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Identification of circRNA-Interacting Proteins by Affinity Pulldown

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Abstract

Circular RNAs (circRNAs) comprise a vast class of covalently closed transcripts, generated primarily via backsplicing. Most circRNAs arise from full or partial exons, but they can also arise from introns, and from combinations of introns and exons. While high-throughput RNA-sequencing analysis has identified tens of thousands of circRNAs expressed in different tissues and growth conditions, the function of circRNAs has only been described for a handful of them. As most circRNAs appear not to encode peptides, their function is presumed to be linked to their interaction with a range of molecules, particularly other nucleic acids (notably microRNAs) and proteins. A major impediment to identifying circRNA-associated molecules is a lack of suitable methodologies capable of analyzing specifically circRNAs and not their linear RNA counter-parts with which they share most of their sequence. Here, we describe a flexible and robust method for identifying the proteins that associate with a given circRNA. The affinity pulldown assay is based on the use of a biotinylated antisense oligomer that recognizes the circRNA-specific junction sequence. Following pulldown using streptavidin beads, the proteins are eluted from the circRNP (circribonucleoprotein) complex and identified by mass spectroscopy; validation by Western blot analysis and other methods would then confirm the identity of the circRNA-associated proteins. We present a detailed step-by-step protocol, tips to optimize the analysis, troubleshooting suggestions, and assistance in interpreting the results. In sum, this protocol enables the discovery of proteins present in circRNPs, a critical effort toward elucidating circRNA function.

Keywords

circRNAs; Backsplice junction; Ribonucleoprotein complex; circRNA-Protein; Antisense oligomer

1 Introduction

With less than 2% of the transcribed genome serving as a template for protein synthesis, a vast majority of expressed transcripts is thought to participate in other cellular processes [1]. Among this massive class of noncoding RNAs are circular (circ)RNAs, a family of covalently closed transcripts. Generally believed to arise from the splicing

³-It is highly recommended that the ASO pulldown experiments be validated using alternative methods such as RNP IP (RIP) or biotin-RNA pulldown analyses.

machinery through backsplicing, circRNAs are ubiquitous in the cytoplasm and nucleus, are evolutionarily conserved, and display tissue- and development-specific expression patterns [2–7]. Until recently, circRNAs were considered to be rare byproducts of splicing, far less abundant than the linear parental mRNAs; however, now we know that this expansive class includes tens of thousands of circRNAs, and that thousands of circRNAs may be found in a given cell [8].

A circRNA generally shares its sequence fully with its parent linear RNA; the only segment that is unique to the circRNA is the point of covalent union –the circRNA junction. Accordingly, the methods that have been developed to isolate and characterize the function of circRNAs exploit both their circular nature and their junction sequences [9]. As most circRNAs do not appear to encode proteins, their function has been proposed to be linked to their interaction with other molecules. Most of the interactions studied to date have implicated microRNAs [10] and in some cases, a function as “microRNA sponges” has been described for abundant circRNAs. However, many proteins also bind circRNAs and the function of these proteins may be modulated by the interaction with circRNAs, as shown for transcription factors and RNA-binding proteins (RBPs) that influence mRNAs posttranscriptionally [11–15].

Affinity RNA pulldown assays can specifically extract ribonucleoprotein (RNP) complexes from a mixed population of molecules. The general strategy is to use a biotinylated antisense oligomer (ASOs) designed to recognize the RNA sequence, incubate the ASO with a mixed lysate containing the RNP, pull it down using streptavidin-coated beads, and purify and identify the associated molecules. In the case of circRNPs, the ASO is designed to hybridize with the unique junction sequence on the circRNA in order to reduce or exclude pulling down the linear RNA. The proteins associated with the circRNA can be studied using methods such as mass spectrometry and Western blotting, while the RNAs present in the pulldown material can be studied by methods such as sequencing and RT-qPCR analysis. The basic schematic of this method is illustrated in Fig. 1. For low-abundance circRNAs, moderate overexpression of the circRNA before pulldown may enhance the identification of the interacting partners.

This method was successfully used in recent studies to identify proteins bound to circRNAs. ASOs against *circFoxo3* uncovered that the circRNA associated with ID1, E2F1, FAK, and HIF-1 α [13, 14], ASOs that pulled down *circPABPN1* revealed its interaction with HuR [12], and ASOs directed at *circSamd4* were used to find the association of the circRNA with transcription factors PURA and PURB [15]. We have formulated a detailed protocol, adjustments to improve detection, possible limitations, and other points for consideration.

2 Materials

It is highly recommended that all steps of this protocol be performed in RNase-free conditions by using RNase-free glassware, plasticware, and solutions.

2.1 General Reagents

1. Cultured cell line; for example, mouse C2C12 myoblasts (ATCC, USA).

2. Culture media; for C2C12 myoblasts, Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific).
3. Serum for cell culture; for C2C12 myoblasts, fetal bovine serum (FBS) (Thermo Fisher Scientific) and horse serum (Thermo Fisher Scientific).
4. Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific).
5. Cell scrapers.
6. DNase/RNase-free 1.5-mL microcentrifuge tubes.
7. Vortexer.
8. Microcentrifuge (Eppendorf).
9. NanoDrop spectrophotometer (Thermo Fisher Scientific).
10. Tube rotator.
11. Magnetic separation stand (New England Biolabs).

2.2 Preparation of Biotinylated Antisense Oligomer (ASO)

Biotinylated ASOs are designed against the circRNA junction to pull down the circular RNA.

3'-biotin-labeled antisense DNA oligomers (ASOs; 20–40 nucleotides in length) are designed against the splice junction and custom-made from Integrated DNA Technologies (IDT). It is highly recommended that the ASO sequence be BLASTed against the NCBI database to exclude the recognition of other possible target RNAs. As a negative control, 3'-biotin-labeled sense DNA oligomer or a scrambled oligomer (SO; IDT) can be used. If the secondary structure of the target circRNA junction is available, shorter oligomers (e.g., 20–22 nucleotides in length) can be designed to target the single-stranded regions.

2.3 Cell Lysis and Binding

1. Polysome extraction buffer (PEB) (20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.5% NP-40). For mitochondrial proteins, replace 0.5% NP-40 with 0.5% Triton X-100 and 1% NP-40.
2. 2× TENT binding buffer [20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 500 mM NaCl, 1% v/v Triton X-100].
3. RiboLOCK RNase inhibitor (40 U/μL) (Life Technologies, # EO0384).
4. Halt™ Protease Inhibitor Cocktail (100×) (Life Technologies # 87786).

2.4 Pulldown of circRNAs and Elution of Proteins

1. Streptavidin Dynabeads (Thermo Fisher Scientific).
2. 2× Laemmli buffer (Bio-Rad).

2.5 RNA Isolation, Reverse Transcription, and qPCR

1. Tripure (Thermo Fisher Scientific).
2. Random primers (Thermo Fisher Scientific).
3. dNTP mix (Thermo Fisher Scientific),
4. Ribolock RNase inhibitor (Thermo Fisher Scientific).
5. Maxima Reverse Transcriptase (Thermo Fisher Scientific).
6. MicroAmp® Optical 96-Well Reaction Plate (Thermo Fisher Scientific).
7. MicroAmp® Optical Adhesive Film (Thermo Fisher Scientific).
8. MPS 1000 Mini Plate Spinner.
9. KAPA SYBR® FAST qPCR mix (Thermo Fisher Scientific).
10. circRNA-specific divergent sets of PCR primers.
11. Thermomixer (Eppendorf).
12. Veriti® 96-well Thermal Cycler (Thermo Fisher Scientific).
13. QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific).

3 Methods

3.1 Preparation of Cell Lysate

1. Seed ~five million C2C12 cells per 150-mm dish, culture in DMEM supplemented with 10% FBS and antibiotics at 37 °C in a 5% CO₂ incubator.
2. Allow the cells to grow for 24–48 h until they reach 70–90% confluency and then replace with differentiation media (DMEM with 2% horse serum). At 72 h after inducing differentiation, remove media, wash cells with cold DPBS and use a cell scraper to collect them into a 1.7-mL tube. Use at least one 150-mm dish per each pulldown or 30–60 million cells, depending upon the abundance of the circRNA of interest (see **Note 1**).
3. Harvest cell pellets, and lyse cells with 1 mL of ice-cold PEB supplemented with a cocktail of protease/phosphatase inhibitor, along with RNase inhibitor (200 U).
4. Mix well by flicking the tubes. Incubate the tubes on ice for 15–20 min, and continue to mix by flicking the tubes intermittently.
5. Centrifuge at $14,000 \times g$ at 4 °C for 15 min.
6. Collect supernatant (whole-cell lysate) into a new tube and discard the cell pellet.

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¹It is helpful to start with at least 50–80 million cells or 800 µg to 1 mg proteins per pulldown, as the pulldown itself is not an efficient process. The inclusion of protease/phosphatase inhibitor cocktail along with RNase inhibitors is strongly encouraged in all key steps while performing the assay.

3.2 Binding of circRNPs to Biotinylated SO/ASO (All Steps on Ice)

1. Transfer 700 μ L (approximately 0.8–1 mg) of cell lysate into a fresh microfuge tube. At this point remove a small fraction (1–10%) of the lysate to use as input for Western blot analysis.
2. Add 700 μ L of 2 $\times$ TENT Buffer supplemented with 2 μ L of Ribolock and protease/phosphatase inhibitor cocktail.
3. Add 1–3 μ L of 100 μ M 3'-biotin-labeled ASO. Prepare a separate tube for control oligomer (sense or scrambled oligomer, SO) pulldown side-by-side.
4. Incubate at 4 $^{\circ}$ C for 2 h with rotation.

3.3 Preparation of Streptavidin Magnetic Beads and circRNP Purification

1. Wash 50 μ L streptavidin Dynabeads with 500 μ L ice-cold PBS once, then wash with 1 $\times$ TENT (diluted 1:1 with PEB). After the wash, incubate tubes on the magnetic stand for 1 min. Remove the supernatant by aspiration and repeat this step four additional times, aspirating the remaining buffer after each wash.
2. Add the lysate containing ASOs (from Subheading 3.2) into prewashed beads and incubate at 4 $^{\circ}$ C for 1 h with rotation.
3. Once the lysate and beads have rotated for 1 h, place the tubes on the magnetic stand for 1 min. Remove the supernatant by aspiration without touching the beads.
4. Wash the beads with 1 $\times$ TENT (diluted in 1:1 in PEB) four times, incubating on the magnetic stand for 1 min between washes, removing the wash buffer each time.
5. After the last wash, spin down the beads with any captured molecules, and remove any remaining buffer. The sample can temporarily be stored at -80° C for further steps. At this stage, the beads can be subjected to a variety of analyses to identify the molecules bound to the beads. However, first and foremost it must be confirmed that the circRNA of interest is enriched in the beads (Subheading 3.4). Afterward, one can proceed to protein isolation (Subheading 3.5).

3.4 RNA Isolation, Reverse Transcription and qPCR

It is extremely important to check that the designed ASO specifically pulled down the circRNA of interest (and not other RNAs) (Fig. 2a).

1. Resuspend the beads with 100 μ L PEB, and add 1 mL TRIzol directly into both the "Input" material (1–10% of total material) and to 100 μ L of PEB containing beads. Mix by pipetting, and incubate at room temperature for 5 min. Add 200 μ L chloroform to each tube and vortex the samples vigorously.
2. Centrifuge the tubes at $12,000 \times g$ for 15 min at 4 $^{\circ}$ C, transfer $\sim$ 500 μ L of supernatant to a new tube, and add an equal volume of isopropanol and 1 μ L GlycoBlue into every tube.

3. Centrifuge the tubes at $12,000 \times g$ for 10 min at 4 °C, and discard the supernatant.
4. Add 1 mL ice-cold 75% ethanol, and centrifuge at $7500 \times g$ for 5 min at 4 °C. Discard the supernatant and air-dry the tubes.
5. Resuspend the RNA pellet in 20 μ L of RNase-free water.
6. Add 1 μ L dNTP mix (10 mM) and 1 μ L random hexamers (150 ng/ μ L) to 12 μ L of the RNA resuspended in water.
7. Incubate at 65 °C for 5 min followed by 4 °C for 5 min using a Thermal cycler.
8. Add 1 μ L Maxima Reverse Transcriptase (200 U/ μ L), 1 μ L RNase inhibitor (40 U/ μ L), and 4 μ L 5 $\times$ RT reaction buffer provided with the reverse transcriptase kit.
9. Incubate samples at 25 °C for 10 min, followed by 50 °C for 30 min, and lastly 85 °C for 5 min using a Thermal cycler to synthesize cDNA.
10. Mix 5 μ L of diluted cDNAs, 5 μ L of SYBR green master mix, and divergent primers (2.5–10 μ M) designed to amplify circRNA of interest, as well as primers used to amplify (a) housekeeping control RNAs, to ensure even loading of samples; (b) the linear RNA from which the circRNA originated, to ensure that the ASO did not enrich this RNA; (c) other circRNAs, to ensure that only the circRNA of interest, and not other circRNAs, is enriched.
11. After completion of RT-qPCR, calculate all Ct values, and compare between control sense or scrambled oligomer (SO) with specific circRNA ASO. Normalize data using housekeeping RNAs (*GAPDH* mRNA, *ACTB* mRNA, *18s* rRNA, etc.) and assess if the specific circRNA was enriched in the ASO pulldown but not the SO pulldown, and compare with the values of other RNAs associated with beads listed in **step 10**.

3.5 Preparation of Protein for MS Analysis and Immunoblotting Validation

After confirming that the assay optimally pulled down the circRNA of interest (Subheading 3.4), one can proceed further to protein isolation and analysis. Mass spectrometry (MS) has become the method of choice for high-throughput detection, identification, and quantitation of proteins. The workflow of sample preparation, technical processing and software analysis varies depending on researcher preferences and MS facilities. The quality of protein sample preparation significantly impacts the MS results. We recommend using SDS-Laemmli buffer at 95 °C for 5 min for maximum protein elution and directly sending the samples for analysis by using standard liquid chromatography-mass spectrometry (LC-MS/MS) which can be handled by most proteomics facilities in different institutions.

1. To isolate proteins bound to the biotinylated ASO, add 50 μ L 2 $\times$ Laemmli buffer containing β -mercaptoethanol (Bio-Rad Catalog # 1610737) to the beads, heat at 95 °C for 5 min and spin down the beads.

2. Place beads in magnetic stand and remove as much of the eluate as possible without touching the beads. Load 25 μ L on SDS-PAGE gels and keep the remaining 25 μ L for future Western blot analysis and validation steps.
3. Stain gels with colloidal blue or silver, cut distinguishable bands from the gel, and process them according to sample preparation instructions for mass spectrometry analysis. A commonly used protocol for MS analysis is the in-gel digestion method.
4. Send the samples collected from Subheading 3.5, **step 3** for mass spectrometry analysis.
5. After the mass spec facility returns the data (Fig. 2b), those proteins displaying maximum peptide counts, potentially implicated in cellular processes, and having RNA-binding properties may be selected for further validation by Western blotting analysis using sample groups “input,” “control sense/scrambled oligomer (SO),” and “specific pulldown fractions (ASO).” Other criteria for selection of candidate proteins for validation may also be employed (see **Notes 2–4**).

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References

1. Atianand MK, Fitzgerald KA (2014) Long non-coding RNAs and control of gene expression in the immune system. *Trends Mol Med* 20:623–631 [PubMed: 25262537]
2. Hansen TB, Jensen TI, Clausen BH et al. (2013) Natural RNA circles function as efficient microRNA sponges. *Nature* 495:384–388 [PubMed: 23446346]
3. Floris G, Zhang L, Follesa P, Sun T (2017) Regulatory role of circular RNAs and neurological disorders. *Mol Neurobiol* 54:5156–5165 [PubMed: 27558238]
4. Memczak S, Jens M, Elefsinioti A et al. (2013) Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495:333–338 [PubMed: 23446348]
5. Salzman J, Chen RE, Olsen MN et al. (2013) Cell-type specific features of circular RNA expression. *PLoS Genet* 9:e1003777 [PubMed: 24039610]
6. Cocquerelle C, Mascrez B, Hetuin D et al. (1993) Mis-splicing yields circular RNA molecules. *FASEB J* 7:155–160 [PubMed: 7678559]
7. Nigro JM, Cho KR, Fearon ER et al. (1991) Scrambled exons. *Cell* 64:607–613 [PubMed: 1991322]
8. Rybak-Wolf A, Stottmeister C, Glazar P et al. (2015) Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol Cell* 58:870–885 [PubMed: 25921068]
9. Pandey PR, Rout PK, Das A et al. (2018) RPAD (RNase R treatment, polyadenylation, and poly (A)+ RNA depletion) method to isolate highly pure circular RNA. *Methods* 155:41–48 [PubMed: 30391514]

².For validation experiments, it is highly encouraged to include control groups in which the samples are incubated with RNase A and RNase R, so that total RNA and linear RNAs are degraded, respectively. The association of specific proteins should be carried out in untreated and RNase R-treated fractions.

⁴.For nuclear circRNA or if a stronger lysis buffer is needed to isolate circRNAs, crosslinking between circRNAs and RBPs can be considered, because crosslinking enables the complexes to endure stronger lysis conditions, binding of denatured molecules, and more stringent washes. The ASO/SO design is the same as in Subheading 2.2. The cross-linking and pull-down processes can follow ChIRP-MS protocol [16, 17].

10. Panda AC, Grammatikakis I, Kim KM et al. (2017) Identification of senescence-associated circular RNAs (SAC-RNAs) reveals senescence suppressor CircPVT1. *Nucleic Acids Res* 45:4021–4035 [PubMed: 27928058]
11. Li X, Liu CX, Xue W et al. (2017) Coordinated circRNA biogenesis and function with NF90/NF110 in viral infection. *Mol Cell* 67:214–227 [PubMed: 28625552]
12. Abdelmohsen K, Panda AC, Munk R et al. (2017) Identification of HuR target circular RNAs uncovers suppression of PABPN1 translation by CircPABPN1. *RNA Biol* 14:361–369 [PubMed: 28080204]
13. Du WW, Yang W, Liu E et al. (2016) Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. *Nucleic Acids Res* 44:2846–2858 [PubMed: 26861625]
14. Du WW, Yang W, Chen Y et al. (2017) Foxo3 circular RNA promotes cardiac senescence by modulating multiple factors associated with stress and senescence responses. *Eur Heart J* 38:1402–1412 [PubMed: 26873092]
15. Pandey PR, Yang JH, Tsitsipatis D et al. (2020) circSamd4 represses myogenic transcriptional activity of PUR proteins. *Nucleic Acids Res* 155:41–48
16. Chu C, Chang HY (2018) ChIRP-MS: RNA-directed proteomic discover. *Methods Mol Biol* 1861:37–45 [PubMed: 30218357]
17. Chen YG, Chen R, Ahmad S et al. (2019) N6-Methyladenosine modification controls circular RNA immunity. *Mol Cell* 76:96–109 [PubMed: 31474572]

Fig. 1.
Schematic of circular RNA-antisense oligo pulldown

Fig. 2.

Validation of circRNA interacting proteins. **(a)** Biotinylated antisense oligomers (ASOs) complementary to the junction of *circSamd4* and control (Ctrl) were incubated with C2C12 in GM as well as in DM. After affinity pulldown using streptavidin beads, the levels of *circSamd4* enrichment in ASO pulldown samples were assessed by RT-qPCR analysis of *circSamd4* (relative to the enrichment of *GAPDH* mRNA, a transcript that does not bind the ASOs and encodes a housekeeping protein) in the pulldown samples. Enrichment in *Samd4* mRNA was monitored in parallel. **(b)** Top 5 proteins shared in the mass spec datasets from mouse and human myoblast pulldowns. **(c)** Validation in C2C12 of other proteins identified as being selectively enriched in *circSamd4* and *circSAMD4* ASO pulldowns. Following ASO pulldown, the presence of candidate proteins was assessed by Western blot analysis. Input, 5 µg. (Figure is reproduced from ref. 15)