EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit

Base Catalog # P-2037

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit is a complete set of optimized reagents designed to quickly enrich RNA binding protein-specific RNA complex directly from cells to profile interactions between proteins and RNA via RT-PCR or next generation sequencing using Illumina platforms.

Starting Materials: Starting materials can include various mammalian cell samples such as culture cells from a flask or plate, primary cells, or rare cell populations isolated from blood, body fluid, fresh/frozen tissues, and specific cells sorted from entire cell populations and embryonic cells, etc.

Input Amount of Cells: The amount of cells can be 5×10^4 to 1×10^6 cells per reaction. For optimal preparation, the cell input amount should be 5×10^5, although the results for strong binding proteins can be obtained with as few as 20,000 cells.

Antibodies: Antibodies should be RIP-grade in order to recognize the proteins that are bound to RNA or other proteins. If you are using antibodies that have not been validated for IP, then an appropriate control antibody, such as anti-SNRNP70, should be used to demonstrate that the antibodies are suitable for RIP.

Internal Controls: Both negative and positive controls for EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit use are provided in this kit.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the tube or vials. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

© EigenTek Group Inc. All rights reserved. Products are for research use only.

KIT CONTENTS

Component	24 Reactions Cat# P-2037-24	Storage upon receipt
WB (Wash Buffer)	30 ml	4°C
PB (Permeabilization Buffer)	4 ml	RT
PIC (Protease Inhibitor Cocktail)*	30 µl	4°C
RI (RNase Inhibitor)*	30 µl	-20°C
RCE (RNA Cleavage Enhancer)	300 µl	RT
CEM (Cleavage Enzyme Mix)*	60 µl	-20°C
Positive Control Ab (SNRNP70,1 mg/ml)*	12 µl	-20°C
Non-Immune IgG (1 mg/ml)*	25 µl	4°C
PDB (Protein Digestion Buffer)	5 ml	RT
Proteinase K (10 mg/ml)*	100 µl	4°C
Affinity Beads*	100 µl	4°C
RPS (RNA Purification Solution)	600 µl	RT
RNA Binding Beads*	60 µl	4°C
Elution Buffer	1 ml	RT

* *Spin the solution down to the bottom prior to use.*

SHIPPING & STORAGE

The kit is shipped in two parts: the first part at ambient room temperature and the second part on frozen ice packs at 4°C.

Upon receipt, store the components according to the temperatures in the table above away from light.

Note: *Check if **WB (Wash Buffer)** and **PB (Permeabilization Buffer)** contain salt precipitates before use. If so, briefly warm at room temperature or 37°C and shake until the salts are re-dissolved.*

All components of the kit are stable for 6 months from the date of shipment when stored properly.

MATERIALS REQUIRED BUT NOT SUPPLIED

Equipment

- ☐ Vortex mixer
- ☐ Microscope and cell counter

© EigenTek Group Inc. All rights reserved. Products are for research use only.

- ☐ Thermocycler with 48 or 96-well block
- ☐ Centrifuge, including desktop centrifuge (up to 14,000 rpm)
- ☐ Rotator or rolling shaker
- ☐ Magnetic device (96-well PCR plate format)
- ☐ Adjustable pipette and pipette tips
- ☐ 0.2 ml PCR vials
- ☐ 1.5 ml microcentrifuge tubes
- ☐ Hemocytometer

Reagents

- ☐ PBS
- ☐ Trypan blue
- ☐ Antibodies of interest
- ☐ Cell sample
- ☐ 100% ethanol
- ☐ 100% isopropanol
- ☐ Distilled water

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit is tested against predetermined specifications to ensure consistent product quality. EpigenTek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply call our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: EpigenTek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Be sure to use the latest User Guide for this kit, which can be accessed online at www.epigentek.com/datasheet.

Usage Limitation: The EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit and methods of use contain proprietary technologies by EpigenTek.

© EpigenTek Group Inc. All rights reserved. Products are for research use only.

A BRIEF OVERVIEW

Enrichment of protein-complexed RNA in vivo followed by qPCR and/or next-generation sequencing offers an advantageous tool for studying epitranscriptome-wide protein-RNA interactions. The major methods used for a long time to achieve this goal are traditional RIP (RNA immunoprecipitation) followed by PCR (RIP-PCR) or sequencing (RIP-Seq). These methods have been widely used but are unable to achieve high resolution, need crosslinking, and suffer from poor reproducibility and a complicated process. In particular, these methods are time-consuming (from 8 hours to 2 days) and costly.

To address these issues, EpigenTek has developed a new assay, the EpiNext™ CUT&LUNCH *(Cleavage Under Target and Liberate Unique Nucleic Complex Homogenously)* RNA Immunoprecipitation (RIP) Kit, for studying in vivo protein-RNA interaction, which integrates all advantages of the currently used RIP and CUT&RUN, a new method for protein-DNA interaction profiling. The EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit has the following advantages and features:

- **Extremely Fast 3-Step Protocol**: Start directly from intact cells without needing lysis and crosslinking. The entire procedure of the assay can be finished within 1 h and 50 min, minimizing RNA damage and loss of disassociated RNA binding components and preserving native protein-RNA structure.

- **Enhanced Specificity and Minimized Background:** Unique nucleic acid cleavage enzymes ensure low sequence bias by cleaving or removing RNA sequences at both ends of the target protein/RNA complex without affecting RNA occupied by the target protein. The liberated non-specific protein-RNA complexes are selectively eliminated, allowing for the recovery of only the antibody-bound complexes. As a result, target protein-enriched regions can be reliably achieved and identified through high-resolution mapping.

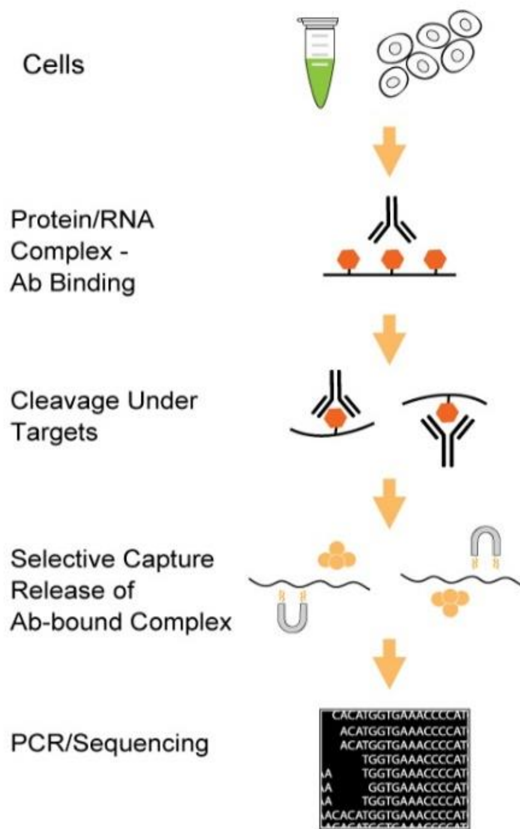
- **Low Input Materials:** Robust unbound RNA cleavage and immunocapture are all processed in the minimal time. This method uses both cells and tissues and allows for maximal degradation protection of the target protein with minimal sample loss. As a result, the input cell amount can be as few as 20,000 cells, <5% of the minimal amount required by traditional RIP.

- **Wide Suitability**: Ideal for cultured cells as well as fresh and frozen tissues from various species for different RNA binding proteins while maintaining both assay specificity and sensitivity.

- **Highly Convenient** The kit contains all required components, making the EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit both convenient and cost-effective.

© EpigenTek Group Inc. All rights reserved. Products are for research use only.

PRINCIPLE & PROCEDURE



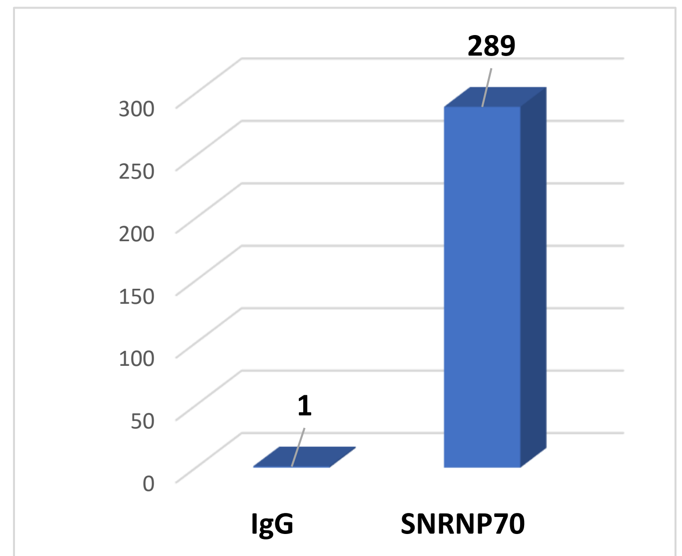


Fig 2. Target protein/RNA enrichment using the EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit: SNRNP70/RNA complex was captured by the positive control antibody from 200,000 MCF-7 cells. Non-immune IgG was used as the negative control. The enriched RNA was purified and fluorescently quantified by Qubit.

Fig 1. Schematic Procedure of the EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit.

The EpiNext™ CUT&LUNCH RNA Immunoprecipitation (RIP) Kit provides all necessary reagents to enrich RNA binding protein-specific RNA complexes, enabling profiling of protein-RNA interactions from various cell samples via qRT-PCR or NGS. In this assay, cells are permeabilized and treated with a RIP-grade antibody specific to the protein of interest. A unique nucleic acid cleavage enzyme mix then selectively cleaves RNA sequences flanking the target protein regions, removing non-specific RNA-protein complexes. Only antibody-bound complexes are selectively retained. The RNA fragments in the captured protein/RNA complexes are purified and can be directly used for qRT-PCR or cDNA library construction to profile interactions between proteins and RNA.

© EpigenTek Group Inc. All rights reserved. Products are for research use only.

Procedure overview and time table

Steps	Required time
Antibody binding to target	50 min
Enzyme cleavage	5 min
Target recovery and purification	45 min

REACTION PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

1. Preparation of Working Buffers

Prepare **Working PB buffer** by adding 1 µl of **PIC** (**Protease Inhibitor Cocktail)** to every 1 ml of **PB (Permeabilization Buffer).**

2. Cell Collection and Antibody Capture

a. Isolate or collect the cells according to your own successful methods. These cells could include culture cells from a flask or plate, primary cells, or rare cell populations isolated from blood, body fluid, and fresh/frozen tissues, specific cells sorted from entire cell populations and embryonic cells, etc. Count the cells using a hemocytometer by trypan blue staining. Total 2-3 X 10^6 cells may be needed for each standard assay (including RIP sample, IgG negative control, antibody positive control and “Input”).

b. Wash cells with PBS by centrifugation at 2500 rpm for 5 min. Discard the supernatant.

c. Add 80-100 µl of **Working PB buffer** to re-suspend the cell pellet and count the cells using a hemocytometer by trypan blue staining. Adjust the cell concentration to be 2.5 X 10^4/µl if possible.

d. Prepare antibody-binding reaction by adding the reagents to 0.2 ml PCR tubes according to the following and mix well:

Reagents	Sample	Positive Control	Negative Control
Working PB buffer	76-78 µl	78 µl	78 µl
Your Antibodies	2-4 µl	0	0
Control Antibody	0	2 µl	0
Non-Immune IgG	0	0	2 µl
Total Volume	**80 µl**	**80 µl**	**80 µl**

Note: *The final amount of each component should be (a) antibodies of interest: 2 µg/tube; (b) positive control antibody: 2 µg/tube; and (c) non-immune IgG: 2 µg/tube.*

*Shake the **Affinity Beads** to completely suspend them before use.*

© EpigenTek Group Inc. All rights reserved. Products are for research use only.

e. Transfer 20 µl of the suspended cells into each PCR tube, respectively as sample, positive control, and negative control. Mix and rotate on a rotator or rolling shaker at room temperature (RT) for 30 min. Meanwhile, transfer the 20 µl of the remaining cells to a 0.2 µl PCR tube and use it as an “Input” (no antibody control).

   The “Input” can be cleaved according to the following steps:

   Incubate at RT for 50 min and spin down 10 sec in a mini centrifuge (6000-7000 rpm) to pellet cell debris. Transfer supernatant to a new 0.2 ml PCR tube and add 1 µl of **RCE** (RNA Cleavage Enhancer)***, 1 µl of CEM (Cleavage Enzyme Mix)*** *and incubate at room temperature for 2 min. Then go to Step 4b for RNA Release/Purification.*

f. After the 30 min incubation, spin down 10 sec in a mini centrifuge (6000-7000 rpm) to pellet cell debris. Transfer supernatant to a new 0.2 ml PCR tube, add 4 µl of **Affinity Beads**, then rotate and incubate at RT for an additional 20 min.

3. Cleavage Reaction

a. After the 20 min incubation, open the tube and add 2 µl of **RCE** (RNA Cleavage Enhancer)**,** and 1 µl of **CEM (Cleavage Enzyme Mix),** then rotate and incubate for additional 5 min.

b. Place the PCR tubes in the magnetic stand and wash each reaction tube three times with 150 µl of **WB (Wash Buffer),** and once with 150 µl of **PDB (Protein Digestion Buffer**). The wash can be performed as follows:

   Place the tubes in the magnetic stand for 1-2 min to pellet the beads. Remove and discard the solution from each reaction tube, then add 150 µl **of WB (Wash Buffer)** to the reaction tubes. Resuspend the beads by gently pipetting up and down several times. Ensure the pellets are completely resuspended and the beads are not clinging to the pipette tips after pipetting. Place the tubes back in the magnetic stand for 1-2 min to pellet the beads. Then remove and discard the solution from each reaction tube and repeat the wash step as required.

4. RNA Release/Purification

a. Prepare **Protein Digestion Solution** by mixing **Proteinase K** with **PDB** (**Protein Digestion Buffer**) at a 1:10 dilution (e.g. 1 µl of **Proteinase K** + 9 µl of **PDB (Protein Digestion Buffer**).

b. Remove the tubes from the magnetic device after the last wash. Add 20 µl of **Protein Digestion Solution** to each reaction tube and “Input“ tube. Mix and incubate at 55°C for 15 min in a thermocycler (without heated lid).

c. Put the tubes on the magnetic device until the solution is clear (about 2 min). Carefully transfer the solution from each sample to an unused PCR tube and mark it.

d. Add 20 µl of the **RPS (RNA Purification Solution)** into each tube containing the solution followed by adding 40 µl of 100% isopropanol. Add 40 µl of the **RPS (RNA Purification Solution)** into “Input” tube followed by adding 80 µl of 100% isopropanol.

© EpigenTek Group Inc. All rights reserved. Products are for research use only.

e. Resuspend **RNA Binding Beads** by vortexing. Add 2 µl of the resuspended beads to each tube. Mix thoroughly by pipetting up and down at least 10 times.

f. Incubate for 5 min at room temperature to allow the RNA to bind to the beads.

g. Put the PCR tubes in the magnetic device until the solution is clear (about 2 min). Carefully remove and discard the supernatant. *(Caution: Be careful not to disturb or discard the beads that contain the RNA).*

h. Keep the PCR tubes in the magnetic device and add 150 µl of freshly prepared *90% ethanol* to the tubes, then carefully remove and discard the ethanol.

i. Repeat Step 4h once for a total of two washes.

j. Resuspend the beads in 12 µl of **Elution Buffer**, and incubate at room temperature for 5 min to release the RNA from the beads.

k. Capture the beads by placing the tubes in the magnetic device until the solution is completely clear (about 1 min).

l. Transfer 12 µl from each sample to a new 0.2 ml PCR tube for immediate use or store at -20°C.

Note:

1) The enriched RNA sample concentration can be quantified with Qubit RNA quantification method and then used to construct cDNA library for NGS analysis or used to perform simple qRT-PCR assay.

2) For the enrichment fold comparison of the positive control/negative control with qRT-PCR, the primers specific for the positive target region of SNRNP70 such as U1 snRNA can be designed. For data analysis after real time PCR, Fold Enrichment (FE) can be calculated by simply using a ratio of amplification efficiency of the positive control RNA sample over that of the negative control sample with appropriate primers.

$$FE = 2^{(NC\ CT - PC\ CT)} \times 100\%$$

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
Little or no enriched RNA from samples	Poor enrichment with antibody: some antibodies used in the reaction might not efficiently bind to the protein.	Increase the antibody amount and use validated RIP-grade antibodies.

110 Bi County Blvd. Ste. 122, Farmingdale, NY 11735
Tel: 1-877-374-4368 ■ Fax: 1-718-484-3956 ■ E-mail: info@epigentek.com ■ Web: www.epigentek.com
© EigenTek Group Inc. All rights reserved. Products are for research use only.

	Inappropriate cleavage condition.	The cleavage time may be too short or too long to cause the RNA fragment to be >1000 bps or <20 bps, respectively. Ensure the cleavage time and cleavage enzyme amount are correct.
	Improper storage of the kit.	Ensure that the kit has not exceeded the expiration date. Standard shelf life, when stored properly, is 6 months from date of shipment.
No difference in signal intensity between negative and positive controls	Insufficient washing.	Check if washing recommendations at each step are performed according to the protocol. If the signal intensity in the negative control is still high, washing stringency can be increased in the following ways: 1) Increase wash time at each wash step: after adding **WB (Wash Buffer)**, leave it in the wells for 2 min and then remove it. 2) Add an additional one wash with **WB (Wash Buffer)**, respectively: the provided volume of **WB (Wash Buffer)** is sufficient for 4 extra washes for each sample.
	For RT-PCR verification, too many PCR cycles were used: plateau phase of amplification caused by excessive number of PCR cycles in endpoint PCR may mask the difference of signal intensity between negative control and positive control.	Decrease the number of PCR cycles (i.e., 32-35 cycles) to keep amplification at the exponential phase. This will reduce high background in endpoint PCR and allow differences in amplification to be seen. Real time PCR is another choice in such cases.
Poor results in downstream qPCR	Little or no PCR product even in the positive control.	Ensure that all PCR components were added and that a suitable PCR program is used (PCR cycle should be >35).
		PCR primers and probes were not appropriate or were incorrectly designed. Ensure the primer and probes are suitable for amplification of the RNA region of interest and the target regions to be amplified are less than 100 bps.
		Ensure the amount of template DNA used in PCR was sufficient.
	Significant non-specific PCR products.	Failed RNA cleavage. Ensure that all steps of the digestion and cleanup protocol were followed and that input RNA amount is within the recommended range.
		Primers and probes are not specific for target genes. Check the primer and probe design.

© EpigenTek Group Inc. All rights reserved. Products are for research use only.

RELATED PRODUCTS

RNA Isolation and Cleanup

P-9105 EpiQuik™ Total RNA Isolation Fast Kit
P-9107 EpiQuik™ Viral RNA Isolation Fast Kit
P-9108 EpiMag™ Viral RNA Isolation Kit (Magnetic Beads)
P-9109 EpiMag™ 96-Well Viral RNA Extraction Kit (High Throughput)

Methylated RNA Quantification and Enrichment Reaction

P-9003 Methylamp™ RNA Bisulfite Conversion Kit
P-9005 EpiQuik™ m6A RNA Methylation Quantification Kit (Colorimetric)
P-9008 EpiQuik™ m6A RNA Methylation Quantification Kit (Fluorometric)
P-9009 MethylFlash™ 5-mC RNA Methylation ELISA Easy Kit (Fluorometric
P-9015 MethylFlash™ Urine N6-methyladenosine (m6A) Quantification Kit (Colorimetric)
P-9018 EpiQuik™ CUT&RUN m6A Enrichment Kit

Modified RNA Library Prep

P-9007 EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina)
P-9016 EpiQuik™ CUT&RUN m6A-Seq Kit

NGS Barcode

P-1060 EpiNext™ NGS Barcode (Index) Set-12

110 Bi County Blvd. Ste. 122, Farmingdale, NY 11735
Tel: 1-877-374-4368 ■ Fax: 1-718-484-3956 ■ E-mail: info@epigentek.com ■ Web: www.epigentek.com
© EpigenTek Group Inc. All rights reserved. Products are for research use only.