RNA pull-down Kit

Catalog# JKR23004

Instruction Manual (For Two Groups)

Sufficient reagents for 6T RNA pull-down assays per kit.
Store at -20 & 4°C

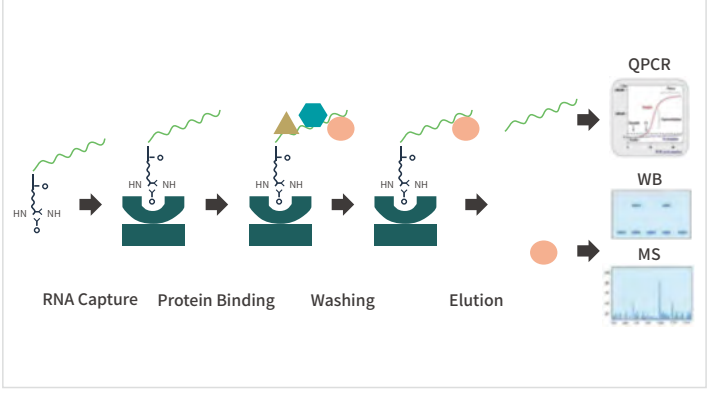
Table of Contents

1.Experimental Principle

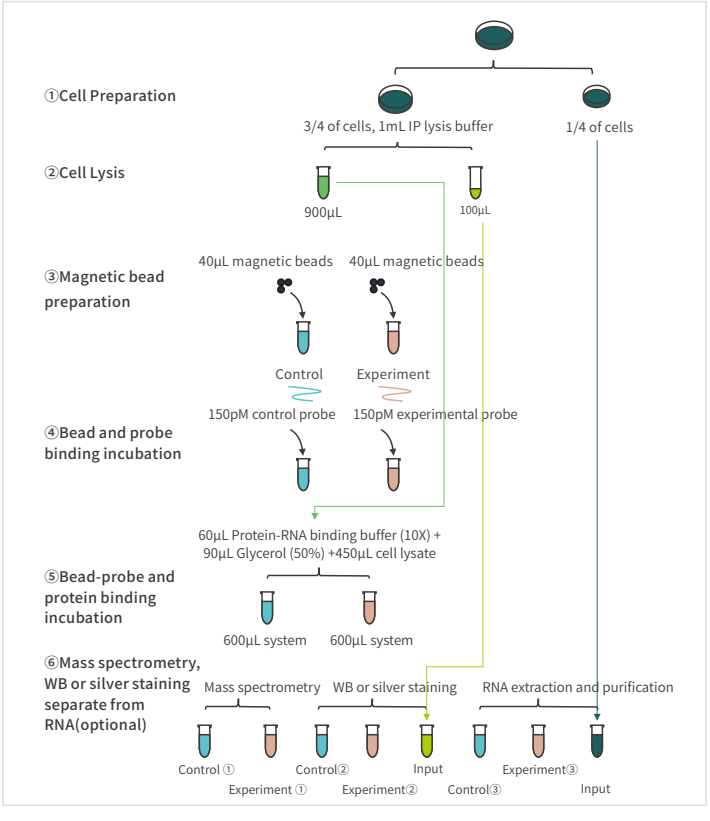
RNA pull-down is a technique used to study RNA-protein/RNA interactions within cells. First, the RNA is labeled (e.g., with biotin), then incubated with cell lysate to form RNA-RNA/protein complexes, which are subsequently used to detect bound RNA or proteins. After elution of the complexes, methods such as quantitative PCR (RNA pull-down-qPCR) or high- throughput sequencing (RNA pull-down-seq) are employed to identify whether the target RNA interacts with certain RNA molecules, while Western blot (pull-down-WB) and mass spectrometry (pull-down-MS) techniques are used to detect whether the target RNA interacts with specific proteins.

2.Experimental Procedure

2.1 Experimental Flowchart



2.2 Control Setup Flowchart



3.Kit Components

Component	Volume (6T)	Storage Temperature
Nucleic-Acid Compatible Streptavidin Magnetic Beads	270μL	4°C
RNA binding buffer	6mL	4°C
Wash Buffer I	20mL	4°C
Protein-RNA binding buffer (10X)	600μL	4°C
IP lysis buffer	9mL	4°C
Protease inhibitor (100 X)	35μL	-20°C
Glycerol (50%)	800μL	4°C
Wash Buffer II	10mL	4°C
Solution I	600μL	4°C
Elution Buffer	800μL	4°C protected from light
DEPC water	400μL	4°C

Special note 1: Please prepare reagents such as 75% ethanol, Trizol, chloroform, isopropanol, 1x PBS, reverse transcription kit, and fluorescent dye yourself.

Special note 2: 6T refers to 6 single-group (1 experimental group or 1 control group) immunoprecipitation experiments; the subsequent steps include one experimental group and one control group, consuming 2T of reagents.

4.Operating Steps

4.1 Sample lysis

4.1.1 Cell Samples

① Lysis buffer preparation: Take 990 μL of IP lysis buffer, add 10 μL of Protease inhibitor (100X), and mix well;

② Collect 2x10^7 cell samples, add 2 mL of PBS to wash the cells, centrifuge and discard the supernatant to collect the cell pellet, reserve 1/4 of the cell sample for subsequent RNA extraction and purification in the Input group (store in nuclease-free EP tubes at -80°C);

③ Add lysis buffer to the remaining cell pellet, resuspend, and pipette up and down 10 times to ensure thorough cell lysis. Incubate on ice for 30 min , vortexing every 5 min

for 10 sec each time.

④ After lysis, perform ice-bath sonication for 5 min using an ultrasonic cell disruptor at 20% power, with 3 sec of sonication and 3 sec of intervals.

⑤ Centrifuge at 4°C, 12,000 rpm for 10 min , collect the supernatant. Add IP Lysis Buffer to the supernatant to bring the total volume to 1 mL, mix well, and divide into three portions: 450 µL (control group), 450 µL (experimental group), and 100 µL (Input). Store the Input sample at -80°C for later use.

4.1.2 Animal Tissue

① Lysate preparation: Take 990 µL of IP lysis buffer, add 10 µL of Protease inhibitor (100X), and mix well;

② Take fresh or cryopreserved tissue (approximately 0.3 g), place it in a sterilized and pre-cooled mortar, add liquid nitrogen and quickly grind into a powder, collect about 1/4 of the sample reserved for subsequent RNA extraction of the Input group (store in nuclease-free EP tubes at -80°C);

③ Add 800 µL of lysis buffer to the remaining sample in the mortar, continue grinding on ice for 5-10 min until the sample becomes a fine homogeneous slurry, transfer to a new EP tube, then add the remaining 200 µL of lysis buffer to the mortar to collect residual sample, and similarly transfer it to the same EP tube;

④ Allow the EP tube containing the sample slurry to fully lyse on ice for 30 min , vortexing every 5 min for 10 sec each time;

⑤ After lysis is complete, perform ice-bath sonication using a cell disruptor for 8-10 min at 20% power, with 3 sec of sonication and 3 sec of intervals.

⑥ Centrifuge at 4°C and 12,000 rpm for 10 min , collect the supernatant, then add IP Lysis Buffer to the supernatant to bring the volume to 1 mL, mix well, and divide into three portions: 450 µL (control group), 450 µL (experimental group), and 100 µL (Input). Store the Input sample at -80°C for later use.

4.2 Magnetic Bead and RNA Binding

① Magnetic bead preparation: Take two 1.5 mL nuclease-free EP tubes labeled as control group and experimental group, take the Nucleic- Acid Compatible Streptavidin Magnetic Beads from the kit, mix thoroughly, and add 40 µL of magnetic beads to the EP tubes;

② Place the two EP tubes on a magnetic stand, let stand for 1 minute, and aspirate and discard the protective solution;

③ Wash the magnetic beads with 200 µL of Wash Buffer I, vortex the EP tube to resuspend the beads, centrifuge briefly at low speed in a microcentrifuge, place on a

magnetic stand, let stand for 1min, and aspirate to discard Wash Buffer I; repeat this step for a total of 3 times;

④ Add 300 μL of RNA binding buffer to the washed magnetic beads, and vortex the EP tube to resuspend the beads;

⑤ Add 150 pmol of biotin-labeled experimental probe to the experimental group EP tube, add 150 pmol of biotin-labeled control probe to the control group EP tube, and mix gently by pipettin;

⑥ Place on a silent mixer and incubate with rotation at room temperature for 2 hours.

4.3 RNA-Bead Complex Binding with Protein/RNA

① Place the two EP tubes that have completed RNA-bead incubation on a magnetic rack, and aspirate and discard the supernatant;

② Wash the beads with 200 μL of Wash Buffer I, vortex the EP tube to resuspend the beads, centrifuge briefly at low speed in a microcentrifuge, place on the magnetic rack, let stand for 1 minute, then aspirate and discard Wash Buffer I; repeat this step a total of 3 times;

③ According to the table below, sequentially add the corresponding volumes of reagents to the washed EP tubes;

④ Vortex to mix, then incubate with stirring at 4°C overnight.

Reagent	Reference volume of reagent added (μL)
Protein-RNA binding buffer (10X)	60
Glycerol (50%)	90
Protein lysate prepared in step 4.1	450

Note: If RNA binds to RNA, the beads in step ④ of 4.4 should not be divided; use all for RNA extraction.

4.4 Washing of RNA-binding protein complexes

① Place the EP tube from 4.3, containing the protein-RNA-bead incubation, on the magnetic rack, and aspirate and discard the supernatant;

② Wash the beads with 200 μL of Wash Buffer I, resuspend the beads by vortexing the EP tube, centrifuge briefly at low speed in a microcentrifuge, place on the magnetic rack, let stand for 1min, aspirate and discard Wash Buffer I, and collect the beads;

③ Wash the beads with 200 μL of Wash Buffer II, resuspend the beads by vortexing the EP tube, centrifuge briefly at low speed in a microcentrifuge, place on a magnetic stand, let stand for 1min, aspirate and discard Wash Buffer II, and collect the beads.

This step is performed a total of 3 times;

④ Add 200 μL of Wash Buffer II to the washed EP tube, mix well, and aliquot a certain volume into corresponding tubes (if there are 2 or more downstream experiments, the beads need to be divided into separate tubes, with both control and experimental groups set up; for each type of downstream experiment, mark one tube accordingly. It is recommended to allocate volumes according to WB/silver staining: qPCR: mass spectrometry = 3:5:2).

⑤ Place the EP tube containing separated magnetic beads into a microcentrifuge for low-speed centrifugation, then place it on a magnetic stand, let it stand for 1 min, aspirate and discard Wash Buffer II, and collect the magnetic beads.

4.5 Protein Elution (Downstream for WB or Silver Staining, Optional)

① Add 100 μL of Elution Buffer to each of the two washed EP tubes, mix well, and boil in water for 10 min ;

② After low-speed centrifugation in a microcentrifuge, place on a magnetic rack, let stand for 1 minute, and transfer the supernatant to newly labeled EP tubes;

③ Add 10 μL of 6× Loading Buffer to the two new EP tubes; also add 10 μL of 6× Loading Buffer to the 100 μL Input reserved after adding lysis buffer in step 4.1, mix well, and store at -20°C.

4.6 RNA Extraction (Downstream for QPCR, Optional)

① Add 500 μL of Trizol to each of the two washed EP tubes, and also add 500 μL of Trizol to the EP tube reserved with 1/4 cells from step 4.1, vortex to mix, let stand for 5 min , add 100 μL of chloroform, vortex to mix, and centrifuge at 4°C 14000rpm for 10 min ;

② Transfer the upper aqueous phase (approximately 250 μL) to a new EP tube, add 50 μL of solution I and 500 μL of isopropanol, vortex to mix, and precipitate RNA at -80°C overnight;

③ Take out the overnight precipitated EP tubes and thaw at 4°C, centrifuge at 4°C 14000rpm for 10 min , and discard the supernatant;

④ Add 1 mL of 75% ethanol, vortex to mix, let stand at 4°C for 5 min, centrifuge at 4°C 14000 rpm for 10 min, discard the supernatant; repeat this step 3 times in total;

⑤ Open the lid and air-dry at room temperature for 3-5 min, add an appropriate amount (10-20 μL) of DEPC water to dissolve the RNA.

4.7 Mass spectrometry (downstream is mass spectrometry, optional: if the mass spectrometry sample is protein solution, it can be directly taken from step 4.5, and this step is not required)

① Add 200 μL of pre-chilled 1X PBS to the washed EP tube to wash the magnetic beads, perform magnetic separation, discard the wash solution; repeat this step 3 times in total;

② Store at -20°C for mass spectrometry identification.

5. Frequently Asked Questions

Q1: How to prevent RNA degradation throughout the experiment?

All reagents and consumables used in the experiment must be treated to remove RNase.

Q2: Is in vitro transcription necessary to synthesize RNA probes for RNA pull-down?

In vitro transcription is one method to obtain RNA, offering higher purity compared to chemical synthesis. Therefore, pull-down experiments typically use target RNA obtained via in vitro transcription. When the target RNA sequence exceeds 2000 bp, in vitro transcription often becomes challenging. In such cases, we can design and synthesize a short target RNA probe; by allowing the probe to bind to the target RNA and then to the protein, we can circumvent this issue.

Q3: After transcription, RNA typically has a low OD value. Can it still be used? How should quantification be performed?

If the OD value is low, RNA purification can be carried out; even without purification, adding more RNA in the experiment is also feasible. For quantification, agarose gel electrophoresis is commonly used, where RNA concentration can be estimated based on marker concentrations. Alternatively, instruments can measure RNA concentration, but RNA obtained via in vitro transcription usually reaches above 2 μg/μL. Moreover, pull-down experiments do not require precise quantification, as an excess of probe is typically added.

Q4: Regarding sample processing, should protease inhibitors or RNase inhibitors be added during cell or tissue lysis? Are any other treatments needed? What should be noted during the operation?

Cell lysis requires the addition of protease and RNase inhibitors, and sonication is recommended. Additionally, the entire protein lysis process should ideally be performed

on ice.

Q5: What are the considerations for lncRNA primer design? Or how does it differ from ordinary primer design?

For in vitro transcription amplification primers, simply add the T7 promoter sequence to the 5' end of the forward primer.

Q6: Are there any recommended silver staining kits?

Either Thermo Fisher's or our Genecreate's will do.

Q7: Are proteins identified by mass spectrometry primarily filtered based on scores? What score threshold is considered valid for this filtering?

After pull-down enrichment and mass spectrometry analysis, unreliable proteins are removed, and only credible proteins are delivered, with no explicit scores provided. For ranking, the number of characteristic peptides identified for each protein is generally used as a reference.

Q8: Does lncRNA pull-down combined with mass spectrometry typically reveal multiple interacting proteins? How should one select which protein to pursue for further study?

The number of RNA-binding proteins varies; filtering is based on the proteins actually identified. The selection principle is determined by the research direction or relevant functions of such proteins.

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