
High background signals or all spots visible	Too much HRP-second Antibody Cocktail	Prepare these signal enhancing components precisely as instructed
	Membranes dried out	Do not let the membranes dry out during the experiment. Cover the incubation tray with the lid to minimize evaporation
	Too High of Sample Protein Concentration	Increase dilution of the sample or load less protein
	Exposed Too Long	Decrease exposure time
	Insufficient Washing	Ensure all the wash steps are carried out and the wash buffer is removed completely after each wash step

	Non-specific binding	Ensure the blocking buffer is stored and used properly.

RayBio® ELISA Kits

Over 3,000 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits.html for details.

This product is for research use only.