

Pierce™ Magnetic RNA-Protein Pull-Down Kit

20164

2509.0

Number**Description**

20164

Pierce Magnetic RNA-Protein Pull-Down Kit, contains sufficient reagents for 20 desthiobiotinylation reactions with 50pmol of RNA and 20 pull-down reactions using 50pmol of labeled RNA and 50µL of magnetic beads

Kit Contents:

Pierce RNA 3' End Desthiobiotinylation Kit (20163); store at -20°C

RBP Enrichment Module (20164Y); store at 4°C

Pierce Nucleic-Acid Compatible Streptavidin Magnetic Beads, 1mL, supplied at 10mg/mL in ultrapure water with 0.05% sodium azide

RNA Capture Buffer (1X), 10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA

20mM Tris, 5mL, pH 7.5

Protein-RNA Binding Buffer (10X), 1mL, 0.2M Tris (pH 7.5), 0.5M NaCl, 20mM MgCl₂, 1% Tween™-20 Detergent

Wash Buffer (1X), 10mL, 20mM Tris (pH 7.5), 10mM NaCl, 0.1% Tween-20 Detergent

Biotin Elution Buffer, 1.5mL

Glycerol, 50%, 0.5mL

HuR Monoclonal Antibody (Mouse), 50µL, sufficient for detection of five Western blots

RNA Controls (20164Z); store at -20°C

Positive RNA Control (AR RNA), 250pmol (in 25µL), sufficient for five labeling and pull-down reactions

5'-CUGGGCUUUUUUUUCUCUUUCUCUCCUUUCUUUUUCUUCUCCCCUCCCUA-3'

Negative RNA Control [poly(A)₂₅ RNA], 250pmol (in 25µL), sufficient for five labeling and pull-down reactions

Storage: Upon receipt store Product No. 20163 and 20164Z at -20°C; store Product No. 20164Y at 4°C. Product No. 20163 is shipped on dry ice. Product No. 20164Y and 20164Z are shipped with an ice pack.

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Introduction

The Thermo Scientific Pierce Magnetic RNA-Protein Pull-Down Kit provides reagents to efficiently enrich RNA-binding proteins (RBPs) using RNA end-labeled with desthiobiotin and Thermo Scientific Pierce Nucleic-Acid Compatible Streptavidin Magnetic Beads. Desthiobiotinylated target RNA directly enriches RBPs (or complexes) and provides an alternative to antibody capture of protein-RNA complexes. The kit offers several advantages, including validated controls for labeling and the pull-down assay and compatibility with downstream applications such as Western blotting and mass spectrometry (MS).

The Thermo Scientific Pierce RNA 3' End Desthiobiotinylation Kit uses T4 RNA ligase to attach a single desthiobiotinylated cytidine biphosphate to the 3' end of the RNA strand. End-labeling at the 3' end does not interfere with RNA structure and is, therefore, more desirable than random incorporation of labeled ribonucleotides. Each labeling reaction was designed for 50pmol of RNA; however, the labeling reactions may be scaled (1pmol to 1nmol have been tested), if necessary. The labeling reaction requires a 20-fold excess of desthiobiotinylated nucleotide with reaction incubation times from 30 minutes at 37°C for less complex RNA to overnight at 4-16°C for longer or more complex RNA. Optimization of the labeling efficiency for complex RNA structures is achieved by altering the RNA to nucleotide ratio, increasing the incubation time or by adding DMSO to the labeling reaction to relax the RNA structure.

The control system for the pull-down assay uses 3' untranslated-region androgen receptor (AR) RNA, poly(A)₂₅ RNA and mammalian cell lysate. The proximal 3' untranslated region (UTR) of AR RNA contains UC-rich regions for HuR and Poly(C) Binding Proteins (CP1 and CP2). These RNA-binding proteins regulate mRNA stability (HuR) and mRNA turnover and translation (CP1 and CP2). The negative control RNA [poly(A)₂₅ RNA] does not contain HuR or poly(C) BP binding sites. Incubation of A431 lysate with labeled AR UTR RNA enriches HuR RBP; incubation with only poly(A)₂₅ RNA or beads does not enrich HuR RBP (Figure 1). However, the control system is versatile, and other cell lysates will work as sources of HuR.

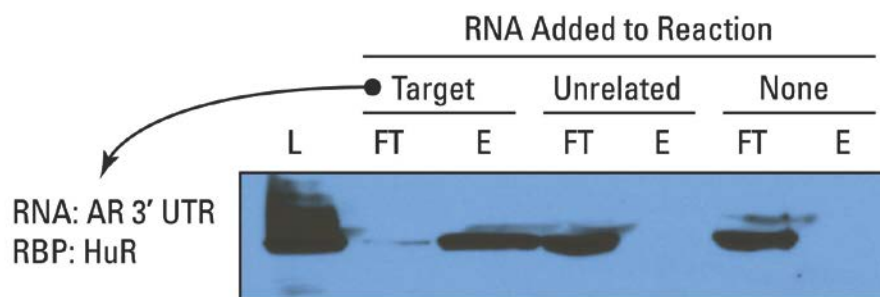


Figure 1. Androgen receptor 3'-UTR RNA specifically pulls down HuR.

AR 3'-UTR RNA and poly(A)₂₅ RNA (50pmol) were labeled using desthiobiotinylated cytidine biphosphate and T4 RNA ligase using the kit procedure. Labeled RNA was captured using 50µL of streptavidin magnetic beads in RNA Capture Buffer for 30 minutes at room temperature. Beads were washed twice in 20mM Tris (pH 7.5), once in Protein-RNA Binding Buffer and 40µg of A431 extract was added. Samples were incubated for 45 minutes at 4°C, washed three times with Wash Buffer and eluted after 15 minutes of incubation at 37°C with Biotin Elution Buffer. RNA pull-down specificity was assessed by Western blotting with samples normalized by volume and bands detected using Thermo Scientific SuperSignal West Pico Substrate (Product No. 34080) and a 2-minute film exposure (L = lysate load; FT = flow-through; E = eluate).

Procedure Summary

The procedure for enrichment of RBPs has been optimized for ease of use (Figure 2). The RNA is first bound to the beads to orient the RNA for protein binding. RNA-bound beads are then equilibrated in Protein-RNA Binding Buffer before protein lysate is added. Beads are washed by adding the appropriate buffer, vortexing and separating on a magnetic stand. Additional salt, reducing agent or detergent may be added to the buffers to alter stringency. Samples may be eluted using non-denaturing Biotin Elution Buffer or SDS-PAGE Loading Buffer.

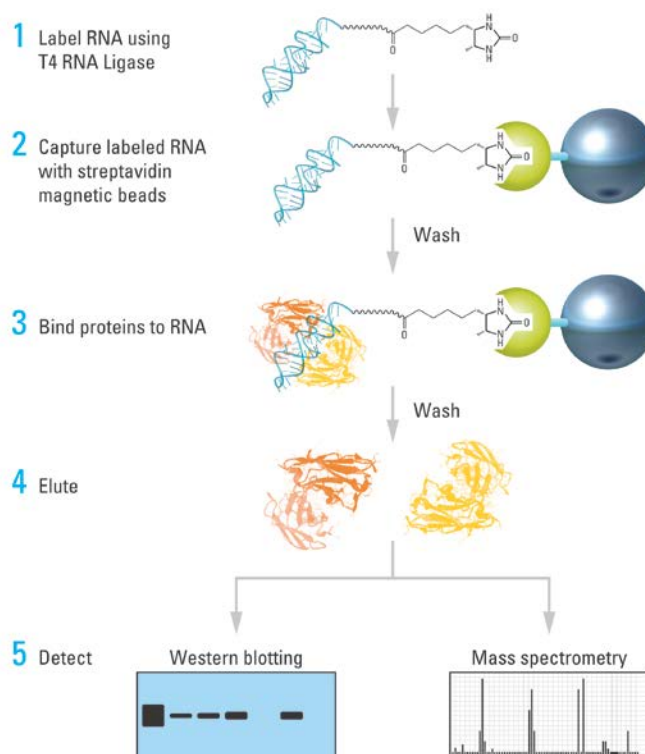


Figure 2. Procedure summary schematic for the Thermo Scientific Pierce Magnetic RNA-Protein Pull-Down Kit.

Important Product Information

- Complete instructions for RNA labeling are included in the instruction booklet for the Pierce RNA 3' End Desthiobiotinylation Kit (Product No. 20163).
- Maintain a nuclease-free environment during the procedures and when working with the RNA intended for labeling. Wear gloves and only use reagents and plastics compatible with nucleic-acid manipulations.
- The Pierce Nucleic Acid-Compatible Streptavidin Magnetic Beads are compatible with mass spectrometry because of their low nonspecific binding. Do not freeze or dry the streptavidin magnetic beads. Freezing or drying will cause the beads to aggregate and lose binding activity.
- To minimize protein degradation, include protease inhibitors (e.g., Thermo Scientific Halt Protease Inhibitors or Pierce Protease Inhibitor Mini Tablets) in the cell lysate preparation.
- Boiling the magnetic beads in SDS-PAGE Reducing Sample Buffer is acceptable for single-use applications. Boiling will cause bead aggregation and loss of binding activity.

Additional Materials Required

- Target RNA for labeling
- Heated mixer or chiller
- Chloroform:isoamyl alcohol (24:1)
- Ethanol, absolute
- Cell Lysis Buffer (for preparation of cell lysate)
- Alternate Elution Buffer: SDS PAGE Reducing Sample Buffer (optional)
- Tris-buffered saline containing 0.05% Tween™-20 Detergent (TBS-T)
- Magnetic separation stand
- Western blotting reagents
- Nitrocellulose (Product No. 88014) or PVDF (Product No. 88585)
- Goat Anti-Mouse IgG (H+L), HRP conjugated (Product No. 31430)
- SuperSignal™ West Pico Chemiluminescent Substrate (Product No. 34080)
- Electrophoresis apparatus

Procedure for Enrichment of RNA-Binding Proteins Using RNA

Note: Maintain nuclease-free conditions. Clean the work area, wear gloves and only use reagents and plastics compatible with nucleic acids.

A. Pre-Washing Streptavidin Magnetic Beads (Optional)

Note: Nucleic acid-compatible beads were validated without pre-treatment of the beads.

1. Resuspend the beads in the original vial by gentle swirling or rotation.
2. Remove the amount to be treated and transfer to a nuclease-free tube.
3. Place tube on a magnetic stand to collect the beads against the sides of the tube.
4. Wash the beads twice with a 2X volume of 0.1M NaOH, 50mM NaCl (nuclease-free).
5. Wash the beads once in 100mM NaCl.
6. Continue with equilibration of magnetic beads for RNA capture (Section D).

B. Preparation of Cell Lysate

- Cell lysates may be prepared using standard lysis buffers (e.g., Thermo Scientific Pierce IP Lysis Buffer, M-PER Mammalian Protein Extraction Reagent and T-PER Tissue Protein Extraction Reagent).
- Protein(s) translated *in vitro* using human *in vitro* expression kits (e.g., Thermo Scientific 1-Step Human IVT Kits, Product No. 88881-9) may also be used to test a protein's ability to bind to an RNA target.
- Ensure the cell lysate protein concentration is greater than 2mg/mL, such that there is significant dilution into the Binding Reaction Buffer. If high salt or detergent interferes with the binding reaction, lysates may be buffer exchanged using Thermo Scientific Zeba Desalting Columns.

C. Label Target RNA

- Label the target RNA using the included Thermo Scientific Pierce RNA 3' Desthiobiotinylation Kit, Product No. 20163. Basic reaction components and amounts are provided in Table 1.

Table 1. Basic reaction components and protocol notes for the Thermo Scientific Pierce RNA 3' End Desthiobiotinylation Kit.

Component	Volume (μ L)	Final Concentration
Nuclease-free Water	3	-
10X RNA Ligase Reaction Buffer	3	1X
RNase Inhibitor	1	40U
Control RNA or Test RNA	5	50pmol
Biotinylated Cytidine Bisphosphate	1	1nmol
T4 RNA Ligase	2	40U
PEG 30%	15	15 %
Total Volume	30	-

Note: Add the PEG 30% to the reaction last and mix well. Incubate at the desired temperature for the appropriate time. For the pull-down reaction, extract labeled RNA with an equal volume of chloroform:isoamyl alcohol, followed by ethanol precipitation.

D. Binding of Labeled RNA to Streptavidin Magnetic Beads

Note: Use a range of 25-100pmol of RNA per 20-50 μ L of magnetic beads. The instructions below use a scale of 50pmol of RNA to 50 μ L of beads.

Note: Positive and negative RNA controls have been included in the kit. To determine specificity of user-supplied RNA, use a positive, negative and lysate-only control.

1. Add 50 μ L of streptavidin magnetic beads to a 1.5mL microcentrifuge tube.
2. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.
3. Wash with an equal volume of 20mM Tris (pH 7.5). Resuspend beads by pipetting or vortexing.
4. Repeat Steps 2 and 3.
5. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.
6. Add an equal volume of 1X RNA Capture Buffer. Resuspend beads by pipetting or vortexing.
7. Add 50pmol of labeled RNA to the beads. Mix gently by pipetting.
8. Incubate for 15-30 minutes at room temperature with agitation.

E. Binding of RNA-Binding Proteins to RNA

Note: The Protein-RNA Binding Buffer was developed as a starting point for binding reactions. Additional reagents may also be added to the supplied binding buffer to enhance binding affinity and specificity. User-developed binding buffers are also compatible with this kit.

1. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.
2. Wash with an equal volume of 20mM Tris (pH 7.5). Resuspend beads by pipetting or vortexing.
3. Repeat Steps 1 and 2.
4. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.
5. Dilute 10X Protein-RNA Binding Buffer to 1X (i.e., 10 μ L into 90 μ L of ultrapure water for each reaction).
6. Add 100 μ L of 1X Protein-RNA Binding Buffer to the beads and mix well.

- Prepare a Master Mix of RNA-Protein Binding Reaction (Table 2).

Table 2. Reaction components for the Master Mix of RNA-Protein Binding Reaction.

Reagent	Volume (μL) per 100 μL reaction for control	Range
10X Protein-RNA Binding Buffer	10	5-20 μL
50% glycerol	30	0-50 μL
Additional salts, etc. (variable)	x	x μL
Lysate (protein conc. > 2mg/mL)	1-30	20-200 μg
Nuclease-free water	to 100	to 100 μL

- Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.
- Add 100 μL of Master Mix to the RNA-bound beads. Mix by pipetting or gentle vortexing.
- Incubate 30-60 minutes at 4°C with agitation or rotation.

F. Washing and Elution of RNA-Binding Protein Complexes

- Place the tube into a magnetic stand to collect the beads against the side of the tube. Transfer the supernatant to a tube for later analysis.
- Wash with equal volume of 1X wash buffer (100 μL).
- Repeat Steps 1 and 2 two additional times. Save wash supernatants for analysis, if desired.
- Place the tube into a magnetic stand to collect the beads against the side of the tube. Transfer the supernatant to a tube for later analysis.
- Add 50 μL of Elution Buffer to the beads and mix well by vortexing. Incubate 15-30 minutes at 37°C with agitation.
- Place the tube into a magnetic stand to collect the beads against the side of the tube.
- Remove supernatant for downstream analysis.
- If the downstream application is Western blotting, add reducing sample buffer to samples to 1X.
- Heat the eluted samples for 5-10 minutes at 95-100°C. Electrophorese samples on a gel or store at -20°C until use.
- For the control reaction, apply 30 μL per lane.

G. Western Blot Analysis

Note: The control reactions have been optimized for use with SuperSignal West Pico Chemiluminescent Substrate. Include a cell lysate lane as a control to verify that the Western blot is properly functioning.

- Separate the proteins by SDS-PAGE and transfer to nitrocellulose membrane.
- Block the membrane in TBS-T containing 5% bovine serum albumin (BSA) at room temperature for 1 hour.
- Prepare a solution containing 10 μL of anti-HuR antibody in 10mL of TBS-T containing 5% BSA.
- Incubate the membrane in the anti-HuR antibody for 4 hours at room temperature or overnight at 4°C.
- Wash the membrane 5 times for 5 minutes each with TBS-T.
- Dilute the Goat Anti-Mouse IgG (H+L) (HRP-conjugated, 1:20,000) into TBS-T containing 5% BSA.
- Incubate the membrane in the diluted Goat Anti-Mouse IgG at room temperature for 1 hour.
- Wash the membrane 5 times for 5 minutes each with TBS-T.
- Incubate the membrane with chemiluminescent substrate at room temperature (e.g., SuperSignal West Pico Chemiluminescent Substrate).
- Immediately expose the membrane to X-ray film or a CCD camera for the appropriate exposures.

Note: The HuR band is located at ~36kDa.

Troubleshooting

Problem	Possible Cause	Solution
Degraded RNA	Nuclease-free environment was compromised	Clean work area and ensure all plastics are nuclease-free and from unopened packages
	mRNA from <i>in vitro</i> transcription reaction was degraded	After transcription, ensure RNA is intact by gel electrophoresis
Inefficient recovery of RNA-binding protein	Binding reaction was not optimized	Optimize incubation time, temperature, salt and detergent for binding reactions
		Titrate amount of labeled RNA to protein lysate
		Use a more concentrated lysate
	Insufficient amount of magnetic beads used for capture	Increase amount of magnetic beads for capture
	Insufficient amount of RNA used for capture	Increase amount of labeled RNA in reaction
Confirm good ligation efficiency		
No recovery of RNA-binding protein	Insufficient amount of target protein in the sample	Increase amount of sample
	Sample was not compatible with binding reaction	Buffer exchange sample using Zeba™ Desalting Columns
	Binding reaction was not optimized	Optimize incubation time, temperature, salt and detergent for binding reactions
		Titrate amount of labeled RNA to protein lysate
		Use a more concentrated lysate
RNA binding protein had low affinity for labeled RNA	Add crosslinking reagent (e.g., UV, etc.) after protein has bound RNA	
High nonspecific binding of RNA-binding protein	Binding reaction was not optimized	Optimize incubation time, temperature, salt, and detergent for binding reactions
	Insufficient washing stringency	Increase stringency of wash buffer; add salt and/or detergent
	Ratio of labeled RNA to lysate was not optimized	Titrate labeled RNA with protein lysate
		Reduce the concentration of lysate to ~2mg/mL
Low signal on Western blot	Insufficient signal	Increase amount of secondary antibody
		Use a more sensitive chemiluminescent detection (e.g., SuperSignal Dura or SuperSignal Femto Chemiluminescent Substrate)
	Poor antibody quality	Pre-screen antibody with cell lysate
		Include cell lysate as a control on Western blot
	Protein was insufficient in lysate	Increase amount of sample
		Identify alternate source of protein
		Use a more concentrated lysate

Related Thermo Scientific Products

20163	Pierce RNA 3' End Desthiobiotinylation Kit
20160	Pierce RNA 3' End Biotinylation Kit
88881-9	1-Step Human <i>In Vitro</i> Translation Kits
88859-69	pT7CFE1 Cell-Free Expression Vectors

General References

Yeap, Bu. B., *et al.* (2002). Novel binding of HuR and Poly(C)-Binding Protein to a conserved UC-rich motif within the 3' untranslated region of the androgen receptor messenger RNA. *J Biol Chem* **277**:27183-92.

Khanam, T., *et al.* (2006). Poly(A)-binding protein binds to A-rich sequences via RNA-binding domains 1+2 and 3+4. *RNA Biology* **3**:170-7.

Product References

Brown, R., *et al.* (1989). Protein measurement using bicinchoninic acid: Elimination of interfering substances. *Anal Biochem* **180**(1):136-9.

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