Engibody®
BIOTECHNOLOGY

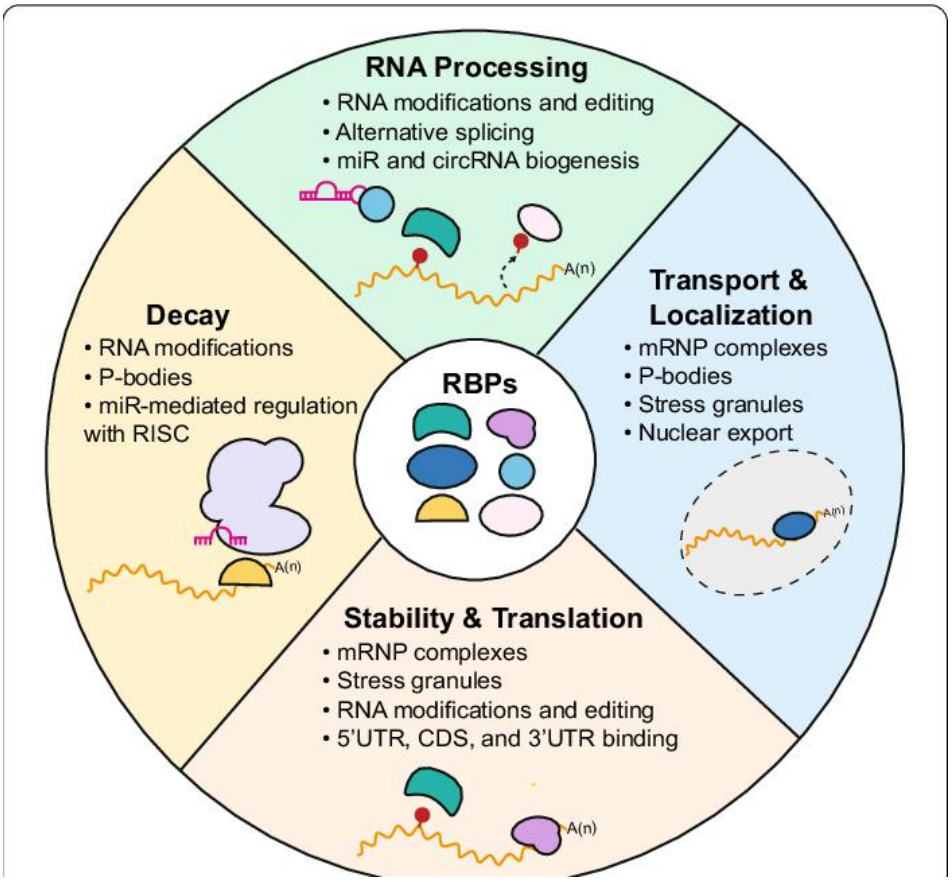
PRODUCT DATA SHEET

Smart-RNA-Pull™ RNA Pull-down Kit

(RNA Protein Pull-down)

Cat no: IF9608 Size: 20 Reactions

RNA-RBP In Vitro Pull-down



Version: 2024

Content

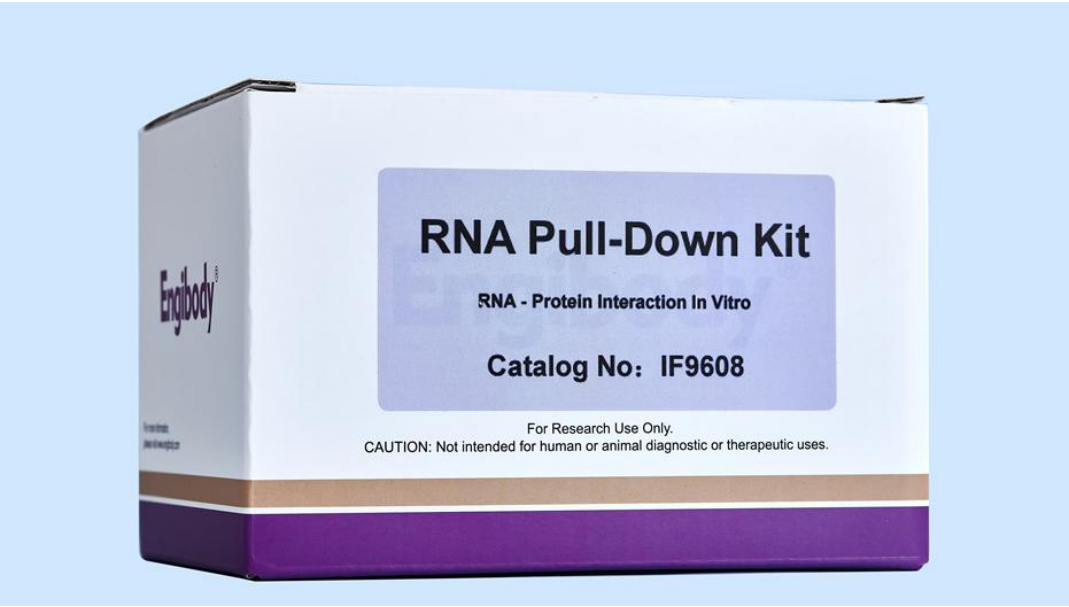
Advantages of Smart-RNA-Pull™

- This kit is suitable for adherent cells, suspended cells, animal tissues, plant tissues, and purified recombinant proteins. It is suitable for various length and complexity RNA probes labeled with terminal biotin. Proteins that are endogenous, overexpressed, or translated in vitro are suitable as sample types.

- This kit uses biotin labeled RNA probes to directly capture the target protein. Pull down does not require antibodies or centrifugation. After completing the RNA labeling reaction, very little manual operation time is required (less than 3 hours).

- This reagent kit is suitable for the interaction between RBP and mRNA, RBP and LncRNA, RBP and miRNA, RBP and circRNA, as well as downstream Western blot verification detection of known proteins and mass spectrometry identification of unknown proteins.

- This manual is a comprehensive technical manual for RNA Pull down experiments, summarizing detailed information on the interaction between RNA and various proteins. After reading this nanny level manual, all difficulties will be easily solved, and RNA Pull down is within your grasp.



Part 1: Background

RNA protein interaction

In fact, 95% of the human genome does not encode proteins, but produces a large number of non coding RNAs (ncRNAs). These RNAs play an important regulatory role in the growth and development of life, are closely related to HIV / AIDS, leukemia, diabetes and other diseases, and participate in the regulation of stem cell development and epigenetics. RNA protein complexes drive post transcriptional regulation of gene expression in almost all cellular processes, including splicing, nuclear export, mRNA stability, and protein translation. Therefore, the understanding of post transcriptional regulatory networks and mechanisms depends on determining the changes in RNA binding in these processes.

RNA binding proteins control many aspects of cell biology by binding to single stranded RNA binding motifs (RBMs). However, RBM can be hidden in its local RNA structure, thereby inhibiting the interaction between RNA and protein.

The research on the interaction between RNA and protein has attracted more and more attention of scientists, and has become a hot spot of epigenetic research.

At present, the hotspots of RNA research are mainly mRNA, lncRNA, miRNA and circRNA.

The magnetic beads RNA protein pull-down capture kit provides researchers with a simplified and efficient method, using terminal biotin labeled RNA as bait to enrich proteins interacting with RNA.

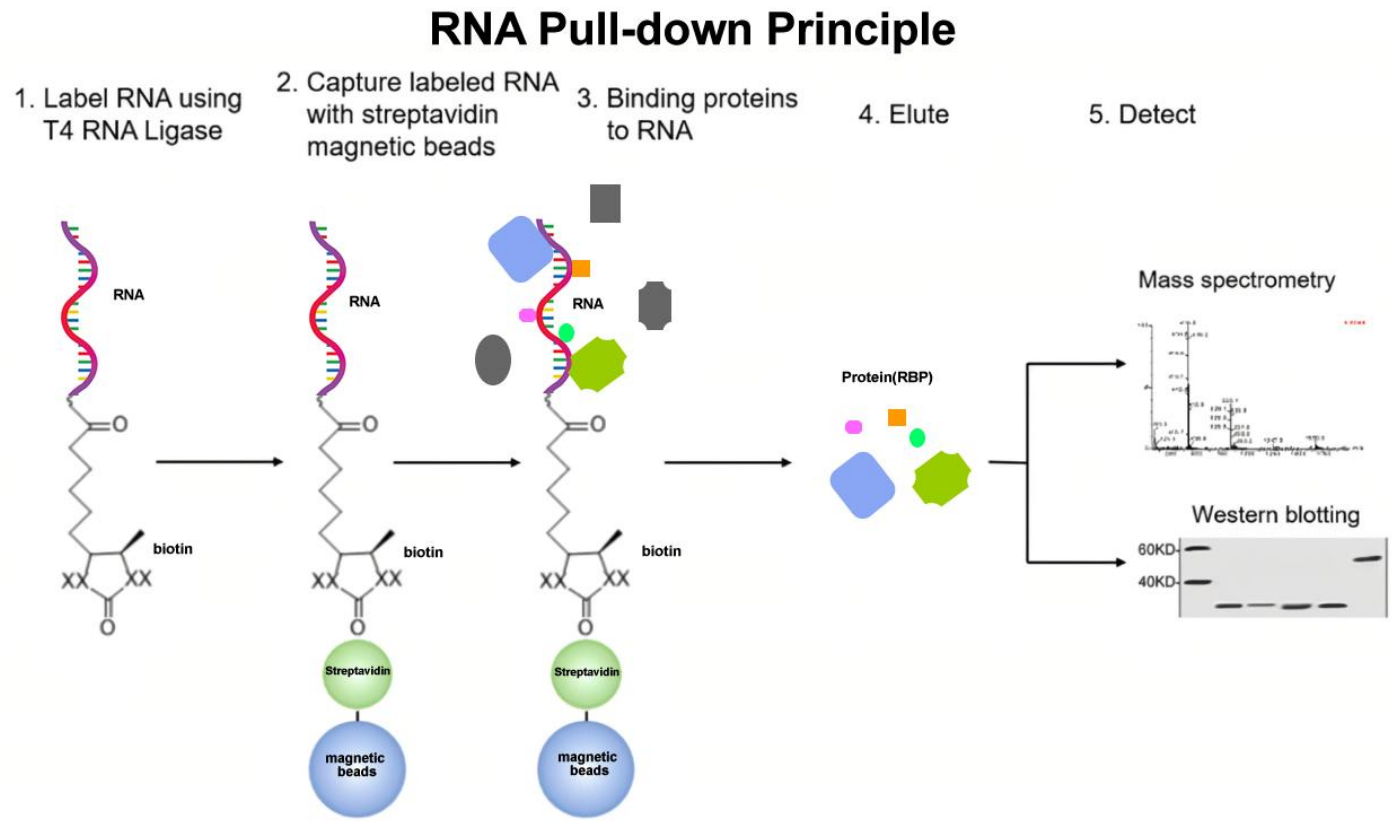
It is applicable to the interaction between RBP and mRNA, RBP and lncRNA, RBP and miRNA, RBP and circRNA.

Western blot for known proteins and mass spectrometry identification for unknown proteins.

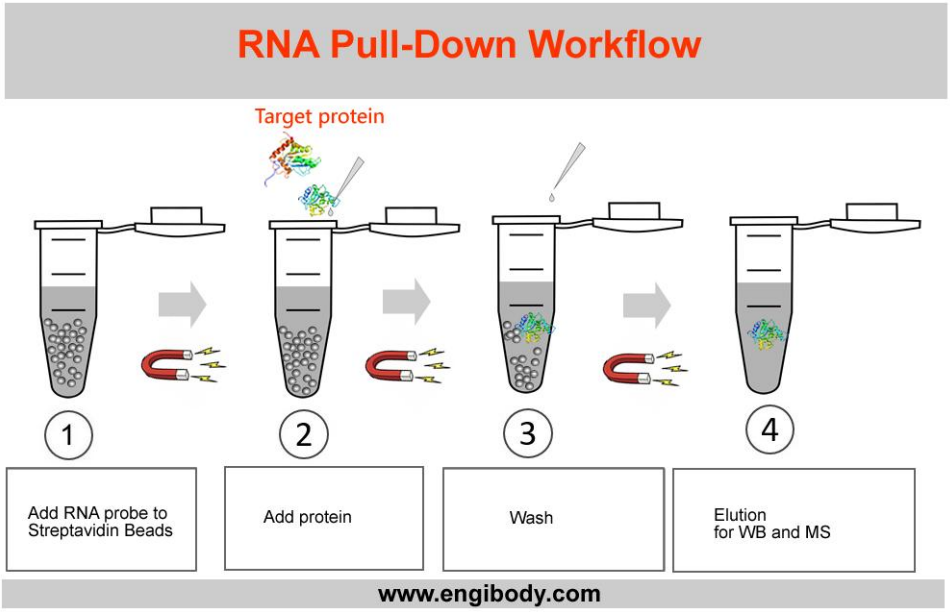
Part 2: Technical Principle and Process

Technical Principle:

When the RNA probe immobilized on the streptavidin magnetic beads is mixed with the protein sample and incubated, the protein in the sample that can bind to the RNA probe is captured by the probe and forms a protein-probe complex with the probe. After washing and elution, the target protein is obtained. For unknown proteins, mass spectrometry can be used for identification, and for known proteins, WB experiments can be used for verification.



Technical Process:



Part 3: Experimental Group and Control

Experimental grouping

Treated samples in the experimental group and Control samples in the control group

In the experimental design, a minimum of one group each for the treated group and the control group should be set up, with a minimum of three biological replicates per group. Based on the varying drug concentrations or treatment durations in the experimental groups, multiple experimental groups with different gradients can be established.

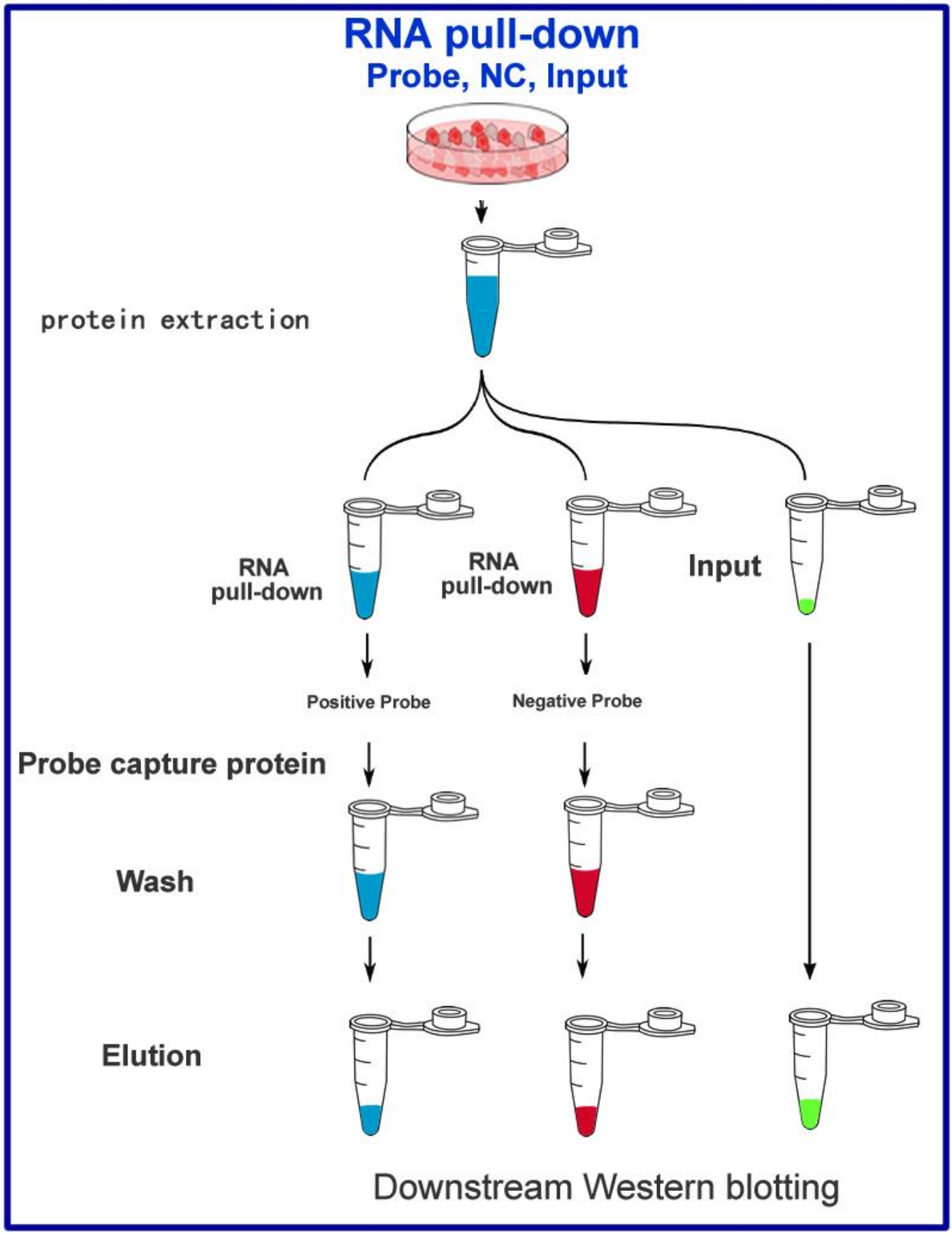
Control

1. Negative control:

It is necessary to set up a negative probe, which can be a mutant probe, an anti-sense probe, or a poly-A probe.

2. Input control:

It is necessary to set up a protein sample that does not undergo RNA probe-protein binding, washing, elution, and other pull-down steps, and is directly used for downstream WB


RNA pull-down
Probe, NC, Input
protein extraction
RNA pull-down
RNA pull-down
Input
Positive Probe
Negative Probe
Probe capture protein
Wash
Elution
Downstream Western blotting

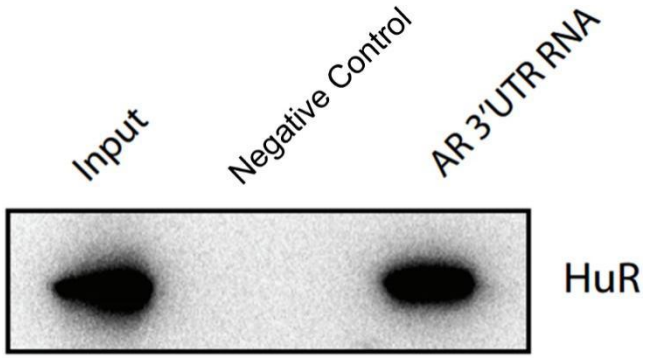
Part 4: RNA Pull-down Results Display

The most classic results of RNA and RBP interactions are shown below:

Two RNA probes are required for each target protein. For example, the RNA binding protein HuR can bind to the Androgen receptor 3′-UTR RNA, so each set of probes contains two probes, as shown below:

A. Biotin-labeled wild-type AR 3′-UTR RNA probe
5′-CUGGGCUUUUUUUUUCUCUUUCUCUCCUUUCUUUUUCUUCUUCCCUCCCUA-3′
250pmol (in 25μL)

B. Poly-A negative control probe
poly(A)25 RNA, 250pmol (in 25μL)



RNA Pull-Down and WB assay of the HuR example

Part 5: Components of RNA Pull-down Kit

ITEM	NAME	SIZE		NOTE
The following components need to be stored at 4 ° C upon receipt.				
IF9304	Streptavidin Magnetic Beads Note: The surface of Streptavidin magnetic beads has been pre coated with nucleic acid oligo, which can minimize non-specific binding to the probe to the greatest extent possible	1 mL	+4 °C	Magnetic beads should not be frozen or dried. Do not centrifuge at high speed
R-pull-002	1×RNA Capture Buffer	5 mL	+4 °C	
R-pull-003	1×Magnetic Beads Wash Buffer	20 mL	+4 °C	
R-pull-004	10×Protein-RNA Binding Buffer	1 mL	+4 °C	
R-pull-005	R-pull 1×Wash Buffer	15 mL	+4 °C	
R-pull-006	R-pull Elution Buffer (Denaturing)	1.5 mL	+4 °C	
R-pull-007	R-pull Elution Buffer (non-denaturing, solution A)	4 mL	+4 °C	
R-pull-008	R-pull Elution Buffer (non-denaturing, solution B)	0.5 mL	+4 °C	
The following components need to be stored at RT upon receipt.				
IF9217	QuickStain™ One-Step Protein Gel (PAGE) Blue Stain	50 mL	RT	
The following components need to be stored at -20 ° C upon receipt.				
R-pull-010	DNase I（Enzyme and 10×Buffer）	100 U	-20 °C	Used for the removal of DNA from cell lysate
R-pull-011	RNase Inhibitor	50 μL	-20 °C	
R-pull-012	tRNA (10 mg/mL)	50 μL	-20 °C	
R-pull-013	50% Glycerol	500 μL	-20 °C	
R-pull-014	R-pull Loading Buffer（5×）	1 mL	-20 °C	For SDS-PAGE
AT0098	Goat anti mouse Conjugated HRP	100 μL	-20 °C	For WB
AT0097	Goat anti rabbit Conjugated HRP	100 μL	-20 °C	For WB

Part 6: Required Instrument and Consumables

The instrument, consumables and reagents listed below are to be provided by the user

General Reagents

Name	Brand	Cat no	Note
NE-PER™ Nuclear and Cytoplasmic Extraction Reagents	Thermo	78835	For protein preparation
DEPC-treated Water			It can prevent the degradation of RNA probes
Protease Inhibitor Cocktail	ROCHE		
Short-strand RNA probe (3' terminal biotin labeling)		custom synthesis	For miRNA probe
Long-sttrand RNA probe (prepared by T7 in vitro transcription)			For mRNA probe and lnc RNA probe
T7 in vitro transcription and biotin labeling kit	Engibdoy	IF9670	For RNA in vitro transcription and biotin labeling
Ready-to-use Bradford protein assay kit (including ready-to-use standard BSA, compatible with detergent)		ZJ104	Faster than the BCA method, incubate at room temperature for 3-5 minutes
Biotin 3' End RNA Labeling Kit (T4 RNA Ligase + Cytidine-5'-diphosphate)	Engibdoy	IF9504	For RNA biotinylation (length range 22 - 450 nt)
Biotin 3' End RNA Labeling Kit (T4 RNA Ligase + Cytidine-5'-diphosphate)	Thermo	20160	For RNA Biotinylation (length range 22 - 450 nt)

Instrument and Consumables

Name	Brand	Cat no	Note
Cell scraper			
Doune's glass homogenizer	Sigma	D9063	
Cell Strainer	fisher		70 μm
Orbital shaker	SCILOGEX		
Vortex mixer			

rotating mixer			
micro high-speed freezing centrifuge			
Low Speed Centrifuge			
thermostatic metal bath			
ultraviolet photometer	Nanodrop		
Chemiluminescence imaging instrument		6100	
Vertical electrophoresis tank	WIX		
Transfer electrophoresis tank	WIX		
RNase-free centrifuge tube			
RNase-free tips			

Experimental Protection:

- Please wear lab coats, masks and gloves during the experiment to avoid inhaling reagents.
- Protease Inhibitor Cocktail is toxic, avoid inhalation and skin contact.
- When preparing DEPC water, it is important to note that DEPC itself is a potential carcinogen and has strong irritation to the eyes and airway mucosa. Therefore, appropriate safety measures are required when preparing DEPC water.

Part 7: RNA Pull-down Protocol

Three keys for successful RNA Pull-down

1、To prevent the degradation of RNA probes by exogenous and endogenous RNA enzymes, it is necessary to wear masks, gloves, and hats throughout the entire process and frequently change them. All glassware must be baked in a dry oven at 200°C for more than 2 hours. Materials that cannot be baked, such as plastic, need to be treated with DEPC water and then cleaned with nuclease-free water.All reagents or devices used for RNA should be designated for RNA use only, and an independent RNA experimental operation area should be established.

Ubiquitous RNA enzyme

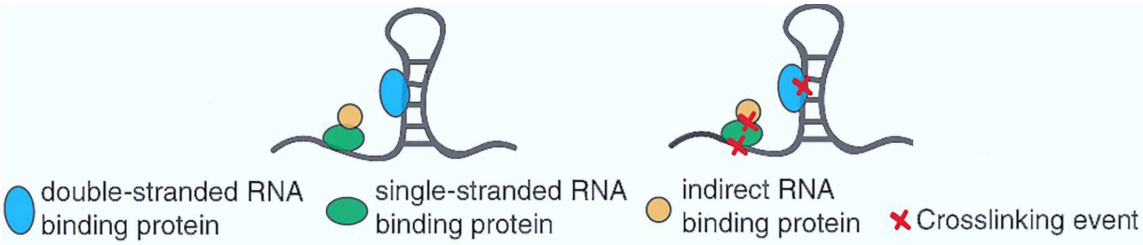
RNase is an endonuclease that hydrolyzes RNA, mainly cutting the phosphodiester bond between nucleotides.RNAse molecules are very stable, with disulfide bonds in their structure, and their activity does not require the presence of divalent cations. Therefore, RNAse is not prone to denaturation, and even after high temperatures or the use of denaturants, it is prone to renaturation.RNA enzymes are divided into endogenous and exogenous types, with endogenous RNA enzymes being released simultaneously when cells rupture. Therefore, eliminating the effects of endogenous RNA enzymes is a crucial step in RNA experiments.Exogenous RNA enzymes are widely distributed, and RNA enzymes are present in the air, human skin, hair, and saliva, which is an important reason for the degradation of RNA.

2、Principles for the Design and Optimization of RNA Probe Sequences

1） Selection of probe RNA sequence: Different types of RNA have different requirements for selecting target binding sequences. Select RNA sequences that contain RBP protein binding sites (such as the iron response element IRE in the 5'UTR or 3'UTR region of mRNA molecules).

2） Double-stranded or single-stranded RNA probes: If the binding of RBP protein to RNA occurs on double-stranded RNA, the designed RNA probe should also pay attention to the formation of double-stranded secondary structure in the target region.

The following figure shows three ways of RNA and protein binding: binding to the single-stranded region of RNA, binding to the double-stranded region of RNA, and indirect binding to RNA.



3） Probe sequence length: The length of RNA probes should be determined based on the type of target RNA. miRNA probes are usually full-length sequences ranging from 22 to 30 base pairs.The lncRNA probes and mRNA probes are longer and vary in length.

4） GC content: The GC content should be between 40-60% to ensure the stability and solubility of the probe.Excessively high or low GC content may affect the specificity and binding efficiency of the probe.

5） Probe purity: The probe should have high purity to avoid non-specific binding and background interference.The probe can be purified by HPLC or PAGE.

3、Different preparation strategies for different types of RNA probes

1） miRNA probes are usually full-length sequences, ranging from 20 to 30 base pairs.It can be produced through chemical synthesis, and then labeled with biotin using T4 RNA ligase.

The information of the labeling kit used is as follows:

Biotin 3' End RNA Labeling Kit (T4 RNA Ligase + Cytidine-5'-diphosphate)	Engibdoy	Cat no： IF9504	For RNA biotinylation (length range 22 - 450 nt)

2） LncRNA probes and mRNA probes are relatively long, and are generally prepared using T7 in vitro transcription.

The information of the labeling kit used is as follows:

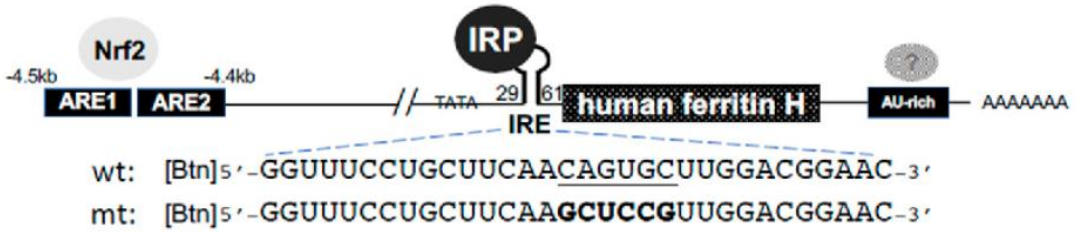
T7 in vitro transcription and biotin labeling kit	Engibdoy	IF9670	For RNA in vitro transcription and biotin labeling

Note：

If necessary, the following positive (wild type) and negative (mutant type) control RNA probes can be synthesized and labeled (the highly conserved mRNA 3' UTR or 5' UTR iron response element IRE and iron regulatory protein IRP1, IRP2 binding are the most classic RNA sequence and protein binding interactions.)

Biotinylated IRE RNA, 50μL, 125nM

The sequence is shown in the following



4、maintenance of natural protein conformation

If it is a cytoplasm or nuclear protein extract, it is necessary to ensure that there is not too much denaturant such as SDS during extraction to maximize the retention of the natural conformation of the protein. If protein denaturation occurs and the spatial structure disintegrates, it will be difficult to bind with RNA probes.

If it is to express purified recombinant proteins, it is necessary to select an expression system. Prokaryotic proteins can be expressed in E. coli expression systems, eukaryotic proteins can be expressed in yeast systems, and mammalian proteins are best expressed in insect or HEK293 cells, as this allows the expressed proteins to closely resemble their in vivo state, which is beneficial for the success of RNA Pull-down.

Preparation before the experiment

1、Formulate a reasonable experimental plan

Design positive probe sequences and negative control probe sequences according to the research purpose, and develop Input, NC, and wild-type probe sets.

2、Reagent Preparation

- If the downstream is a Western blotting experiment, all the instruments, reagents, and consumables for the WB experiment need to be prepared.
- If the downstream is MS mass spectrometry identification, it is necessary to prepare the instrument reagents and consumables for SDS-PAGE gel running

3、Sample preparation

- Animal cells: 20 million cells per sample (the number of cells in two 15cm culture dishes when the cells have grown to 80%-90% confluence)
- Animal viscera tissue: about 400 mg per sample, preferably fresh tissue
- Plant young tissues: about 700 mg per sample, preferably fresh tissues

Experimental Procedure:

Question:

How much protein and labeled probe is required for RNA Pull-down experiments?

Answer:

- **Protein**

For each specific binding protein and probe, the required amount of purified protein, cytosolic or nuclear protein extract is as follows:
Generally, the amount of purified protein used is between 0.5-5μg, and using crude cytoplasmic or nuclear protein extracts requires 5-20μg of protein to initially form specific complexes.

- **RNA probe**

For every 25-50μL of streptavidin magnetic beads, 50-100 pmol of biotin-labeled RNA probe is used.

Purified proteins, cytosolic or nuclear protein extracts should be stored at -80°C, and probes should be stored at -80°C to prevent degradation.

Both the probe and the binding protein should avoid multiple freeze-thaw cycles.

Step 1：Preparation of protein sample

The protein sample types for RNA Pull-down can be total protein extracted from tissues, cytoplasmic protein, nuclear protein, or purified recombinant protein. Proteins translated in vitro can also be used to test the ability of proteins to bind to RNA targets.

1.1、Extraction of cytoplasm, nuclear proteins, or expression and purification of recombinant proteins

- Adherent cultured cells and suspension cultured cells can directly use the nuclear protein extraction kit to extract cytosolic proteins or nuclear proteins. It is

recommended to use NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Product No. 78835).

- Plant and animal tissues need to be placed on ice in a clean petri dish and quickly cut into millimeter-sized pieces with small scissors. Then, they are homogenized in liquid nitrogen and extracted with a nuclear protein extraction kit to prepare for use.
  Note: Cutting tissue needs to be operated on ice for no more than 20 minutes to prevent protein degradation.

- Some proteins with low natural abundance in cells are best expressed and purified in vitro (prokaryotic proteins can be expressed in E. coli expression systems, eukaryotic proteins can be expressed in yeast systems, and mammalian proteins are best expressed in insect or HEK293 cells). After obtaining high-concentration and high-purity target proteins, RNA Pull-down experiments can be performed.

1.2、The protein concentration was determined using the ready-to-use Bradford protein assay kit (detergent compatible, product number ZJ104). The protein was added in equal amounts for subsequent experiments.The obtained protein sample should be stored in a refrigerator at -80 degrees.

Ensure that the protein concentration of the cell lysate is greater than 2 mg/mL, so that the protein abundance in the binding reaction buffer is sufficient. If high salt or detergent interferes with the binding reaction, a desalting column can be used to remove interfering substances.

Note:
The obtained protein sample needs to be divided into three parts (5% of the total volume is taken as Input, and the remaining sample is divided into two equal parts). The first part is the wild-type probe pull-down group, the second part is the negative control probe pull-down group, and the third part is the Input group.

The first and second protein samples are subjected to the subsequent pull-down step, while the third Input group sample is left at -20 degrees and will be directly used for the subsequent WB experiment.

Step 2：RNA probe preparation

- **Biotin-labeled mRNA 5′ or 3′UTR probes and lncRNA probes**

Long RNA single strands require the use of a commercial T7 in vitro transcription and biotin labeling kit (Engibody, Cat no: IF9670) to biosynthesize and complete biotin labeling.

- **Biotin-labeled miRNA probe**

miRNA probes are short, and synthetic companies can chemically synthesize biotin Labeled miRNA single strands.

- **Some popular RBP proteins have commercial RNA pull-down probes that can be purchased and used directly.**

For each target protein, two RNA probes need to be designed. For example, the RNA binding protein HuR can bind to the Androgen receptor 3′-UTR RNA, so each set of probes contains two probes, as shown below:

A、Biotin-labeled wild-type AR 3′-UTR RNA probe

B、Biotin-labeled antisense AR 3′-UTR RNA negative control probe or poly-A negative control probe

The specific sequence is as follows:

Positive RNA Control (AR 3′-UTR RNA):
250pmol (in 25μL), sufficient for five labeling and pull-down reactions
5′-CUGGGCUUUUUUUUUCUCUUUCUCUCCUUUCUUUUUCUUCUUCCCUCCCUA-3′

Negative RNA Control：
poly(A)$_{25}$ RNA, 250pmol (in 25μL), sufficient for five labeling and pull-down reactions

Secondary structure formation of RNA probes

(This step is optional and only required for probes that bind to proteins through secondary structures such as stem loops, not for probes that bind to proteins through single-stranded structures.)

1. Take wild-type RNA probes labeled with biotin and negative control probe solutions separately, and denature them in a 90 ℃ water bath for 2 minutes.
2. Put it into the PCR machine and set a slow cooling program (70 ℃ -25 ℃) to form RNA secondary structure.

Step 3：Binding of labeled RNA probe to streptavidin magnetic beads

Binding of Biotin-labeled RNA Probe to Streptavidin Magnetic Beads
Note: For every 25-50μL of magnetic beads, use 100-200pmol of RNA. The following steps use a ratio of 100 pmol RNA to 25 μL magnetic beads.

3.1、Add 25 μL streptavidin magnetic beads to a 1.5mL microcentrifuge tube.
3.2、Place the centrifuge tube in the magnetic rack, magnetically separate the magnetic beads on the side of the centrifuge tube, aspirate the supernatant and discard it.
3.3、Wash the magnetic beads with an 250 μL volume of the **Magnetic Beads Wash Buffer** in the kit. Resuspend the magnetic beads using a pipette or vortex.
3.4、Place the centrifuge tube in the magnetic rack, magnetically separate the magnetic beads on the side of the centrifuge tube, aspirate the supernatant and discard it.
3.5、Add an 100 μL volume of 1×**RNA Capture Buffer**. Resuspend the magnetic beads using a pipette or vortex.
3.6、Add 100 pmol biotin-labeled RNA probe to the magnetic beads. Gently wash and mix with a pipette tip.
3.7、 Incubate at room temperature for 15-30 minutes.

Step 4：RNA probe capture reaction for RBP protein（Pull-down Reactions）

Note: The **10×Protein-RNA Binding Buffer** in the Kit is the basic buffer used for the binding reaction.Additional reagents can also be added to the binding buffer to enhance binding affinity and specificity.

4.1、Place the 1.5 mL centrifuge tube (containing magnetic beads bound to RNA probes) from the previous step into a magnetic rack, and use magnetic separation to separate the magnetic beads on the side of the centrifuge tube. Suck out the supernatant and discard it.

4.2、Wash with an 100 μL volume of **Magnetic Beads Wash Buffer**, and resuspend the magnetic beads using a pipette or vortex oscillation method.

4.3、Repeat Washing one time.

4.4、Put the centrifuge tube into the magnetic rack, magnetically separate the magnetic beads on the side of the centrifuge tube, aspirate the supernatant and discard it.

4.5、Preparation of Pull-down (Binding Reaction) System

Prepare the Master Mix for the RNA-Protein binding reaction according to the following table:

The amount in the table is required for every 100μL reaction

Component	Final concentration	Input	Wild type	NC
10×Protein-RNA Binding Buffer	1×	——	10μL	10μL
Purified protein, cytoplasmic protein or nuclear protein extract	——	——	20-200μg	20-200μg
50% glycerol	——	——	30μL	30μL
Optional: tRNA (10 mg/mL)	0.1-10μg	——		
Optional: RNase Inhibitor		——		
If the volume is insufficient, add RNase free water to 100μL. If the protein concentration is too low, it is fine if the	——	0μL	100μL	100μL

total volume exceeds 100μL. Use all of it during pull-down				

Note:

- RNase inhibitor and tRNA are both non-essential components. When the initial results are not ideal and the experimental system for the binding reaction needs to be optimized, these two components can be selectively added, and the optimal concentration can be explored in a gradient concentration manner.

4.6、Add 100μL of the mixed binding reaction system Master Mix to the magnetic beads that have been combined with the RNA probe. Resuspend the magnetic beads with a pipette or gentle vortex.

4.7、Incubate at 25°C for 30-60 minutes with rotation. If the target protein is temperature sensitive, it can be incubated overnight at 4 °C.

Step 5： Washing of RBP-RNA-magnetic bead complex

5.1、Place the centrifuge tube in the magnetic rack, magnetically separate the magnetic beads on the side of the centrifuge tube, aspirate the supernatant, and transfer the supernatant to a new centrifuge tube for later analysis.

5.2、Wash the magnetic beads with an 100μL volume of **R-pull 1× Wash Buffer** in the kit.

5.3、Repeat steps 1 and 2 twice more. If necessary, save the washing supernatant for analysis.

5.4、Place the centrifuge tube in the magnetic rack, magnetically separate the magnetic beads on the side of the centrifuge tube, aspirate the supernatant and transfer it to another centrifuge tube for later analysis.

Step 6： Elution of RNA binding protein (RBP)

Note:

Denaturing elution was used for downstream immunoblotting (WB) and mass spectrometry (MS) identification. Non-denaturing elution is used for downstream enzyme activity detection or protein natural structure analysis.

WB and MS elution (denaturing reduction elution)

1) Prepare R-pull elution (denaturation) working solution

The usage amount for each centrifuge tube is as follows:

Take 6μL of the **R-pull loading buffer (5×)** provided with the kit, and 24μL of the **R-pull elution buffer (denaturing)** provided with the kit. Mix them evenly to obtain a working solution of 30μL.

Please perform a consolidated calculation based on the total number of pull-down tubes, and then prepare the corresponding total amount.

2) Add 30μL of freshly prepared R-pull elution (denaturation) working solution to each pull-down tube after washing. Vortex to mix, resuspend the magnetic beads, and briefly centrifuge to collect the magnetic beads. Place the centrifuge tube containing the target protein-RNA probe complex magnetic beads in a boiling water bath for 5-10 minutes to dissociate the protein, which is now reduced and denatured. Centrifuge momentarily to collect the liquid evaporated onto the tube lid and wall to the bottom of the tube.

3) Use a magnetic rack to separate the magnetic beads, and transfer the supernatant to a new centrifuge tube. This supernatant (approximately 30 μL) contains the target RBP protein. Place on ice for subsequent loading on SDS-PAGE gel for WB or mass spectrometry experiments.

Note: Boiled magnetic beads cannot be reused and should be discarded.

4) Suck 24μL from each Input tube sample that has been left aside for a long time, add 6μL of **R-pull Loading Buffer (5×)**, and obtain a 30μL sample mixture. After boiling, directly use it for SDS-PAGE gel loading in downstream WB or MS mass spectrometry experiments.

Non-denaturing elution

1) Add 50μL of **R-pull elution buffer (non-denaturing, solution A)** to each centrifuge tube, and gently resuspend the magnetic beads using a pipette tip. Incubate at room

temperature for 15-30 minutes, and then place it on a horizontal shaker for gentle circular motion.。

2） At the same time, add about 2.5μL of **R-pull elution buffer (non-denaturing, solution B)** to each new tube used to store eluates (conducting a pre-experiment to confirm how much solution B is needed to achieve a pH of 7.4 for 50μL of solution A).

3） Use a 24-well magnetic rack to magnetically separate magnetic beads, transfer the supernatant to a centrifuge tube that has been added with an appropriate amount of **R-pull elution buffer (non-denaturing, solution B)** in the previous step. Ensure that the pH is 7.4, this supernatant contains the target RBP protein captured by the RNA probe, which should be carefully stored for future use (at –20 °C. Store at –80 °C for long term storage).

Note: If the non-denaturing elution buffer provided in the Kit still cannot fully elute the protein, you can try adding an appropriate amount of RNase A

Downstream Experiment

1. Western Blot

Used for detection of known interacting proteins of RNA probes.

WB experiment is the most common immunological experiment, and conventional methods can be used to detect proteins in the eluate. Alternatively, the 3-hour Lightning WB ultra-fast kit (brand: Engibody, product number: IF9999, name: Lightning-WB 3-hour ultra-fast Western Blotting kit) can be used.

The kit only takes 3 hours to complete the whole process of WB experiment, from sample running to exposure and development, including PAGE preformed gel, rapid electrophoresis buffer, rapid membrane transfer buffer, rapid blocking buffer, rapid antibody incubation buffer, and ultra-sensitive ECL luminescent buffer. It contains a complete set of key reagents for WB experiments, all of which have been specially developed with unique formulations and superior performance.

2. Mass-Spectrometry

Used for screening and discovering unknown interacting protein profiles of specific RNA probes

1、SDS-PAGE

Denaturing reduction electrophoresis.

2、Rapid staining

Use the QuickStain ™ One-Step Protein Gel (PAGE) Blue Stain(in the kit), a rapid staining solution for protein PAGE gel (compatible with mass spectrometry), is used for staining to visualize protein bands.

3、Gel cutting (cutting the gel strip of the target band)

4、Send the gel strip to the MS technology service company for mass spectrometry.

Part 8： Troubleshooting

Trouble	Cause	Solution
The RNA probe has been degraded	1. The environment without nuclease cannot meet the requirements	Clean the experimental area to ensure that all containers and plastic consumables are RNase free
	2. The probe has been degraded	The integrity of RNA molecules is determined by RNA electrophoresis, and the measured value of Nanodrop

Trouble	Cause	Solution
		cannot reflect the integrity of RNA molecular structure
Low-efficiency binding of RNA-binding protein	1. There are problems in the binding reaction system	Optimize the RNA-RBP binding reaction system from the aspects of incubation time, temperature, salt concentration, and detergent content
	2. Insufficient streptavidin magnetic beads	Increase the usage of magnetic beads
	3. Insufficient amount of RNA probe	Increase the usage of probes
	4. Insufficient binding of RNA probe and magnetic beads	Optimize the binding reaction conditions of probes and magnetic beads
RNA-binding protein not bound	1. The target protein in the sample is absent or insufficient in content	Ensure that the protein content is sufficient and use concentrated protein samples
	2. The protein sample and the binding reaction system are not compatible	Use a desalting column to replace the buffer solution of the protein sample
	3. The binding reaction	Optimize the RNA-RBP

Trouble	Cause	Solution
	conditions is not suitable	binding reaction system from the aspects of incubation time, temperature, salt concentration, and detergent content
	4. The binding of RNA-RBP is too fragile	After the binding reaction, a UV crosslinking instrument can be used for crosslinking to immobilize RNA-RBP
High non-specific binding of RNA-binding protein	1. There are problems in the binding reaction system	Optimize the RNA-RBP binding reaction system from the aspects of incubation time, temperature, salt concentration, and detergent content
	2. Insufficient washing	Increase the time and frequency of washing steps, and increase the salt content and detergent content in the washing buffer
	3. Too much protein	Reduce the amount of protein used in the binding reaction
There may also be problems in the WB process	The performance of the primary antibody	Whether the primary antibody can effectively recognize the target and whether there are

Trouble	Cause	Solution
		non-specific binding bands
	Sensitivity of the luminescent liquid	Replace with a higher sensitivity ECL
	Whether the membrane transfer is completed	Adjust the formula and time of the transfer solution

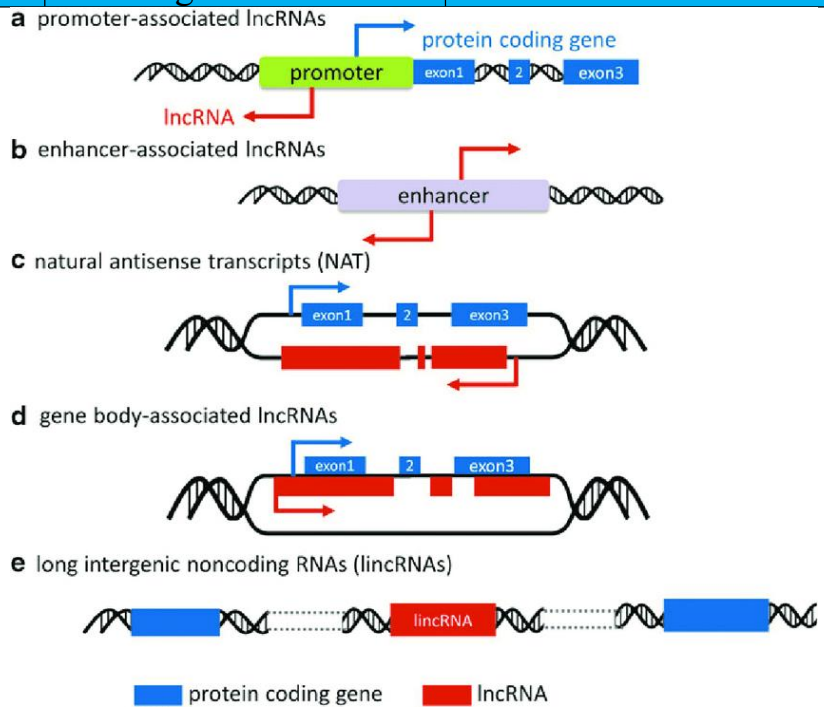
Knowledge of RNA and RBP

Ribonucleic acid (RNA) exists in biological cells, some viruses, and viroids, with a wide variety of types and complex functions. It is generally divided into two categories: coding RNA and non-coding RNA, depending on whether it encodes proteins. Coding RNA usually refers to mRNA, while non-coding RNA includes many types, which can be divided into non-coding large RNA with a length greater than 200 nt, non-coding small RNA with a length less than 200 nt, and special non-coding circRNA.

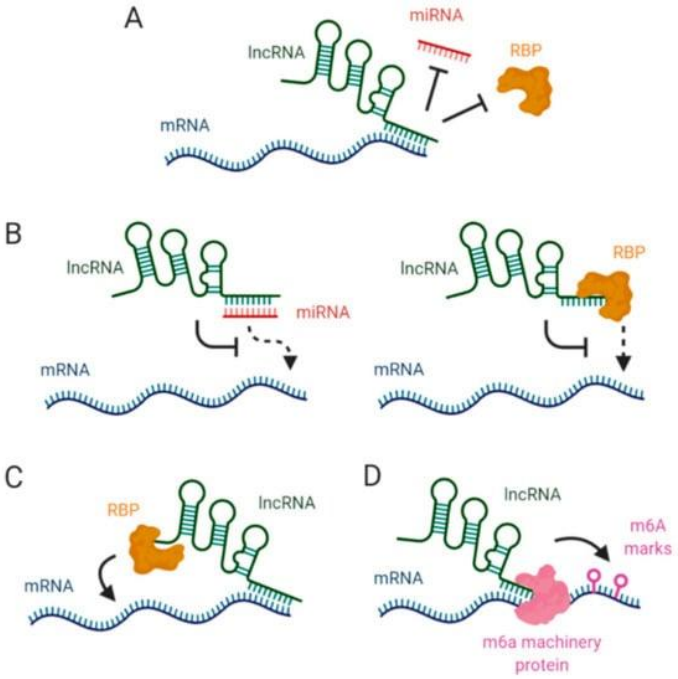
Most RNA molecules have a short life cycle, existing for only a few days within cells, with only a very small number of RNA molecules persisting for several months or even up to two years.A recent study by the German Center for Neurodegenerative Diseases (DZNE) found that in some cell types of the mouse brain, certain RNAs can exist for at least 2 years, which means that they are basically stable throughout the entire life cycle of the mouse after birth.

Summary of RNA Types		
TYPE	NAME	LENGTH
Coding RNA	mRNA	500 ~ 5000 nt
House-keeping non-coding RNA	rRNA	120 ~ 4500 nt
	tRNA	76 ~ 90 nt
	scRNA	100 ~ 300 nt

	snRNA	100 ~ 300 nt
	snoRNA	60 ~ 400 nt
	tiRNA	29 ~ 50 nt
	tRF	16 ~ 28 nt
	TERC	451nt
Regulatory non-coding RNA	lncRNA	greater than 200 nt
	miRNA	21 ~ 23 nt
	circRNA	100 ~ 10000 nt
	eRNA	50 ~ 2000 nt
	piRNA	26 ~ 32 nt
	hnRNP	600 ~ 700 nt
	Endogenous siRNA	20 nt

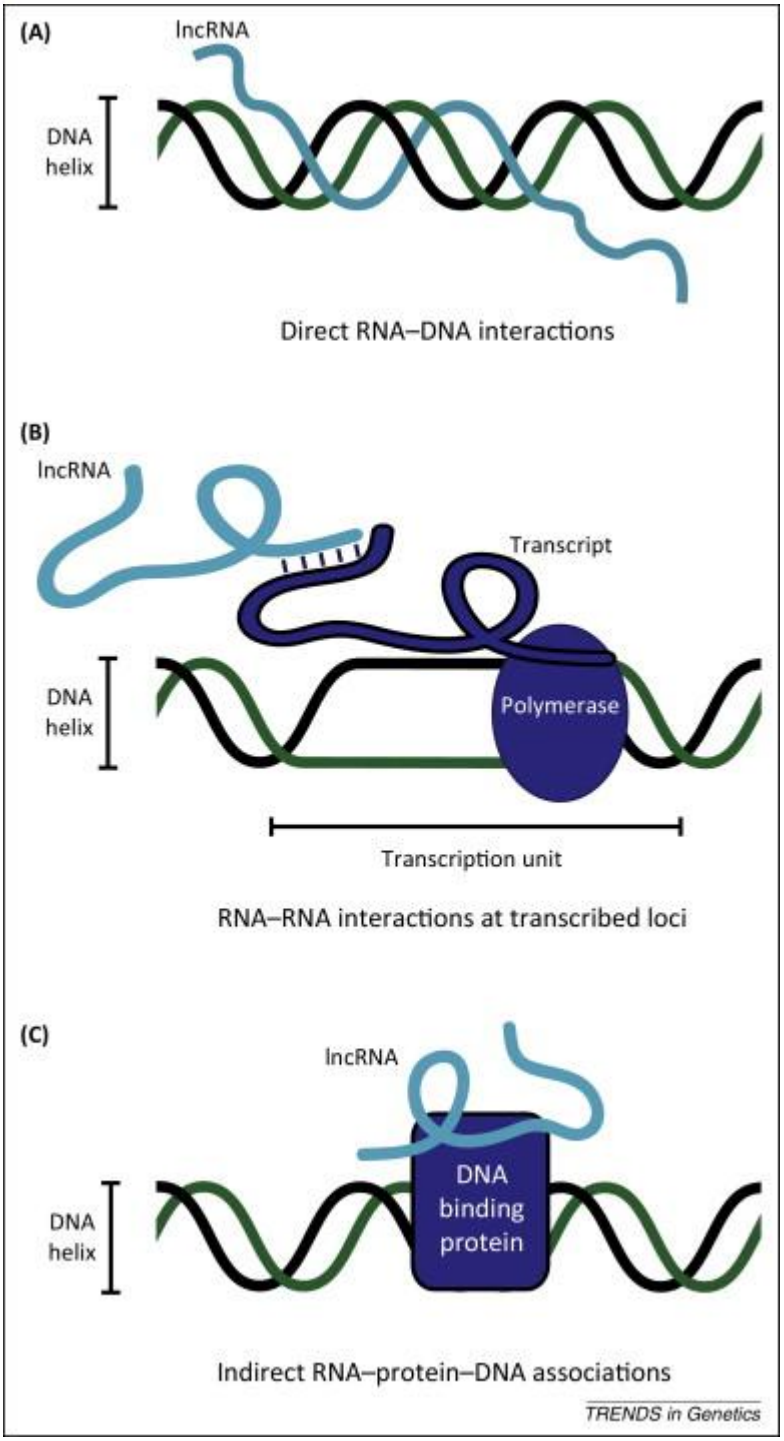


Classification of long noncoding RNA (lncRNA)

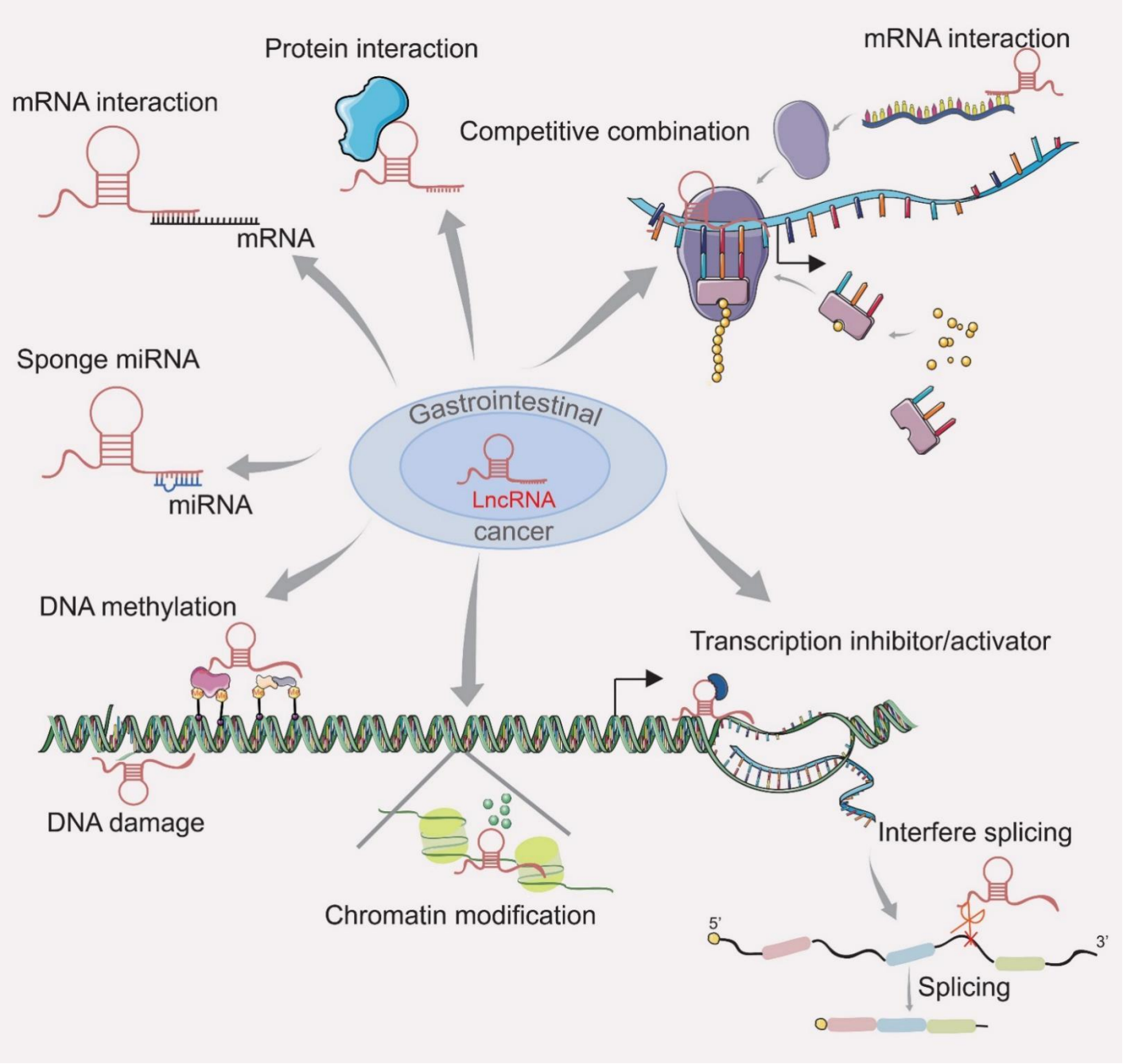


Mechanism of lncRNA-mediated regulation of mRNA stability

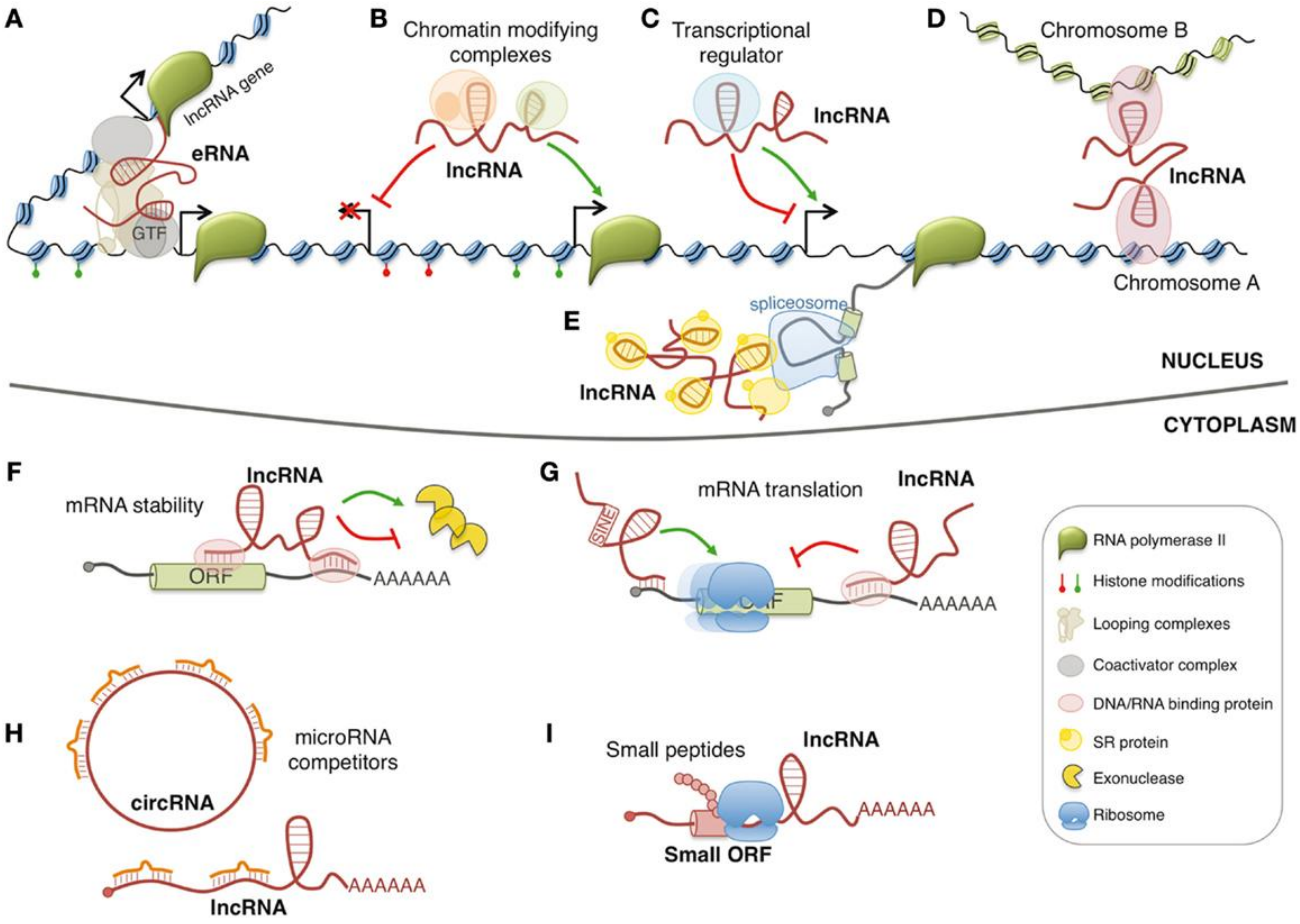
lncRNAs can regulate the stability of mRNA through different mechanisms: (A) direct interaction with miRNA or RBP binding sites in target mRNA;(B) Blocking miRNA or RBP to avoid their interaction with mRNA molecules;(C) Scaffold for enhancing RBP mRNA interaction;(D) Interact with the m6A mechanism to regulate the m6A level of target mRNA.



Different modes of lncRNA binding to chromatin

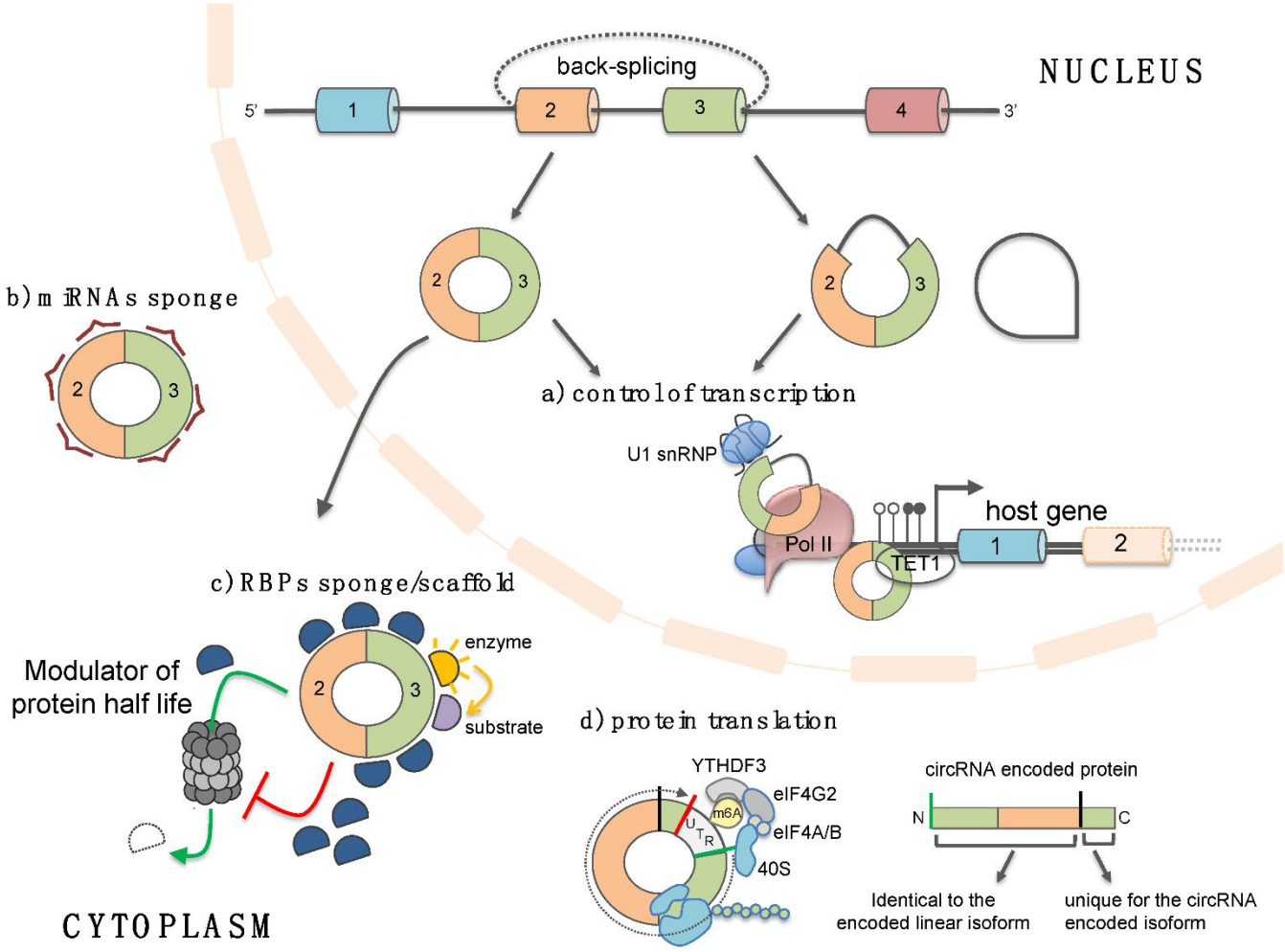


The role of lncRNA in gastrointestinal tumors



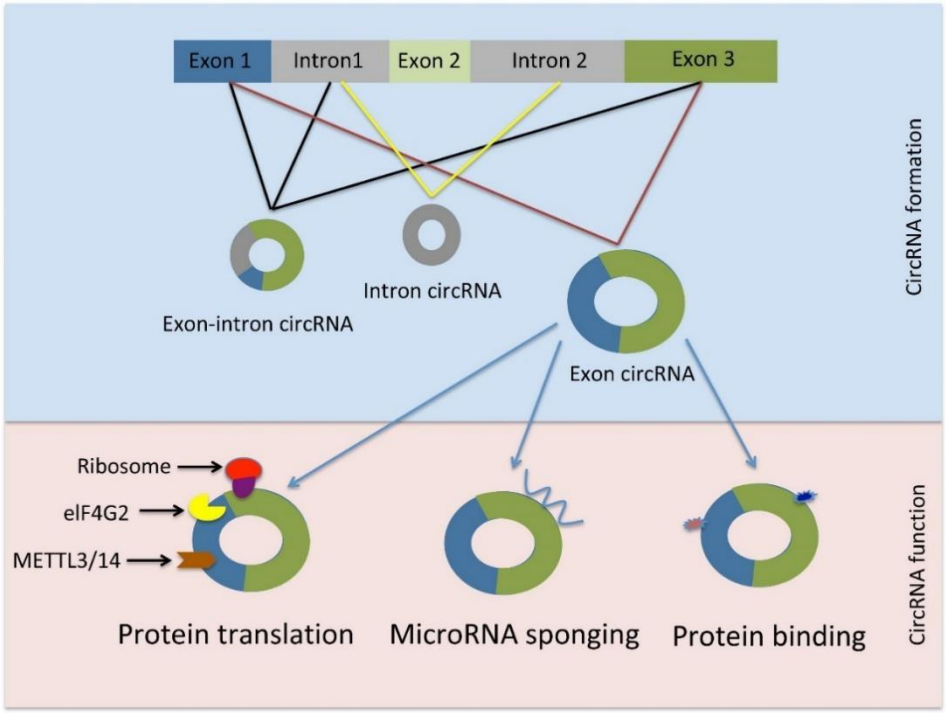
Functional role of lncRNA

Nuclear lncRNAs can regulate transcription by acting as enhancer RNA (eRNA) (A), by recruiting chromatin modifying complexes (B), or by regulating transcription factors activity (C). Moreover, they can regulate gene expression by acting on the spatial conformation of chromosomes (D) or by influencing pre-mRNA splicing (E). Cytoplasmic lncRNAs can regulate mRNA expression by regulating mRNA stability (F), mRNA translation (G), or by competing for microRNA binding (H). In addition, few lncRNAs contain small open reading frames (ORFs) that can be translated in biological active small peptides (I).



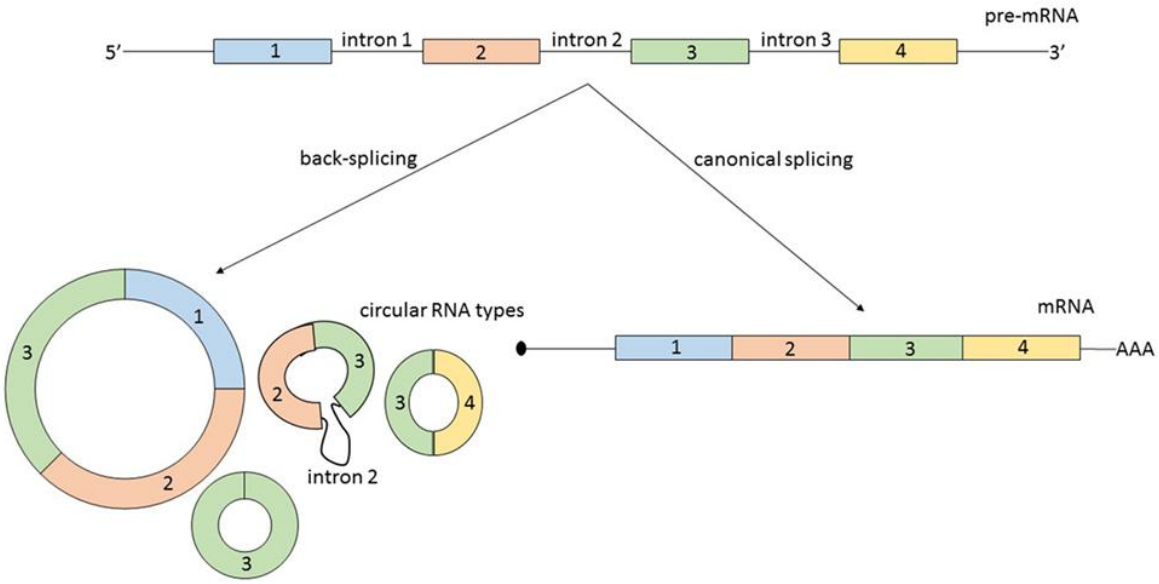
Production and function of circRNA

CircRNAs located in the nucleus can interact with U1 small nuclear ribonucleoprotein (U1-snRNP) and enhance the function of RNA polymerase II (Pol-II) complex, or recruit methyl cytosine dioxygenase TET1 to the promoter region (a) as a regulatory molecule for the transcription of its host gene. When exported into the cytoplasm, circRNA can act as a sponge or bait for microRNAs and RBPs, or regulate the half-life of specific RBPs, either antagonizing (red T-line) or facilitating their proteasome-mediated degradation (green arrow) (b, c). CircRNA has also been shown to function as a protein scaffold (c).By promoting the co-localization of enzymes and their substrates, the reaction kinetics can be enhanced (yellow arrow).Finally, circRNAs with internal ribosome entry site (IRES) elements and AUG sites (green lines) can be translated by CAP-independent mechanisms (dashed arrows; red lines indicate STOP codons).The latter is promoted by the presence of methyladenosine (m6A) and the involvement of the reader proteins YTHDF3 and IRES-specific translation initiation factor eIF4G2 (d).Protein isoforms produced by circRNA translation will have the same partial primary sequence as the linearly encoded protein, while the remaining portion of the polypeptide is unique to the circRNA-encoded isoform.

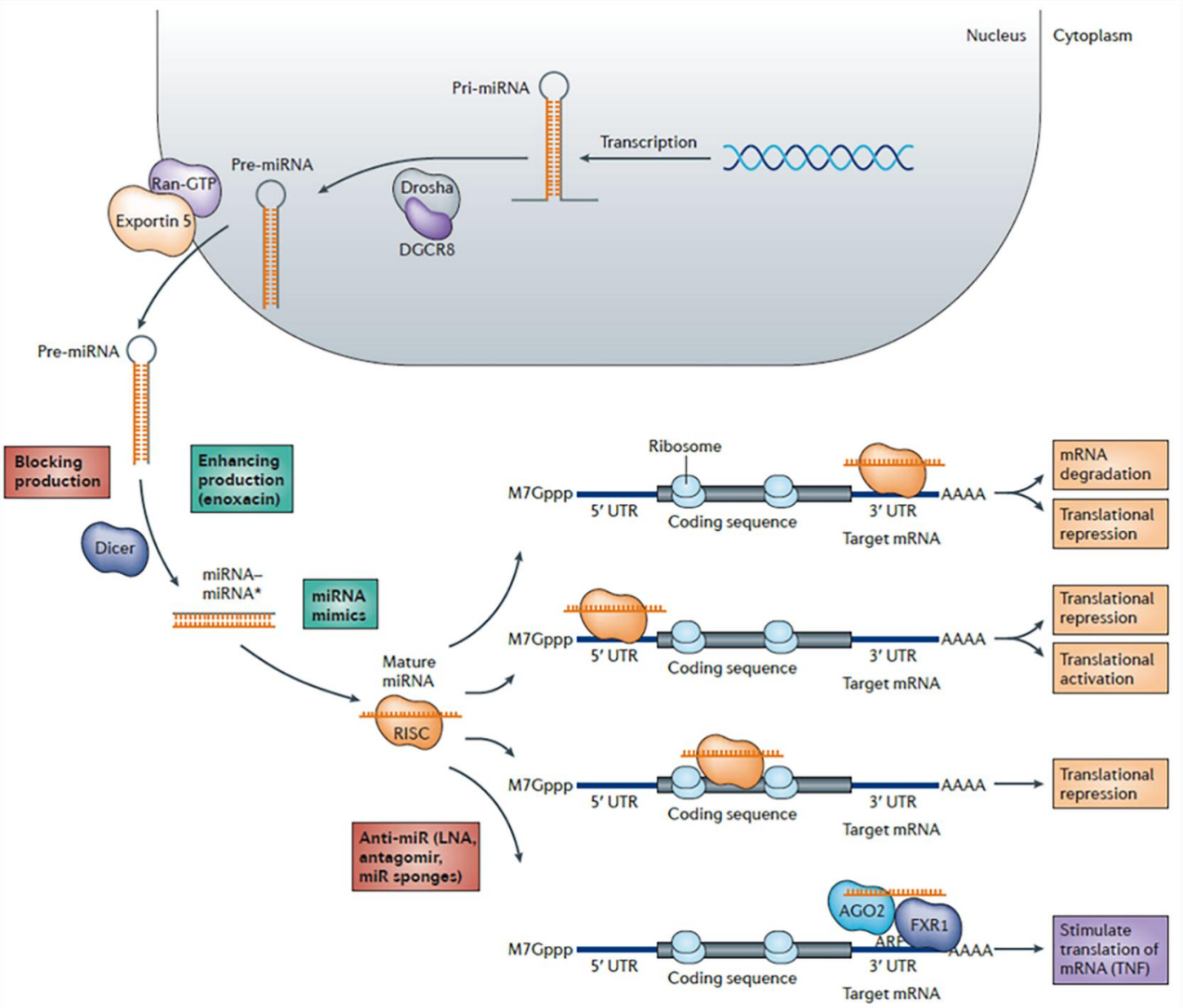


Exonic circRNAs are exported into the cytoplasm and perform multiple functions

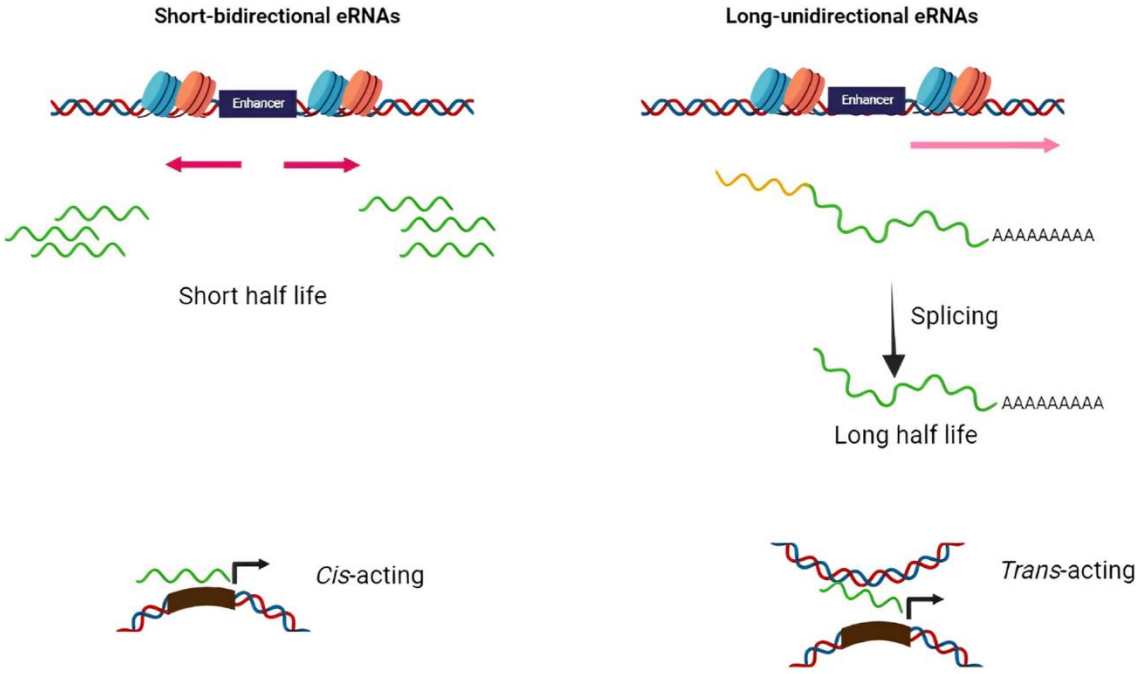
elF4G2, initiation factor eukaryotic translation initiation factor 4 gamma; METTL3, methyltransferase-like 3; METTL14, methyltransferase-like 14.



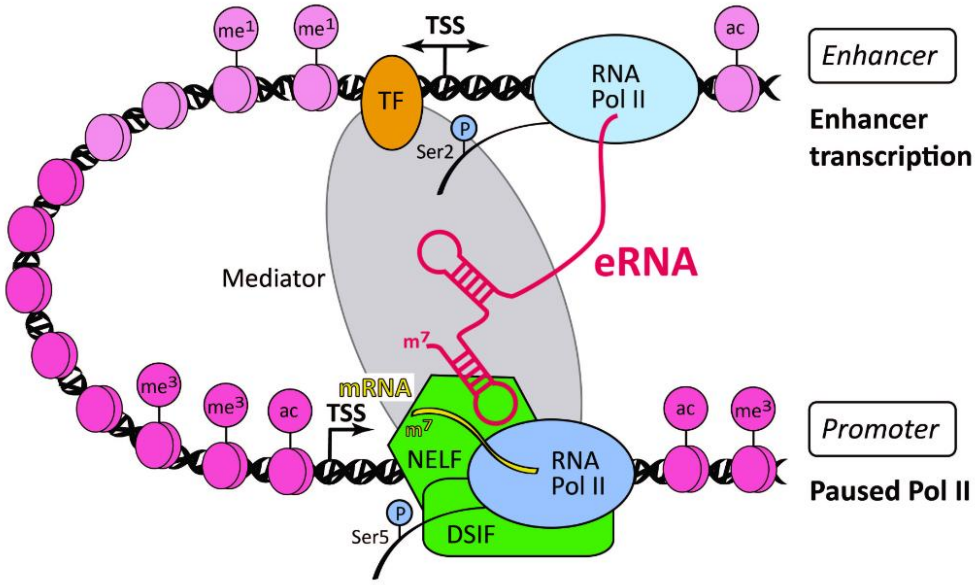
circRNAs are produced from pre-mRNA through back-splicing



The intracellular formation of miRNA and its four regulatory modes on target gene mRNA



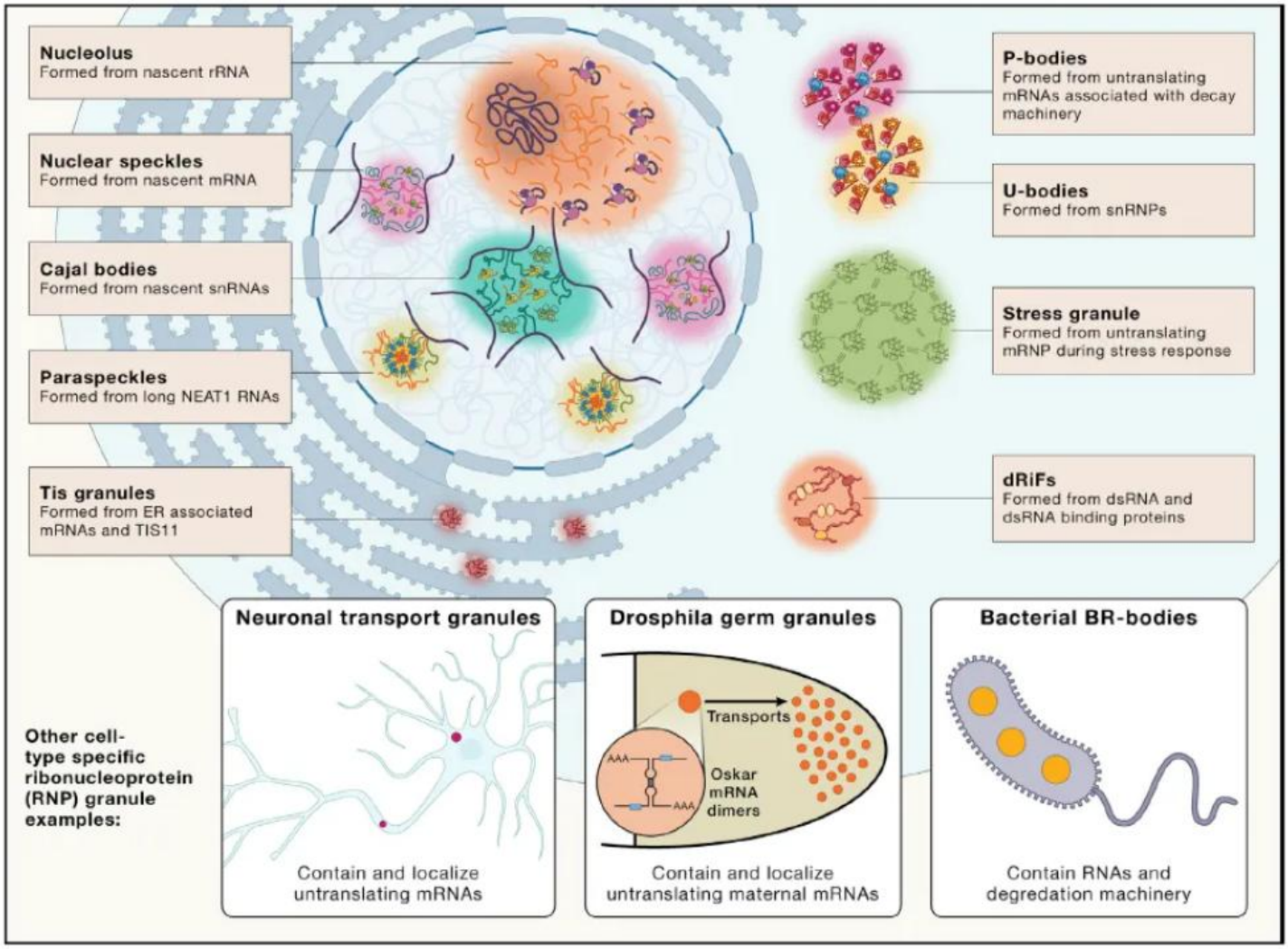
The production method of RNA (eRNA) obtained by enhancer transcription



Transcriptional regulation function of enhancer RNA (eRNA)

The above-mentioned are regulatory RNA-RBP complexes, which are temporary and easily dissociated. They are produced for various regulatory purposes. The following is a structural RNA-RBP complex, also known as RNP particles, which exist in cells for a long time. Nina Ripin and Roy Parker from the University of Colorado wrote a review in the journal Cell titled Formation, function, and pathology of RNP granules, which summarized the types, production methods, and functional roles of this RNP granules.

This paper is well worth reading. The full text link is as follows:
https://www.sciencedirect.com/science/article/abs/pii/S0092867423010279



Types of ribonucleoprotein (RNP) granules

Postscript

List of Epigenetics and biomolecule Interaction Research Kits

Interaction between chromatin and chromatin

Cat no	Name	Application
HI-C-1001	Hi-C kit	Chromatin conformation capture, three-dimensional genome

Study on accessibility of chromatin

Cat no	Name	Application
ATAC-1001-12	ATAC-Seq kit	chromatin accessibility

Interaction between RNA and chromatin

Cat no	Name	Application
ChIRP-2002	ChIRP kit	lnc RNA and chromatin interaction in vivo

Interaction between protein and DNA

Cat no	Name	Application
CUT-RUN-20000-12	UNICUT™ CUT&RUN kit	In situ CUT&RUN
CUT-Tag-40000-12	UNICUT™ CUT&Tag kit	In situ CUT& CUT&Tag
ChIP-1001	Smart-ChIP™ ChIP kit	Classic ChIP
IF9501	EMSA kit	Detection of In vitro DNA-protein interaction
IF9606	DNA Pull-Down Kit	Capture of In vitro DNA-protein interaction
LIB-SEQ-30000-12	Smart-Lib™ DNA library Prep Kit for illumina® (ultrasonic and MNase methods)	DNA library Prep Kit，for ChIP-seq, CUT&RUN
TNLB1001	Smart-Lib-Tn5™ DNA library Prep Kit（Tn5 transposase method)	
LIB-SEQ-30001	Multiplex Oligos for Illumina® (Full Length Adaptors) (for ChIP-seq, CUT&RUN)	
LIB-SEQ-30002	Multiplex Oligos for Illumina® (Unique Dual Indexed Primers)	

	(for ChIP-seq, CUT&RUN)	

■ Interaction between protein and protein

Cat no	Name	Application
IF9058	CoIP kit	protein interaction in vivo
IF9604	GST Pull-Down kit	In vitro protein interaction
IF9602	His Pull-Down kit	In vitro protein interaction
IF9706	Transient CoIP kit (Proximity labeling)	Transient protein interaction in vivo
ENGI-1000	In Cell CoIP Kit (FRET)	Real time interaction of proteins in living cells

■ Interaction between protein and RNA

Cat no	Name	Application
RIP-1001	RIP kit	RNA and protein immunoprecipitation
IF9502	RNA EMSA kit	Detection of In vitro RNA-protein interaction
IF9608	RNA Pull-Down kit	Capture of In vitro RNA-protein interaction
LIB-SEQ-35000-12	Smart-Lib™ RNA library Prep Kit for Illumina® (for RIP，MeRIP and conventional transcriptome sequencing)	RNA library construction kit

■ RNA and DNA interactions

Cat no	Name	Application
DR-IP-1001	DR-IP kit（S9.6 antibody）	Research techniques for the interaction between DNA and RNA to form R-loop

■ Biotin-labeled nucleic acid probe technology

Cat no	Name	Application
IF9503	DNA 3' end Biotin labeling kit	Terminal labeling of short-strand and long-strand DNA (single-stranded and double-stranded with a 3' overhang)
IF9504	RNA 3' end Biotin labeling kit	Terminal labeling of RNA（22-450nt)
IF9670	T7 in vitro RNA transcription and Biotin Labeling kit	Long-strand T7 in vitro RNA transcription and

		biotin labeling

DNA methylation research

Cat no	Name	Application
DIP-1001	meDIP kit	m5C or hm5C DNA methylation immunoprecipitation, without antibody

RNA methylation research

Cat no	Name	Application
meRIP-1001	meRIP kit	General kit without antibody
meRIP-1002	m6A meRIP kit	m6A Methylated RNA immunoprecipitation
meRIP-1003	m5C meRIP kit	m5C Methylated RNA immunoprecipitation
meRIP-1004	m7G meRIP kit	m7G Methylated RNA immunoprecipitation

After ten years of meticulous development, we have established the gold standard for epigenetic research tools!