RNA-Protein Interaction Detection (RaPID)

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Method Article

Keywords: rna,protein,viral,host proteins,mass spectrometry,synthetic biology

Posted Date: February 5th, 2018

DOI: <https://doi.org/10.1038/protex.2018.003>

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Abstract

RNA-Protein interactions play numerous roles in cellular function and disease. Here we describe RNA-protein interaction detection (RaPID), which uses proximity-dependent protein labeling, based on the BirA** biotin ligase, to rapidly identify the proteins that bind RNA sequences of interest in living cells. RaPID displays utility in multiple applications, including in evaluating protein binding to mutant RNA motifs in human genetic disorders, in uncovering potential post-transcriptional networks in breast cancer, and in discovering essential host proteins that interact with Zika virus RNA. To improve the BirA** labeling component of RaPID, moreover, a new mutant BirA** was engineered from *B. subtilis* that enables >1000-fold faster kinetics and >30-fold increased signal-to-noise ratio over the prior standard *E. coli* BirA**, thereby enabling direct study of RNA-protein interactions in living cells on a timescale as short as one minute.

Introduction

RNA-protein interactions are pervasive in biological processes and play a critical role in regulating cellular function^{1,2}. Approximately 5% of the human proteome has been experimentally identified as capable of binding RNA and recent work has revealed several networks of RNA-protein interactions that regulate genomic stability and cellular signaling in addition to gene expression³⁻⁵. Consequently, there is growing interest in utilizing synthetic RNAs as drugs and as tools to control cellular circuits^{6,7}. Discovery and study of RNA-protein interactions has lagged the growing appreciation of their biological importance due to limitations in existing methods. Current methods rely on purifying RNA of interest to discover protein binding partners lead to biases owing to post-lysis re-assortments and generate high false positives (Supplementary Table 1)^{8,9}. RNAs fold and adopt complex structures in cellular environment and such structures are critical in mediating interactions with proteins¹⁰. Methods to study known RNA-protein interactions just as RNA-MaP & RNA-compete are performed in artificial settings that do not provide conditions reflective of RNA-protein interaction in living cells and require specialized equipment (Supplementary Table 1)¹¹. To overcome these technical limitations, a methodology was developed to directly tag and isolate proteins that bind to specific RNA motifs in intact live cells. A modified promiscuous biotin ligase from *E. coli*, BirA**, biotinylates proximal proteins within a range of 10nm and has been used to study protein-protein interactions^{12,13}. Biotinylation of target proteins permits stringent washing and reduces background. Phage biology was leveraged to design a tool utilizing BirA** to biotin tag proteins bound to RNA motifs in living cells followed by streptavidin pull-down and mass spectrometry to identify the proteins, terming it RaPID for RNA-Protein Interaction Detection.

Reagents

- Biotin (Sigma B4501) • Macrosep Advance Spin Filter 3K MWCO 20 mL Capacity 24/pack (VWR 89131-974) • Dynabeads® MyOne™ Streptavidin C1 (Life Technologies #65002) • UltraPure™ SDS Solution, 10% (ThermoFisher #24730020) • DTT, 1M (ThermoFisher P2325) • Sodium Deoxycholate,

10% Solution \ (bioworld #40430018) • UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 \ (ThermoFisher #15567027) • 10% Nonidet P40 \ (Sigma #11332473001) • LiCl 7.5 M Solution \ (ThermoFisher AM9480) • NaCl 5M Solution \ (ThermoFisher AM9759) • HEPES, 0.5M buffer solution, pH 7.5 \ (Alfa Aesar J61275) • Triton X-100 \ (Sigma T8787) • UltraPure™ 0.5M EDTA, pH 8.0 \ (ThermoFisher #15575020) ****REAGENT SETUP**** 4mM Biotin Labeling Solution \ (20x) - Dissolve 50mg of biotin in 50mL of serum-free media - Pipette to completely dissolve biotin - Store in sterile 50mL tube and store at 4C. Can be stored for 2 months. Lysis Buffer \ (0.5M NaCl, 50mM Tris-HCl, 0.2% SDS, 1mM DTT) Wash Buffer 1 \ (2% SDS) Wash Buffer 2 \ (0.1% Na-DOC, 1% Triton X-100, 0.5M NaCl, 50mM HEPES pH 7.5, 1uM EDTA) Wash Buffer 3 \ (0.5% Na-DOC, 250uM LiCl, 0.5% NP-40, 10mM Tris-HCl, 1uM EDTA) Wash Buffer 4 \ (50mM Tris-HCl) Elution Buffer \ (1x LDS, 0.02M DTT, 4mM Biotin)

Equipment

Standard molecular biology lab equipment

Procedure

A. Generate pMotif construct Clone BoxB stem loop \ (GCCCTGAAAAAGGCG) adjacent to RNA of interest
 B. Transfection/Infection Depending on the cell type, transfect or infect BoxB. In this protocol, we will use HEK293T and RaPID labeler plasmid \ (E.coli BirA*) - Day 0 - Plate 300K HEK293T cells per well in a 6 well plate - Day 1 - Transfect 2ug of pMotif plasmid with 0.3ug of RaPID labeler plasmid - Day 2 - Make labeling media. Dilute 20x Biotin \ (4mM Biotin) with DMEM to make labeling media. Aspirate out existing media and add labeling media to each well. - Day 3 - Processing Samples C. Processing Samples Make Lysis buffer fresh. Wash Buffer 1 can be made and stored at room temperature. Wash Buffers 2-4 can be made in bulk and stored at 4C.
 1. Aspirate out labeling media. Add cold 1xPBS and gently mix. Aspirate out 1xPBS
 2. Add 500uL cold 1xPBS to each well and scrape cells. Collect in individual eppendorf tubes
 3. Spin cells down at 500g at 4C
 4. Aspirate out PBS ****STOP Point****: Eppendorf tubes can be stored at -80C for processing later
 5. Lyse cells in each tube with 200uL Lysis buffer at room temperature.
 6. Add 20uL 25% Triton X-100. Mix thoroughly
 7. Add 220uL cold Wash Buffer 4
 8. Sonicate samples thoroughly - 10% for 10s. Ensure lysate is clear after sonication. If lysate is not clear, cool for 5s and repeat sonication
 9. Spin lysate at 15,000 g at 4C for 10 minutes
 10. Carefully transfer supernatant to 3K MWCO filter tube
 11. Spin filter tubes at 1500g at 4C for 1hour
 12. Pipette retentate into new eppendorf tubes.
 13. Estimate protein concentration in each tube and use Wash Buffer 4 to equalize concentration across all samples
 14. After equalizing concentration, pipette 20uL into a new tube and label as Lysate_Sample
 15. Calculate amount of protein in each sample. Calculate the amount of beads required for each sample. Use 50uL of MyOne C1 Streptavidin beads per mg of protein. Ensure a minimum of 30uL of beads is used per sample to capture biotinylated proteins and account for loss of beads during processing.
 16. Wash the MyOne C1 beads in Wash Buffer 4 x 2
 17. Prepare fresh set of tubes and label them Pulldown_Sample. Add equal volume of sample and beads to each tube.
 18. End-over-end rotation for 1hr at room temperature OR overnight at 4C
 19. Place tubes on magnetic stand and aspirate out lysate
 20. Add 1mL of

Wash Buffer 1 to each tube and perform end-over-end rotation for 5 mins 21. Place tubes on magnetic stand and aspirate out Wash Buffer 1 22. Add 1mL of Wash Buffer 1 to each tube and perform end-over-end rotation for 5 mins 23. Place tubes on magnetic stand and aspirate out Wash Buffer 1 22. Add 1mL of Wash Buffer 2 to each tube and perform end-over-end rotation for 5 mins 23. Place tubes on magnetic stand and aspirate out Wash Buffer 2 24. Add 1mL of Wash Buffer 3 to each tube and perform end-over-end rotation for 5 mins 25. Place tubes on magnetic stand and aspirate out Wash Buffer 3 26. Add 1mL of Wash Buffer 4 to each tube and perform end-over-end rotation for 5 mins 27. Place tubes on magnetic stand and aspirate out Wash Buffer 4 28. Add 40uL of Elution Buffer to each tube and heat at 98C for 20 minutes. 29. Run Western Blot for Pulldown & Lysate for samples

Anticipated Results

EDEN15 is an RNA motif that binds to CELF1 protein. Using EDEN15 and scrambled controls, RaPID-Western was performed. Figure 2 shows RaPID-Western results with pulldown and lysates.

References

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Acknowledgements

We thank C.Adams, R.Leib and Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry for help with mass spectrometry. We thank L. Morcom and P. Bernstein for expert administrative assistance and members of the Khavari lab for helpful discussions. This work was supported by the US VA Office of Research and Development, by NIH AR49737, and NIH AR43799 \ (P.A.K.). The project described was supported by Award Number S10RR027425 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources or the National Institutes of Health.

Figures

Figure 1

RaPID Schema

Figure 2

RaPID-Western EDEN15 RaPID-Western of EDEN15 vs two scrambled sequence controls of identical nucleotide composition (scr1 and scr2)

Supplementary Files

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