EpiMark® N6-Methyladenosine Enrichment Kit

NEB #E1610S

20 reactions

Version 2.1_10/21

Table of Contents

The EpiMark N6-Methyladenosine Enrichment Kit Includes:

Each kit contains sufficient reagents to perform 10 x 2-round immunoprecipitations with starting amounts of up to 250 µg of ribosome depleted or poly A+ purified RNA. It contains sufficient reagents for 5 x 2-round immunoprecipitations when using up to 250 µg of total RNA.

N6-Methyladenosine Antibody

M6A Control RNA (100 nM)

Unmodified Control RNA (100 nM)

Required Materials Not Included:

Reaction Buffer
(150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% NP-40 in nuclease free H_2O)

Protein G Magnetic Beads (NEB #S1430)

Magnetic Racks for bead separations (NEB #S1506 or #S1509)

Monarch® RNA Cleanup Kit (10 µg) (NEB #T2030)

Eppendorf® RNA/DNA LoBind microcentrifuge tubes (Sigma catalog #Z666548) or equivalent

RNase-free pipette tips

Powder-free gloves

Nuclease-free water

Optional Materials:

Primers for amplification of control RNAs:

GLuc Forward Primer = 5′- CGACATTCCTGAGATTCCTGG - 3′

GLuc Reverse Primer = 5′- TTGAGCAGGTCAGAACACTG - 3′

CLuc Forward Primer = 5′- GCTTCAACATCACCGTCATTG - 3′

CLuc Reverse Primer = 5′- CACAGAGGCCAGAGATCATTC - 3′

384 well PCR plate (Bio-Rad cat. #HSP-3805)

Optical film (Bio-Rad cat. #MSB-1001)

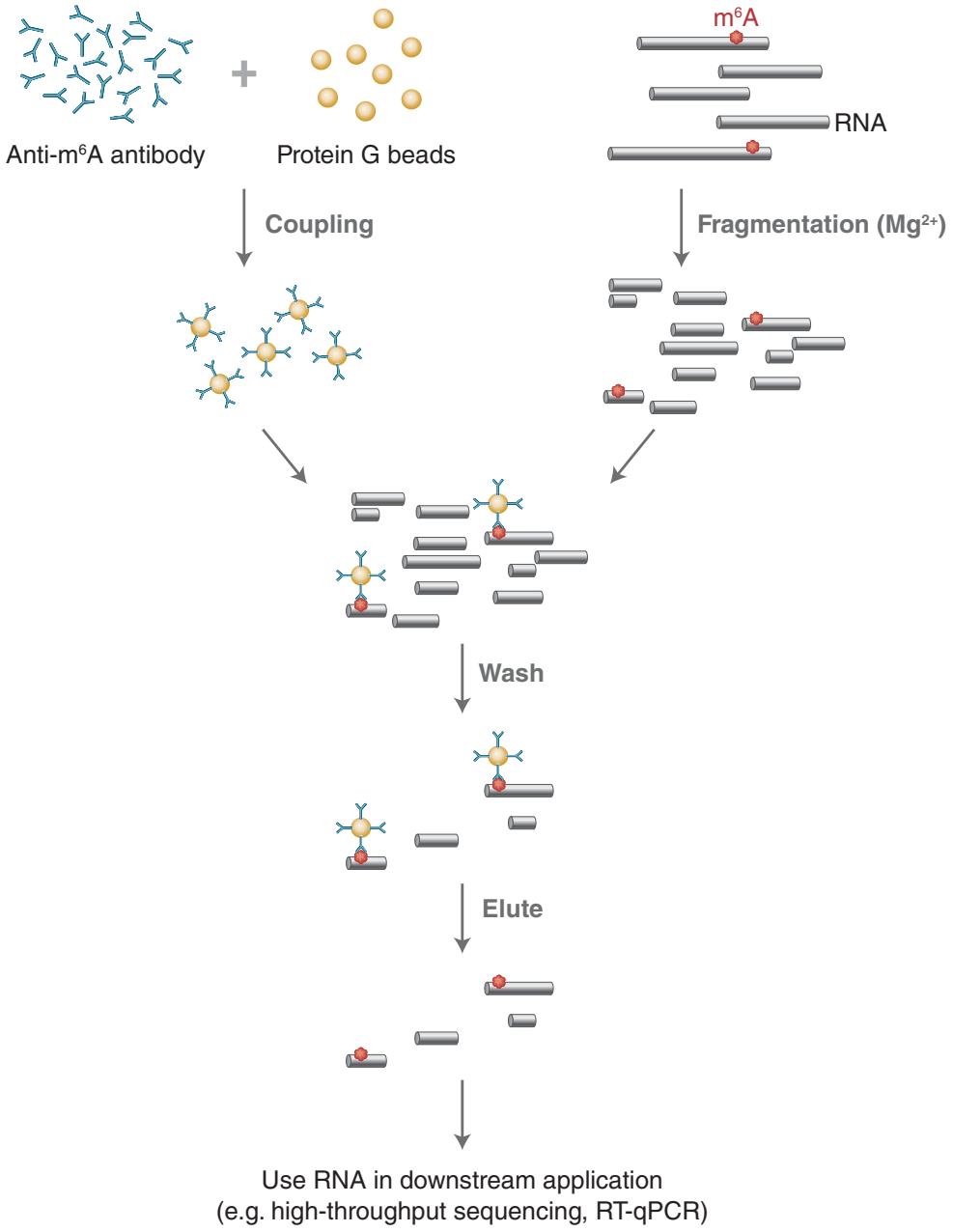
Luna® Universal One-Step RT-qPCR Kit (NEB #E3005)

Introduction

The EpiMark N6-Methyladenosine Enrichment Kit contains a rabbit monoclonal antibody specific for N6-Methyladenosine (m6A). The kit also contains two control RNAs, one with m6A modification (*Gaussia* luciferase) and one without (*Cypridina* luciferase) to monitor enrichment and depletion. The GLuc RNA control was transcribed in the presence of 20% m6ATP and 80% ATP.

This kit can be used to enrich m6A modified RNA in immunoprecipitation protocols for downstream analysis by next-generation RNA sequencing or RT-qPCR. Modified RNA is isolated from a fragmented RNA sample by binding to the N6-Methyladenosine antibody attached to Protein G Magnetic Beads. After multiple wash and clean-up steps, the enriched RNA is eluted in nuclease-free water and is ready for further analysis.

Figure 1. Workflow



Protocols

Immunoprecipitation

Prepare Protein G Magnetic Beads for Binding

1. Vortex Protein G Magnetic Beads prior to use.
2. Pipette 25 µl of Protein G Magnetic Beads (NEB #S1430) into a clean 1.5 ml low-binding microcentrifuge tube. Apply magnetic field to pull beads to the side of the tube. Carefully remove and discard the supernatant.
3. Wash beads twice with reaction buffer:

   Resuspend beads completely in 200 µl Reaction Buffer. Apply magnetic field to pull beads to the side of the tube. Carefully remove and discard the supernatant. Repeat wash a second time.
4. Resuspend beads completely in 250 µl reaction buffer.

Attach N6-Methyladenosine Antibody to Protein G Magnetic Beads

5. Add 1 µl N6-Methyladenosine Antibody to the resuspended beads if working with ribosome depleted or poly A+ purified RNA. In case of total RNA use 2 µl antibody.
6. Incubate with orbital rotation for 30 minutes at 4°C (Do not agitate/shake, avoid foaming).
7. Apply magnetic field to pull beads to the side of the tube. Carefully remove and discard the supernatant.
8. Wash beads twice with Reaction Buffer:

   Resuspend beads completely with 200 µl of Reaction Buffer. Apply magnetic field to pull beads to the side of the tube. Carefully remove and discard the supernatant. Repeat wash a second time.
9. Resuspend beads completely in 250 µl of Reaction Buffer.

Bind RNA to Antibody

10. Add RNA (amount varies; up to 250 µg) to the re-suspended beads.
    To run a control IP instead of adding an RNA sample, make a mix containing 20 nM each of the m6A Control RNA and the Unmodified Control RNA as shown below. The volume may be scaled as required. Add 10 µl of this mix to the re-suspended beads. The leftover mix may be used to prepare the Input RNA control for RT-qPCR.

3 µl	M6A Control RNA (100 nM)
+3 µl	Unmodified Control RNA (100 nM)
+9 µl	Nuclease-free water
15 µl	Control RNA Mix

    Note: If you intend to spike the control RNAs into the RNA sample it is essential to use a much smaller quantity than when doing a control IP on them alone. This is because the m6A control RNA is highly modified and the antibody has a high affinity for it. This will result in the m6A control RNA becoming predominant in the sample after IP. Use of 1 µl of a 1:1,000 dilution of each control RNA (0.1 fmol of each RNA) is recommended. The control RNAs can be spiked in either before or after fragmentation of the RNA sample. If spiked in after, a concentrated stock of the control RNAs should be fragmented and then diluted prior to addition to the sample.
11. Incubate with orbital rotation for 1 hour at 4°C.
12. Briefly spin tubes to ensure no liquid is trapped in the lid, then apply magnetic field to pull beads to the side of the tube.
13. Carefully remove and discard the supernatant.
14. Resuspend each tube of beads completely in 150 µl of Monarch RNA Cleanup Binding Buffer.
15. Incubate at room temperature for 1 min.
16. Apply magnetic field to pull beads to the side of the tube.
17. Carefully collect the eluent and transfer it to a clean 1.5 ml low-bind microcentrifuge tube.

Clean and Concentrate RNA

1. Clean up the RNA using Monarch RNA Cleanup Kit (10 µg) (NEB #T2030).
2. Since the RNA is already in Monarch RNA Cleanup Binding Buffer, proceed to addition of ethanol as per the kit protocol.
3. Follow the remaining kit instructions for clean up and elution

RT-qPCR Protocol to Determine Efficiency of RNA Enrichment after Immunoprecipitation

Reagents for the RT-qPCR reaction are from the Luna Universal One-Step RT-qPCR Kit (NEB #E3005)

RT-qPCR Reaction Setup

1. Set up three reactions for each IP using 2 µl of a 1:50 dilution of the sample as the RNA template and target specific primers.
   If using the Control RNAs, set up three reactions using GLuc primers and three using CLuc primers.
2. Set up one Input RNA control reaction using 2 µl of the input RNA (up to 1 µg). If using the Control RNA Mix (Refer to Section: Bind RNA to Antibody, Step 10), dilute mix 1:5000 and use 2 µl in the reaction.

COMPONENT	20 µl REACTION	FINAL CONCENTRATION
Luna Universal One-Step Reaction Mix (2X)	10 µl	1X
Luna WarmStart RT Enzyme Mix (20X)	1 µl	1X
Forward primer (10 µM)	0.8 µl	0.4 µM
Reverse primer (10 µM)	0.8 µl	0.4 µM
Template RNA	variable	< 1 µg (total RNA)
Nuclease-free Water	to 20 µl	

3. After combining the reaction components, add 8 µl of each reaction to two different wells in a 384 well PCR plate. Seal plate with optical film and briefly spin down plate in a centrifuge.
4. Run RT-qPCR cycling conditions after loading plate into the thermal cycler.

CYCLE STEP	TEMP	TIME	CYCLES
Reverse Transcription	55°C*	10 minutes	1
Initial Denaturation	95°C	1 minute	1
Denaturation	95°C	10 seconds	40–45
Extension	60°C	30 seconds** (+ plate read)	
Melt Curve	60-95°C***	various	1

* **A 55°C RT step temperature** is optimal for Luna WarmStart Reverse Transcriptase. To insure best performance and full WarmStart activation avoid using a temperature of < 50°C.

** For Applied Biosystems real-time instruments use a 60 second extension step.

*** Follow real-time instrument recommendations for melt curve step.

5. Analyze data as per real-time instrument manufacturer instructions.

Appendix:

Sequences of Control RNAs

Unmodified Control RNA (Cypridina Luciferase): 1706 nt

GGAGACCCAAGCTTGGTACCGAGCTCGGATCCGCCACCATGAAGACCTTAATTCT
TGCCGTTGCATTAGTCTACTGCGCCACTGTTCATTGCCAGGACTGTCCTTACGAAC
CTGATCCACCAAACACAGTTCCAACTTCCTGTGAAGCTAAAGAAGGAGAATGTATT
GATAGCAGCTGTGGCACCTGCACGAGAGACATACTATCAGATGGACTGTGTGAAA
ATAAACCAGGAAAAACATGTTGCCGAATGTGTCAGTATGTAATTGAATGCAGAGTA
GAGGCCGCAGGATGGTTTAGAACATTCTATGGAAAGAGATTCCAGTTCCAGGAAC
CTGGTACATACGTGTTGGGTCAAGGAACCAAGGGCGGCGACTGGAAGGTGTCCA
TCACCCTGGAGAACCTGGATGGAACCAAGGGGGCTGTGCTGACCAAGACAAGAC
TGGAAGTGGCTGGAGACATCATTGACATCGCTCAAGCTACTGAGAATCCCATCAC
TGTAAACGGTGGAGCTGACCCTATCATCGCCAACCCGTACACCATCGGCGAGGTC
ACCATCGCTGTTGTTGAGATGCCAGGCTTCAACATCACCGTCATTGAGTTCTTCAA
ACTGATCGTGATCGACATCCTCGGAGGAAGATCTGTAAGAATCGCCCCAGACACA
GCAAACAAAGGAATGATCTCTGGCCTCTGTGGAGATCTTAAAATGATGGAAGATAC
AGACTTCACTTCAGATCCAGAACAACTCGCTAATCAGCCTAAGATCAACCAGGAGT
TTGACGGTTGTCCACTCTATGGAAATCCTGATGACGTTGCATACTGCAAAGGTCTT
CTGGAGCCGTACAAGGACAGCTGCCGCAACCCCATCAACTTCTACTACTACACCAT
CTCCTGCGCCTTCGCCCGCTGTATGGGTGGAGACGAGCGAGCCTCACACGTGCTG
CTTGACTACAGGGAGACGTGCGCTGCTCCCGAAACTAGAGGAACCTGCGTTTTGT
CTGGACATACTTTCTACGATACATTTGACAAAGCAAGATACCAATTCCAGGGTCCC
TGCAAGGAGATTCTTATGGCCGCCGACTGTTTCTGGAACACTTGGGATGTGAAGG
TTTCACACAGGAATGTTGACTCTTACACTGAAGTAGAGAAAGTACGAATCAGGAAA
CAATCGACTGTAGTAGAACTCATTGTTGATGGAAAACAGATTCTGGTTGGAGGAGA
AGCCGTGTCCGTCCCGTACAGCTCTCAGAACACTTCCATCTACTGGCAAGATGGTGA
CATACTGACTACAGCCATCCTACCTGAAGCTCTGGTGGTCAAGTTCAACTTCAAGC
AACTGCTCGTCGTACATATTAGAGATCCATTCGATGGTAAGACTTGCGGTATTTGC
GGTAACTACAACCAGGATTTCAGTGATGATTCTTTTGATGCTGAAGGAGCCTGTGAT
CTGACCCCCAACCCACCGGGATGCACCGAAGAACAGAAACCTGAAGCTGAACGACT
CTGCAATAGTCTCTTCGCCGGTCAAAGTGATCTTGATCAGAAATGTAACGTGTGCCAC
AAGCCTGACCGTGTCGAACGATGCATGTACGAGTATTGCCTGAGGGGACAACAGG
GTTTCTGTGACCACGCATGGGAGTTCAAGAAAGAATGCTACATAAAGCATGGAG
ACACCCTAGAAGTACCAGATGAATGCAAATAGGCGGCC

m6A Control RNA (Gaussia Luciferase): 603 nt

GGAGACCCAAGCTTGGTACCGAGCTCGGATCCAGCCACCATGGGAGTCAAAGTTC
TGTTTGCCCTGATCTGCATCGCTGTGGCCGAGGCCAAGCCCACCGAGAACAACGAA
GACTTCAACATCGTGGCCGTGGCCAGCAACTTCGCGACCACGGATCTCGATGCTGA
CCGCGGGAAGTTGCCCGGCAAGAAGCTGCCGCTGGAGGTGCTCAAAGAGATGGAA
GCCAATGCCCGGAAAGCTGGCTGCACCAGGGGCTGTCTGATCTGCCTGTCCCACAT
CAAGTGCACGCCCAAGATGAAGAAGTTCATCCCAGGACGCTGCCACACCTACGAA
GGCGACAAAGAGTCCGCACAGGGCGGCATAGGCGAGGCGATCGTCGACATTCCTG
AGATTCCTGGGTTCAAGGACTTGGAGCCCATGGAGCAGTTCATCGCACAGGTCGATC
TGTGTGTGGACTGCACAACTGGCTGCCTCAAAGGGCTTGCCAACGTGCAGTGTTC
TGACCTGCTCAAGAAGTGGCTGCCGCAACGCTGTGCGACCTTTGCCAGCAAGAT
CCAGGGCCAGGTGGACAAGATCAAGGGGGCCGGTGGTGACTAAGCGGCC

Reference

1. Schwartz, S. et al. (2013) *Cell* 155, 1409–1421. PubMed ID: 24269006.

Ordering Information

NEB #	PRODUCT	SIZE
E1610S	Epimark N6-Methyladenosine Enrichment Kit	20 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
S1430S	Protein G Magnetic Beads	1 ml
S1506S	6-Tube Magnetic Separation Rack	6 tubes
S1509S	12-Tube Magnetic Separation Rack	12 tubes
E3005S/L	Luna Universal One-Step RT-qPCR Kit	200/500 reactions
E3005X/E	Luna Universal One-Step RT-qPCR Kit	1,000/2,500 reactions
T2030S/L	Monarch RNA Cleanup Kit (10 µg)	10/100 preps

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	2/16
2.0	Apply new manual format. Update Required Materials Not Included and the protocol.	9/20
2.1	Page 4, Step 3 change to “After combining the reaction components, add 8 µl of each reaction to two different wells in a 384 well PCR plate. Seal plate with optical film and briefly spin down plate in a centrifuge.”	10/21

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

This product is covered by one or more patents, trademarks and/or copyrights owned or controlled by New England Biolabs, Inc. For more information about commercial rights, please email us at busdev@neb.com While NEB develops and validates its products for various applications, the use of this product may require the buyer to obtain additional third party intellectual property rights for certain applications.

B CORPORATION™ is a trademark of B Lab, Inc.
ILLUMINA® is a registered trademark of Illumina, Inc.
EPPENDORF® is a registered trademark of Sigma, Inc.
iTAQ™ is a trademark of Bio-Rad Laboratories, Inc.
SYBR® and DYNABEADS® are registered trademarks of Life Technologies Corporation.
MYONE™ is a trademark of Life Technologies Corporation.

Cell Signaling TECHNOLOGY®

N6-Methyladenosine Antibody is produced by Cell Signaling Technology, Inc. and sold by New England Biolabs, Inc.

www.neb.com

NEW ENGLAND BioLabs® Inc.

be INSPIRED drive DISCOVERY stay GENUINE

New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 Telephone: (978) 927-5054 Toll Free: (USA Orders) 1-800-632-5227 (USA Tech) 1-800-632-7799 Fax: (978) 921-1350 e-mail: info@neb.com