Norgene™ Rapid m6A RNA Enrichment Kit

Cat.# 0001

PLEASE READ THIS ENTIRE USER GUIDE CAREFULLY BEFORE USE.

Application: The Norgene™ Rapid m6A RNA Enrichment Kit is specifically designed to enrich RNA fragments containing m6A modifications from low-input RNA samples. It enables the detection of region-specific m6A sites through PCR or allows for comprehensive epitranscriptome-wide m6A profiling using next-generation sequencing on Illumina platforms or other technologies. With its innovative design, optimized protocol, and high-quality components, the kit effectively captures m6A fragments with minimal non-specific background. The enriched RNA is ideally suited for constructing both non-barcoded (singleplex) and barcoded (multiplex) libraries, facilitating high-resolution mapping of m6A regions with reduced bias.

Starting Amount: In general, the amount of mRNA or ribosome-depleted RNA required for each reaction can range from 1 pg to 50 ng. For optimal results, an input amount of at least 10 pg RNA is recommended, although this kit can generate data from as little as single-cell rRNA-depleted RNA. For its unique advantage, this kit is well-suited for detecting very rare and valuable samples, in the case only a small number of cells are available, such as in cancer biopsies or healthy tissue samples.

Starting Materials: The starting material can be poly(A)-selected mRNA or rRNA-depleted RNA from any cell type, including both eukaryotic and prokaryotic cells. For rRNA depletion from very small amounts of intact cells, we strongly recommend using the NEBNext® rRNA Depletion Kit v1/v2 (Human/Mouse/Rat) or the NEBNext® rRNA Depletion Kit (Bacteria), as these kits have consistently delivered the best performance in our experience.

Antibodies: The antibodies we use here have been thoroughly tested to ensure high specificity against m6A RNA fragments.

Precautions: When working with small amounts of starting material, we strongly recommend performing all procedures in a UV-decontaminated laminar airflow (LAF) bench. To prevent cross-contamination, carefully pipette the samples or solutions into the strip wells using filter pipette tips, and always change tips between each liquid transfer. Wear gloves and lab coat sleeves throughout the entire procedure, and if your gloves touch the sample inside, change them immediately.

Post-IP downstream analysis: After completing the procedure, the users can proceed with either reverse transcription qPCR analysis or sequencing. For library construction and sequencing, we recommend using the SMART-Seq® Stranded Kit (Cat. # 634442), as other kits may not be suitable for low-input library preparation.

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KIT CONTENTS

Box 1 is shipped on dry ice at ~ 20 °C, box 2 is shipped at ~ 4 °C with a cold pack.

Component (Box 1)	Volume (10 reactions, Cat. #0001)	Tube size	Storage Upon Receipt
5 X IP buffer	240 µL	1.5 mL tube	-20 °C
1 X IP buffer	2.2 mL	8 mL bottle	-20 °C
m6A Antibody	2.5 µL	1.5 mL tube	-20 °C

Component (Box 2)	Volume (10 reactions, Cat. #0001)	Tube size	Storage Upon Receipt
Washing Buffer 1	4.8 mL	8 mL bottle	4 °C
Washing Buffer 2	2.4 mL	8 mL bottle	4 °C
RNase-free H_2O	5 mL	8 mL bottle	4 °C
Magnetic Beads	15 µL	1.5 mL tube	4 °C
Elution Buffer	500 µL	1.5 mL tube	4 °C

Important: *Before use, freeze-thaw and briefly centrifuge the solution to collect it at the bottom of the tube.*

STORAGE

Upon receipt, store the components at the recommended temperature and protect them from light. When stored properly, the kit remains stable for up to 12 months from the date of shipment.

Important: *Take out all the reagents from –20°C storage and thaw it at room temperature in preparation and then put into 4 °C ready for use.*

MATERIALS REQUIRED BUT NOT SUPPLIED

- Vortex mixer
- Minicentrifuge for 0.2 ml and 1.5 ml microcentrifuge tubes
- HulaMixer
- Magnetic device (Norgene 0.2ml - magnetic separation rack, Cat.# 0009 or similar)
- Adjustable pipette and pipette tips
- Nuclease-free 0.2 ml PCR tubes
- 100% ethanol
- Hielscher UP100H sonicator or similar

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A BRIEF OVERVIEW

N6-methyladenosine (m6A) is one of the most common and abundant chemical modifications found in RNA, primarily in messenger RNA (mRNA). It is a reversible epitranscriptomic modification that plays a critical role in various biological processes, including RNA metabolism, stability, splicing, and translation. m6A is widely present in mammals and is crucial in regulating gene expression, cell differentiation, development, and various pathophysiological processes, such as cancer, neurodegenerative diseases, and immune responses.

The regulation of m6A modification involves a dynamic interplay among "writers," "erasers," and "readers." Writers are methyltransferase complexes that catalyze the addition of methyl groups to specific RNA sites. Erasers are demethylases that can remove the m6A modification, while readers are proteins that recognize and bind to m6A-marked sites, mediating the downstream effects of this modification. This dynamic regulatory mechanism enables m6A to rapidly respond to cellular signals and environmental changes.

Advancements in high-throughput sequencing technologies and the growing focus on RNA modifications have uncovered the essential roles of m6A in gene expression regulation. It has been shown to influence mRNA stability, translation efficiency, and cell cycle control, as well as play a key role in embryonic development, neurodevelopment, stress response, and tumor progression. Due to its significant impact on cellular functions, m6A modification has become a major area of interest in RNA epigenetics and transcriptional regulation, with promising potential applications in medical research and therapeutic strategies.

Currently, various methods have been developed to identify transcriptome-wide m6A profiles, significantly advancing our understanding of m6A. However, these methods are time-consuming and costly. To address these issues, Norgene has developed a new method to fill the researcher needs. The Norgene rapid m6A RNA Enrichment Kit is simple and rapid workflow to enable identification and profiling of RNA m6A methylation sites at transcriptome-wide level. Suitable for downstream RNA library construction and sequencing or qRT-PCR to check methylated RNA transcripts.

HIGHLIGHTS

- Low input: The protocols are processed in the same single-tube, which enables maximum protection of the target m6A-containing fragments and minimized sample loss, allowing the input RNA to be as low as 10 pg.
- Accurate and reproducible: Innovative immunoprecipitation and elution technology ensure efficient and reproducible results across replicates.
- Fast, streamlined procedure: From beginning to end the protocol can be completed in 5 hours.

Steps	Time Required	Stopping Points and Protocol Notes
Antibody-beads preparation	30 mins	Overnight
RNA Fragmentation	~ 1 hours	Time required depends on sample size. Can store fragmented RNA at -80°C
Immunoprecipitation	~ 3 hours	Can store the final beads-RNA complex at -80

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ASSAY PROTOCOL

For the best results, please read the whole protocol prior to starting your experiment.

Starting Materials

Input RNA Amount: The amount of mRNA or ribosome-depleted RNA required for each reaction typically ranges from 1 pg to 50 ng. For optimal results, it is recommended to use at least 10 pg of RNA. However, data can still be obtained from as little as a single cell's rRNA-depleted RNA when using this kit. For efficient rRNA depletion, we strongly recommend using the NEBNext® rRNA Depletion Kit v1/v2 (Human/Mouse/Rat) or the NEBNext® rRNA Depletion Kit (Bacteria), as these kits consistently deliver the best performance in our tests. Finally, the mRNA or ribosome-depleted RNA should be eluted in 80 µl of RNase-free water.

RNA Storage: Store RNA at -20°C for up to one week or at -80°C for long-term storage.

Important: *Total RNA should not be used as the starting material due to the presence of m6A modifications in ribosomal RNA (rRNA), which makes up over 97% of the total RNA. m6A modifications can also be found in other non-coding RNAs. If the study focuses exclusively on mRNA modifications, using poly(A)-selected mRNA as the starting material is recommended. However, for studies that include non-coding RNA modifications, it is advisable to use rRNA-depleted RNA. The choice between poly(A)-selected RNA and rRNA-depleted RNA should be guided by the specific research objectives.*

1. Antibody-beads preparation

1| Create the antibody-beads incubation.

Reagents	Antibody Mix
RNase-Free H_2O	30.0 µl
5x IP buffer	8.0 µl
Anti-m6A antibody	2.0 µl
Total Volume MIX	40.0 µl

Important: *The above table mix is prepared for 10 samples.*

2| Vortex the **magnetic beads** vial few seconds to resuspend the pellet.
3| Transfer 10 µl **beads** to a 0.2 ml tube separately.
4| Place the tube on the magnet to separate the beads from the solution, once the solution is clear, discard the supernatant without disrupting the pellet.
5| Remove the tube from the magnet and wash twice the **beads** by adding 200 µl **1X IP Buffer**. Wash by remove the tube from the magnet, add solution, vortex briefly, pulse-spin, place back in the magnet and discard supernatant.
6| Add the 40 µl **Total Volume MIX** to the 0,2 ml washed **beads**.
7| Incubate with rotation on the rotator at 4°C overnight at low speed (recommended settings: 40 rpm).

2. RNA fragmentation

Option I: Sonication

8| Set a Hielscher UP100H sonicator, fitted with a 2 mm probe, at 0.5 Cycles and 27 % power.
9| Clean probe with 30 sec in **RNase-free water**, 30 sec in **absolute ethanol**.
10| Sonicate samples **X** cycles. Each sonication cycle is 30 seconds sonicating plus 30 sec on ice.

Important: *Shearing conditions have been optimized, proceed with the cycles below.*

Sample Starting Amount	Sonication Cycles
mRNA or rRNA depleted RNAs ≥ 10 ng	2.5 x 30 sec
mRNA or rRNA depleted RNAs 1~10 ng	2 x 30 sec
mRNA or rRNA depleted RNAs ≤ 1 ng	1 x 30 sec

11| Clean probe between sample types with 30 sec in **RNase-free water**, 30 sec in **absolute ethanol** and 30 sec in **RNase-free water**.

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12| After sonication, spin the samples briefly to collect the sample at the bottom of the tube. Remove 7 µl of **the sonicated RNA** and transfer it to a 0.2 ml tube (store at - 80 °C). This will serve as the **Input** RNA for RNA-seq analysis. To the remaining sonicated sample, add 7 µl of **RNase-free water** to bring the final volume to 80 µl.

Important: *After sonication, remember to take an aliquot as the Input sample; it will be used for further analysis in m6A peak calling or RT-qPCR.*

13| Add 20 µl of **5 X IP buffer** to above sample for a final volume of 100 µl.

Option II: Norgene RNA Fragmentation kit (Cat.# 0008)

This kit utilizes heat and exposure to optimized ions at high temperatures. Users have the flexibility to control the size of RNA according to their preferences. Following fragmentation, there's no need for the addition of any stop solution instead of just quick beads purification that is performed on ice. This approach offers a highly efficient and rapid RNA fragmentation strategy. The kit accommodates input amounts ranging from 100 pg to 1 µg of RNA.

3. Inmunoprecipitation

14| Remove the tube with the **bead-antibody complex MIX** from the rotator from STEP 7.
15| Pulse-spin and place on the magnetic rack on the top of ice or cold block, until the solution is clear.
16| Discard the supernatant without disrupting the pellet.
17| Remove the tube from the magnet and wash the **beads** by adding 200 µl cold **1X IP Buffer**, vortex briefly, pulse-spin and place the tube back in the magnet until the solution is clear, discard the supernatant.
18| Perform a second wash by adding 200 µl cold **1X IP Buffer**, vortex briefly to make the beads suspension completely, and aliquot 20 µl into 0.2 ml PCR tubes for each sample.

Important: *The total volume is 200 µl, with 20 µl aliquoted for each sample. This is designed for 10 immunoprecipitated samples as shown in STEP 1.*

19| Place the aliquoted tubes in the magnets and discard the second washing supernatants.
20| Remove from the magnet and resuspend the pellet by adding the **Fragmented RNA Mix** (100 µl) from STEP 13 and resuspend gently.
21| Incubate the tube with rotation on the rotator at 4°C for 2 hours (recommended settings: 40 rpm).
22| After incubation, pulse-spin the tubes and place on the magnetic rack.
23| Remove the tube from the magnet and wash the beads by adding 200 µl **Washing Buffer 1**.
24| Vortex briefly (4x 5 sec) and pulse-spin before place the tube back in the magnet until the solution is clear.
25| Discard the supernatant and remove the tube from the magnet.
26| Wash the beads by adding 200 µl **Washing Buffer 2**, vortex briefly and pulse-spin before place the tube back in the magnet until the solution is clear.
27| Discard the supernatant and remove the tube from the magnet.
28| Wash the beads by adding 200 µl **Washing Buffer 1**.
29| Vortex briefly (4x 5 sec) and pulse-spin before place the tube back in the magnet until the solution is clear, remove supernatant.
30| Add 100 µl **1X IP Buffer** and resuspend the pellet by a brief vortex and pulse-spin.
31| Discard the supernatant and remove the tube from the magnet.
32| Resuspend the pellet in 7 µl **Elution Buffer** and store at – 80 °C for further use.
33| The bead-containing sample can be used directly for reverse transcription, followed by qPCR analysis or library construction. After reverse transcription, briefly centrifuge the sample, place it on a magnetic rack, then transfer the supernatant to a new tube for further use, and discard the beads.

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