3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

m6A (N6-methyladenosine) RNA Enrichment and Quantification Kit

Catalog #: QMERIP-M6A

User Manual
Last Revised: October 14th, 2024

Introduction

N6-methyladensoine (m6A), the methylation of adenosine in the N6 position, is a prevalent and reversible post-transcriptional RNA modification that decorates mRNA, tRNA and rRNA. Recent studies have revealed the crucial roles of m6A RNA modification in multiple cellular processes, including RNA structural stability, folding, interactions with proteins, cell viability, impaired self-renewal ability, cell proliferation, and cell death. Methylated RNA immunoprecipitation (RIP) is a gold standard method to monitor the status and map the location of RNA modifications in specific genes and in transcriptome wide.

The RayBio® m6A (N6-methyladenosine) RNA Enrichment and Quantification Kit (QMERIP-M6A) identifies the abundance and enrichment location of m6A by MeRIP and quantitative real-time PCR. The enriched m6A RNA could also be used for transcriptome-wide profiling of m6A. In this assay, RNA sample is digested into fragments consisting of 100~500 nucleotides followed by incubation with an antibody against m6A conjugated to a magnetic bead. Then, the enriched RNA is released, purified, and eluted for RT-qPCR analysis. Included in the kit is a non-immune IgG control conjugated to a magnetic bead and two pairs of primer controls for Hela cell RNA. QMERIP-M6A uses a fast and streamlined procedure so the assay can be finished in 5 hours with a hands-on time of less than 1 hour. This kit has been validated for human, mouse, and rat m6A enrichment.

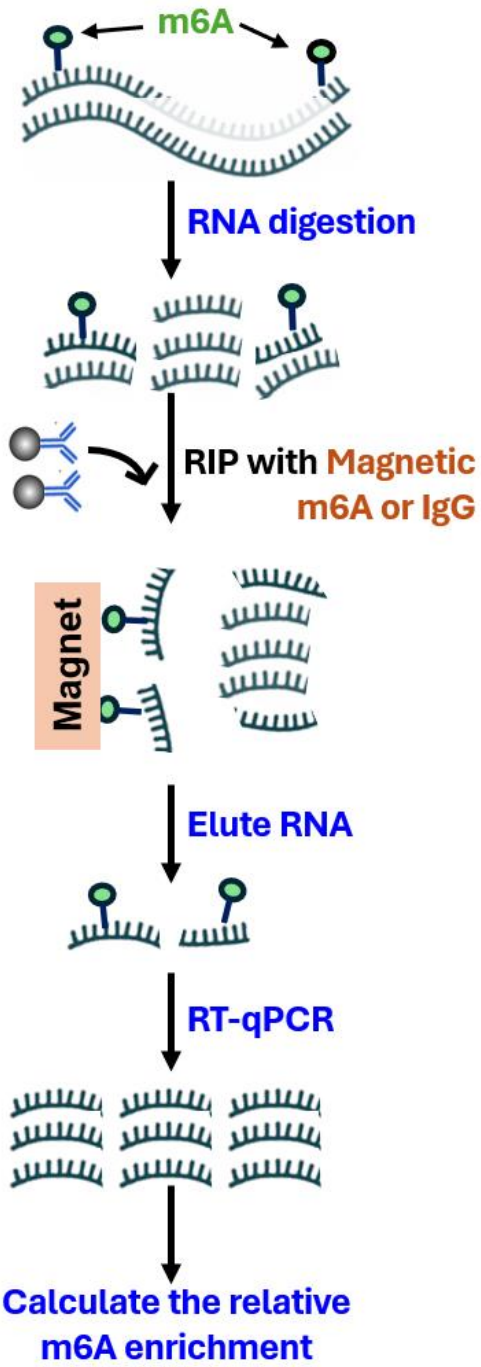
Product Use

QMERIP-M6A is for research use only. It is not approved for application in clinical or in vitro diagnostic procedures.

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

How It Works

Figure1. QMERIP-M6A workflow



RayBiotech
Empowering your proteomics

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

Storage / Stability

The entire kit can be stored at -20°C or -80°C for a period of 12 months prior to opening. After opening, the reagents are valid for at least 6 months if stored as described in the table below. Avoid repeated freeze-thaw cycles.

Kit Components

Name	Catalog #	Size / Qty	Storage After Opening
RNA Digestion Buffer (10X)	RIP-DB	1 mL / 1 tube	-20°C
Magnetic bead-conjugated m6A antibody	RIP-M6A	0.3 mL / 1 tube	-20°C
Magnetic bead-conjugated IgG antibody	RIP-IGG	20 µL / 1 tube	-20°C
RNase Inhibitor	RIP-RI	0.6 mL / 1 tube	-20°C
ACTB (Negative) Primers*	RIP-NP-M6A	20 µL / 1 tube	-20°C
SMAD7 (Positive) Primers*	RIP-PP-M6A	20 µL / 1 tube	-20°C
One-Step RT Master Mix (2X)	RIP-RTM	1 mL / 1 tube	-20°C
IP Buffer (5X)	RIP-IB	30 mL / 1 tube	4°C
Elution Buffer	RIP-EB	4 mL / 1 tube	4°C
0.5 M EGTA	RIP-EGTA	0.2 mL / 1 tube	4°C
NaOH	RIP-NA	0.6 mL / 1 tube	4°C
Nuclease-Free H_2O	RIP-H2O	1 mL / 1 tube	4°C

*Primers included in the kit have only been validated for Hela cell RNA. If running a different RNA sample type, you will need to supply your own primers for the target gene of interest

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

Additional Materials Required

- Fluorescence PCR instrument and compatible PCR plate or tubes
- Sterile nuclease-free pipette tips (barrier tips recommended) and microfuge tubes
- Vortex Mixer
- Magnetic Separator
- Total RNA, mRNA, tRNA or rRNA
- Primers for target gene of interest, with or without probe

Important Notes Before Starting

Please carefully read through the entire protocol before starting this assay. This protocol requires multiple steps but can be finished in one day. The hands-on time is less than 1 hour. The approximate time required for each step is provided in **Table 2** below.

Table 2. Procedure Overview & Estimated Timetable

Protocol Step	Time Required	Stopping Points and Protocol Notes
RNA Digestion	~50 minutes	Can pause after this step and store fragmented RNA at -80°C
RIP and Elution	~1.5 hours	Can pause after this step and store eluted RNA at -80°C
RT-qPCR	~2.0 hours	Continue to data analysis
Data Analysis	~0.5 hours	ΔΔCq Calculations

Reagent Preparation

Place all reagents on ice for 30 minutes to thaw before use. Then, mix gently with a vortex and briefly centrifuge at 1,500x g for 30 seconds to collect contents at the bottom.

RNA Sample Preparation: Extract or purify total RNA, tRNA, rRNA or mRNA from virous cells or tissues. Using high-quality and intact RNA is critical as degraded RNA can affect the quality of the assay. We recommend checking the RNA integrity by Bioanalyzer or agarose gel before starting the assay. The total RNA, tRNA, rRNA or mRNA input amount can range from 5 µg to 50 µg for each sample (this includes the 3 reactions per sample:

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

Input, m6A-IP and IgG-IP). For optimal quantification, we recommend 20 µg per sample for total RNA, and 10 µg for the other RNA types (tRNA, rRNA and mRNA).

Assay Procedure

1. RNA Digestion

1.1. Adjust the RNA sample to a total volume of 45 µL in a 200-µL PCR tube with nuclease-free water. Add 5 µL of 10X RNA Digestion Buffer (RIP-DB). Mix well by pipetting up and down and spin down the tubes to collect contents to the bottom.

1.2. Incubate the RNA samples at 37°C in the thermal cycler block for 30 minutes. Immediately remove the tubes from the block and add 5 µL of 0.5 M EGTA (RIP-EGTA) to each tube. Vortex and spin down the tube and place it on ice.

1.3. (**Optional**) Validate digested RNA size distribution by running 0.5 µg of the digested RNA sample on a 2% (wt/vol) agarose gel or Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Pico kit. The distribution of RNA fragment sizes should be centered around 100~500 bp. Validate digested RNA concentration by NanoDrop.

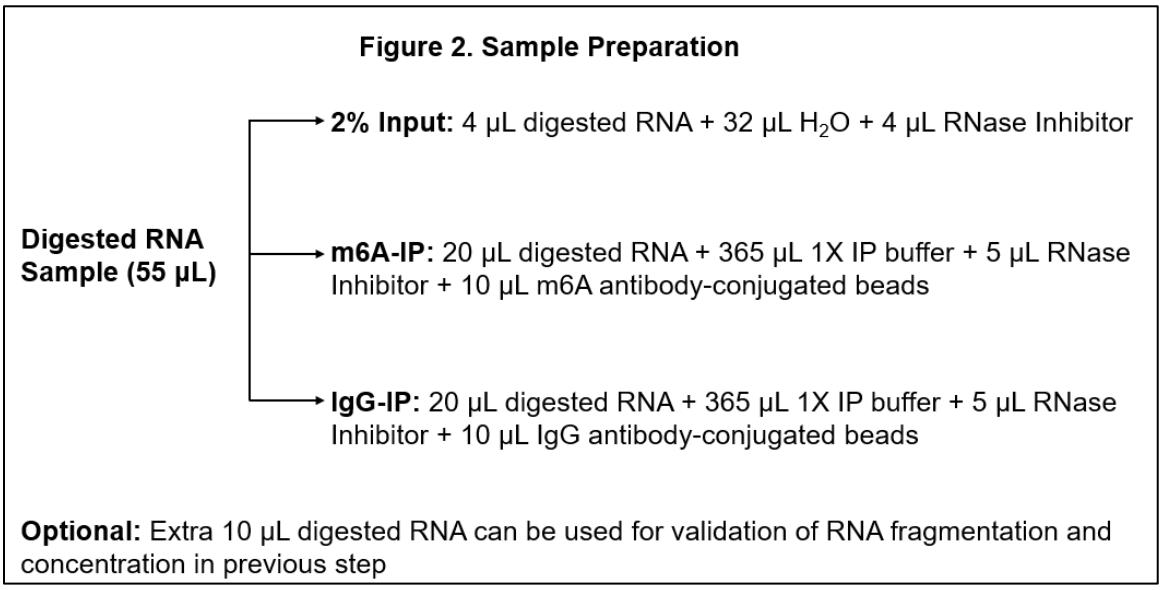
2. m6A RNA Immunoprecipitation (MeRIP)

2.1. **Sample Preparation (see Figure 2 for reference):** For each RNA sample, place three new 1.5 mL Eppendorf tubes on ice and label them “2% Input” (see below for calculation), “m6A-IP”, and “IgG-IP”. Into the “2% Input” tube, pipette 4 µL of digested RNA sample, then add 32 µL of nuclease-free H_2O and 4 µL of RNase inhibitor (RIP-RI). Put the “2% Input” tube on ice or store at -20°C until Step 3.1 of RT-qPCR. For the “m6A-IP” and “IgG-IP” tubes, pipette 20 µL of digested RNA sample into each tube.

2% Input Calculation: [(4 µL of digested RNA / 20 µL digested RNA for m6A-IP reaction / 10 (Dilution factor) x 100% = 2%)]

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

Figure 2. Sample Preparation



2.2. Dilute 5X IP buffer (RIP-IB) with nuclease-free H_2O to a 1X solution. Add 365 µL 1X IP buffer to the "m6A-IP" and "IgG-IP" tubes. Then, add 5 µL RNase inhibitor (RIP-RI) to each tube.

2.3. Thoroughly resuspend the m6A antibody-conjugated beads (RIP-M6A) and IgG antibody-conjugated beads (RIP-IGG) by end-over-end rotation or by pipetting up and down. No clumps of beads should be visible. Add 10 µL m6A antibody-conjugated beads and 10 µL IgG antibody-conjugated beads into the "m6A-IP" and "IgG-IP" tubes, respectively.

2.4. Incubate the "m6A-IP" and "IgG-IP" tubes with rotation at low speed for 60 minutes at room temperature.

2.5. Centrifuge the tubes briefly and place them on the magnetic separator for 1 minute, then carefully remove the supernatant.

Caution: Be careful not to disturb or discard the beads that contain the RNA

2.6. Remove the tubes from the magnet. Add 0.5 mL 1X IP Buffer to each tube and mix gently by pipetting up and down several times to completely resuspend the antibody-

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

conjugated beads. Place the tubes on the magnetic separator for 1 minute, then carefully remove the supernatant.

2.7. Repeat the above step for two additional washes. Be sure to remove the final IP Buffer leaving only the antibody-conjugated beads.

2.8. Remove the tubes from the magnet and place them on ice. Add 30 µL of Elution Buffer and 5 µL of RNase inhibitor (RIP-RI) to each tube and mix by gently pipetting up and down several times to completely resuspend the antibody-conjugated beads.

2.9. Centrifuge the tubes briefly to collect contents to the bottom of the tube. Place the tubes on the magnetic separator for 1 minute.

2.10. Transfer the supernatant containing eluted RNA fragments to new 1.5 mL microcentrifuge tubes. Take special care to not aspirate the beads, as it will increase background noise.

2.11. Add 5 µL NaOH (RIP-NA) into all IP tubes. Continue to RT-qPCR or store all RNA samples at −80°C until further use.

3. RT-qPCR Assay for m6A-RIP

The m6A enriched RNA can now be detected by quantitative RT-PCR assay. This kit includes SMAD7 (SMAD Family Member 7) as a positive control (RIP-PP-M6A) and ACTB (Actin Beta) as a negative control (RIP-NP-M6A) for m6A modification in Hela cells. Calculation of each m6A enriched RNA sample can be performed using the comparative Cq (ΔΔCq) method with three PCR amplicons: 2% Input, m6A-IP and IgG-IP. In addition, we recommend verifying successful RNA retrieval by running a gel after RT-qPCR.

Note: The included SMAD7 and ACTB primers have only been validated for Hela cell RNA. We do not recommend using the included primers with other RNA types.

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

Optional: If you are running a different RNA sample type and you want to ensure the assay is functioning properly, you can include a separate Hela cell RNA sample with the included primers for positive and negative controls.

3.1. Prepare the RT-qPCR reaction mix as stated in **Table 3**. Use enough reagents for two extra reactions to account for loss of volume. We recommend running all RNA samples (each digested RNA sample includes 2% Input, m6A-IP and IgG-IP) in triplicate. So, each RNA sample will have 9 reactions (3 for each condition).

Calculate the number of reactions needed = [1 (2% Input) + 1 (m6A-IP) + 1 (IgG-IP)] x n (number of digested RNA samples) x 3 (triplicates) + 2 (To account for loss of volume).

Example: For 3 samples in triplicate, total reactions = (1+1+1) x 3 x 3 + 2 = 29.

Table 3: RT-qPCR Reaction Components

Components	Volume per Reaction	Master Mix for 29 Reactions
One-Step RT Master Mix (2X)	10 µL	290 µL
Forward primer (10 µM)*	1 µL	29 µL
Reverse primer (10 µM)*	1 µL	29 µL
Nuclease-free Water	6 µL	174 µL
RNA Sample	2 µL	Add to each tube individually
Total Volume	**20 µL**	**522 µL**

*Primers included in the kit have only been validated for Hela cell RNA. If running a different RNA sample type, you will need to supply your own primers for the target gene of interest.

3.2. Prepare a master mix of all components except RNA according to the table. Mix thoroughly by gently pipetting up and down. Collect liquid to the bottom of the tube by brief centrifugation.

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

3.3. Aliquot 18 µL master mix into qPCR tubes or plate. For the best results, ensure accurate and consistent pipetting volumes and minimize bubbles.

3.4. Add 2 µL RNA sample to the appropriate qPCR tubes or plate. Seal tubes with flat, optically transparent caps; seal plate with optically transparent film. Take care to properly seal plate edges and corners to prevent artifacts caused by evaporation.

3.5. Spin tubes or plate briefly to remove bubbles and collect liquid (1 minute at 2,500 – 3,000 rpm).

3.6. Program qPCR instrument with indicated thermocycling protocol (see **Table 4** below).

Use the SYBR (or Taqman™) mode setting on the qPCR instrument. Refer to **Table 4** for SYBR PCR program setup (Use the Applied Biosystems QuantStudio 5 Real-Time PCR System as an example).

Table 4. RT-qPCR program (For SYBR model)

Steps		Temperature (°C)	Time	Cycles
Step 1	Reverse Transcription	55	10 minutes	1
Step 2	Initial Denaturation	95	3 minutes	1
Step 3	Denature	95	10 seconds	40-45
	Anneal, extend, and detect fluorescence	60	60 seconds	
Step 4	Melt Curve (SYBR model)	60-95	Various	1

3.7. Results and Export: In the analysis settings, select automatic baseline, or select according to your lab validation. Then, export the results as an excel file.

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

4. Calculate the Relative m6A Enrichment

4.1. Calculate the mean Cq value (Quantification Cycle Value) for each set of triplicates: 2% Input, m6A-IP and IgG-IP samples. Then, use the comparative ΔΔCq method to analyze the relative m6A enrichment.

4.2. Normalize Cq values of the 2% Input, m6A-IP and IgG-IP samples to the average ΔCq of 2% input samples: Normalized $\Delta Cq = Cq_{IP} - Cq_{Average\ 2\%\ Input}$

4.3. Calculate the percent of 100% Input for each sample: $2^{-\Delta Cq\ (normalized\ IP)} \times 50 \times 100\%$

4.4. The relative m6A enrichment measured by the m6A-IP over the IgG-IP:

$= 2^{-\Delta\Delta Cq} = 2^{-[\Delta Cq\ (normalized\ m6A\text{-}IP) - \Delta Cq\ (normalized\ IgG\text{-}IP)]}$

or

$= 2^{-\Delta Cq\ (normalized\ m6A\text{-}IP)} \times 100\% / 2^{-\Delta Cq\ (normalized\ IgG\text{-}IP)} \times 100\%$

Normally, relative m6A enrichment ≥ 8 means significant enrichment with P value <0.05 by appropriate statistical analysis. See example calculations in Typical Data section below.

Assay Procedure Summary

1. Prepare All Reagents, Samples and Standards as Instructed
2. RNA Digestion
3. m6A RNA Immunoprecipitation (RIP)
4. RT-qPCR Assay for m6A-RIP
5. Calculate the Relative m6A Enrichment

RayBiotech
Empowering your proteomics

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

Typical Data: Example of QMERIP-M6A Analysis:

The individual and mean Cq value for each set of triplicates samples shown in table below.

Samples	Replicates	ACTB			SMAD7		
		2% Input	IgG-IP	m6A-IP	2% Input	IgG-IP	m6A-IP
Sample #1	Rep-1	17.683	28.629	28.509	20.089	31.609	27.340
	Rep-2	16.976	28.992	28.784	19.521	32.041	27.010
	Rep-3	17.644	28.270	28.561	19.906	32.259	27.795
	Average	**17.434**	28.630	28.618	**19.839**	31.970	27.382
Sample #2	Rep-1	17.963	29.129	29.274	20.354	31.580	27.163
	Rep-2	18.629	29.410	28.748	20.354	31.670	26.688
	Rep-3	18.202	28.309	28.020	20.478	32.063	27.814
	Average	**18.265**	28.949	28.681	**20.395**	31.771	27.222
Sample #3	Rep-1	18.509	28.645	28.646	20.635	31.926	28.650
	Rep-2	18.736	29.372	29.224	20.132	32.114	28.137
	Rep-3	18.629	28.940	29.185	20.459	30.235	28.161
	Average	**18.625**	28.986	29.018	**20.409**	31.425	28.316

The average of ACTB Cq $_{2\% \text{ Input}}$ for 3 samples is: (17.434 + 18.265 + 18.625) / 3 = 18.108

The average of SMAD7 Cq $_{2\% \text{ Input}}$ for 3 samples is: (19.839 + 20.395 + 20.409) / 3 = 20.214

Normalized $\Delta Cq = Cq_{IP} - Cq_{\text{Average 2\% Input}}$ shown in the table below.

Samples	ACTB			SMAD7		
	2% Input	IgG-IP	m6A-IP	2% Input	IgG-IP	m6A-IP
Sample #1	-0.674	10.523	10.510	-0.376	11.756	7.168
Sample #2	0.157	10.841	10.573	0.181	11.557	7.008
Sample #3	0.517	10.878	10.910	0.195	11.211	8.102

The percentage of RIP by Input: $2^{-\text{normalized } \Delta Cq} \times 50 \times 100\%$ shown in the table below:

Samples	ACTB			SMAD7		
	2% Input	IgG-IP	m6A-IP	2% Input	IgG-IP	m6A-IP
Sample #1	159.536	3.399	3.429	129.739	1.446	34.778
Sample #2	89.701	2.725	3.282	88.202	1.660	38.855
Sample #3	69.879	2.657	2.598	87.387	2.110	18.196

Result: The relative m6A enrichment = The ratio of percentage of m6A-IP to IgG-IP

The relative m6A enrichment of ACTB in Sample #1 = 3.429 / 3.399 = 1.009

The relative m6A enrichment of SMAD7 in Sample #1 = 34.778 / 1.446 = 24.051

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

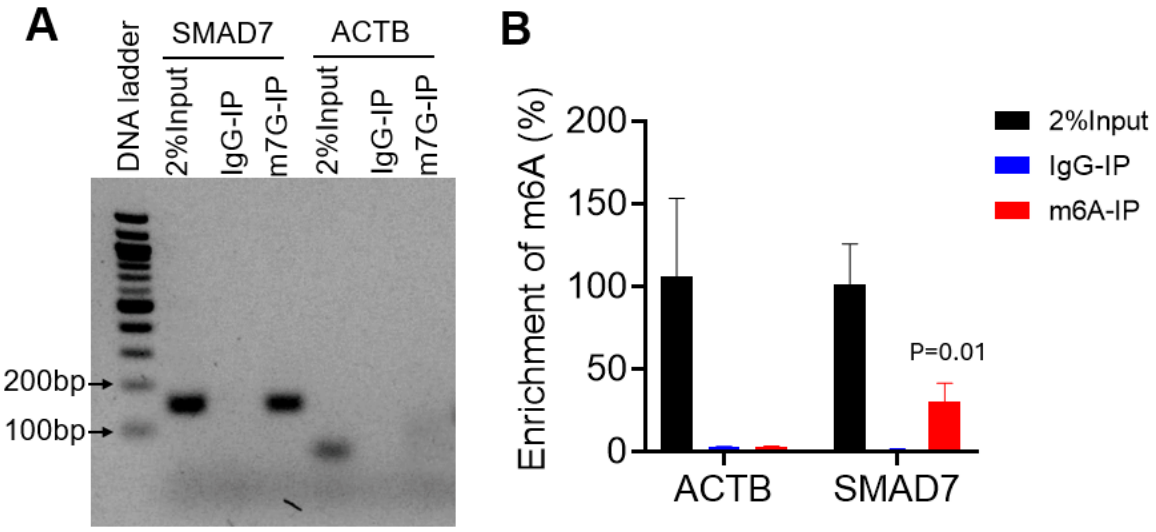


Figure 3. QMERIP-M6A enrichment test by PCR and RT-qPCR. (A) Representative images of agarose gel running after PCR amplification for the enrichment of m6A targeted SMAD7 and ACTB mRNA. (B) Bar graph showing the percentage enrichment of m6A targeted SMAD7 and ACTB mRNA using RT-qPCR assays. 2% Input RNA sample before IP was used as the positive control and IgG-IP was used as the negative control. Almost no PCR products were amplified from the m6A-IP ACTB RNA samples. Error bars, SD (*n* = 3 independent experiments). *** *P*-value <0.001 compared to IgG-IP was calculated using the student's *t*-test.

Precision

Intra-assay CV: <5%

Inter-assay CV: <20%

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
ISO 13485:2016 cGMP

TROUBLESHOOTING

Problem	Cause	Solution
Little or no enriched RNA	Insufficient amount of qualified RNA or m6A-containing RNA	Use a higher amount of RNA
	Poor enrichment with antibody	Increase incubation time of the antibody with RNA to overnight at 4°C
	Inappropriate RNA fragmenting condition	RNA digestion enzyme contained in buffer may be degraded due to improper storage. Ensure the proper storage conditions are used for this component
	Improper primer or probe for PCR	Redesign the primer with/without probe, check m6A occupancy in your targeting region or gene
	RNA degraded	Use RNase inhibitor as recommended in this protocol. Make sure that all working conditions are RNase-free and RNases are not being introduced. Use RNase-inactivating reagents to ensure the work area and materials are RNase-free
	Incorrect temperature and/or insufficient time during RNA release	Ensure that proper incubation times and temperatures described in the protocol are followed correctly
No difference in enriched RNA between m6A-IP and IgG-IP	Improper washing during RIP steps	Check if washing is performed according to the protocol. Increase wash time at each wash step or add an additional wash step
	Not enough fragmentation	Optimize fragmentation conditions to obtain appropriately sized fragments