RiPCA: An Assay for the Detection of RNA-Protein Interactions in Live Cells

Sydney L. Rosenblum[1] and Amanda L. Garner[1,2,3]

[1]Program in Chemical Biology, University of Michigan, Ann Arbor, Michigan
[2]Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, Michigan
[3]Corresponding author: *algarner@umich.edu*

Increasing interest in studying and modulating the interactions between RNAs and their RNA-binding proteins has indicated the need for enabling technologies. Existing means of detecting RNA-protein interactions (RPIs) are often limited to biochemical or post-lysis methods or cell-based methods that require the addition of an RNA-based affinity tag, such as the MS2 hairpin, precluding them from use in detecting small or highly processed RNAs. Taking advantage of bioorthogonal chemistry- and split-luciferase-based technologies, we developed an assay for the detection of RPIs in live cells. This article details the protocol and design considerations for RiPCA, or RNA interaction with Protein-mediated Complementation Assay. © 2022 Wiley Periodicals LLC.

Keywords: cellular assay • protein complementation assay • RNA • RNA-binding protein • RNA-protein interaction

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BASIC PROTOCOL

The human genome contains between 1072 (Sundararaman et al., 2016) and 1542 (Gerstberger, Hafner, & Tuschl, 2014) RNA-binding protein (RBP)-encoding genes, accounting for >7% of annotated protein-coding genes (Gerstberger et al., 2014; Mukherjee et al., 2019; Sundararaman et al., 2016). RBPs are responsible for the regulation of gene expression both co- and post-transcriptionally, playing roles in all aspects of RNA biology, from pre-mRNA splicing, cleavage, and polyadenylation to RNA stability, localization, and editing, as well as in miRNA maturation and translation (Glisovic, Bachorik, Yong, & Dreyfuss, 2008; Van Nostrand et al., 2020). Recent advancements in large-scale technologies, such as next-generation sequencing and protein mass spectrometry, have enabled genome-wide mapping of RNA-protein interactions (RPIs) (Ascano, Hafner, Cekan, Gerstberger, & Tuschl, 2012; Darnell, 2010; Huppertz et al., 2014; Treiber et al., 2017; Van Nostrand et al., 2016; Zhao et al., 2010). The generation of large datasets warrants further investigation to fully understand the functional role of individual RPIs. However, methods to validate RPIs remain limited.

Interest in detecting the interaction between RNAs and their protein binding partners has led to the adaptation and development of several biochemical and cellular techniques to monitor RPIs. Surface plasmon resonance (SPR) (Yang, Wang, & Guo, 2008) and isothermal calorimetry (ITC) (Feig, 2009), for example, can be used to measure RPIs. Reliance on robust expression and purification of RBPs coupled with non-physiological conditions limits the utility of these techniques. Other prominent methods of studying RPIs involve co-immunoprecipitation (co-IP) of either an RNA or a protein of interest.

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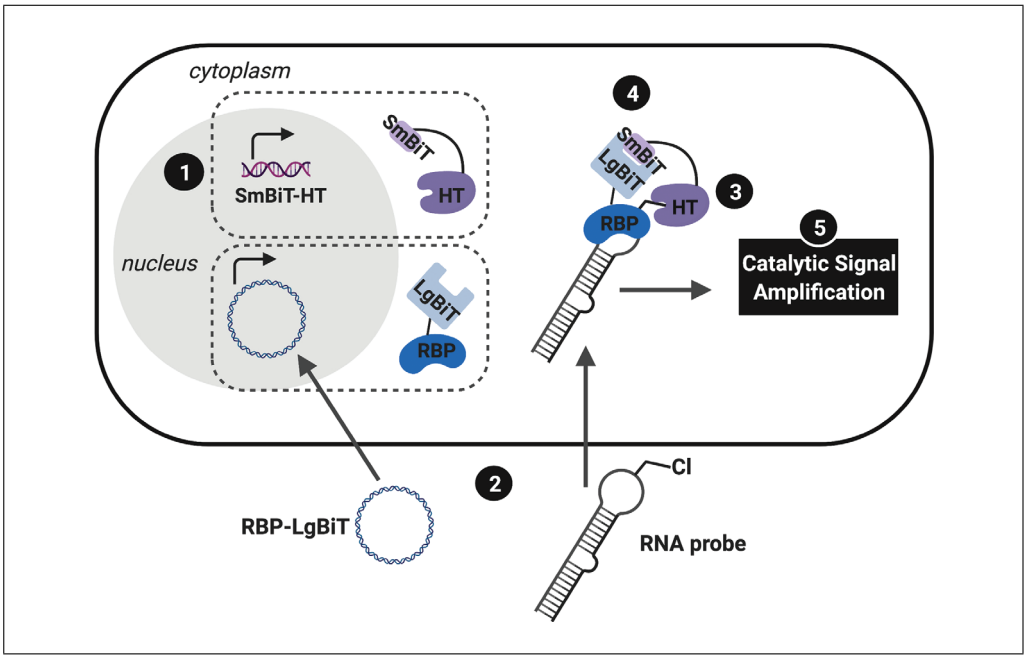


Figure 1 Schematic of RiPCA. Cells stably expressing SmBiT-HT (SmHT) (1) are transiently co-transfected with an RBP-LgBiT-encoding plasmid and functionalized RNA probe (2). The RNA probe becomes covalently modified with SmBiT via HT (3), and association of the RBP and RNA enables reassembly of the BiTs (4), which generates chemiluminescent signal upon treatment with a NanoLuc luciferase substrate (5). Created with BioRender.com. Reproduced from Rosenblum et al. (2021) with permission from the Royal Society of Chemistry.

Even though this allows for RNA-protein complexes to form in cells, artificial buffered conditions during lysis and pulldown may disrupt RPIs or enable non-native interactions (Mili & Steitz, 2004).

Fluorescence resonance energy transfer (FRET)-based assays (Huranova et al., 2009; Lorenz, 2009) circumvent many of these limitations by enabling the detection of RPIs in cells; however, fluorescence-based methods are not easily adapted in all laboratories as they require specialized equipment and are limited by the sensitivity of fluorophores to changes in the local environment (Leavesley & Rich, 2016). Furthermore, many cellular assays and techniques involve tagging of the RNA of interest with an RNA affinity tag, namely the MS2 hairpin, which could impact RNA structure or RBP binding and preclude these methods from use with small RNA species (Graindorge et al., 2019; Huranova et al., 2009; Rackham & Brown, 2004). The protocol presented here describes RiPCA, or RNA interaction with Protein-mediated Complementation Assay, which serves as a useful tool for detecting direct RPIs in live cells.

RiPCA enables detection of cellular RPIs using biorthogonal chemistry and split-luciferase technology, namely the HaloTag (HT) (Los et al., 2008) and NanoBiT (Dixon et al., 2016) systems developed by Promega. RiPCA utilizes a stable cell line (Flp-In™-293) engineered to express the small subunit (SmBiT) of the split luciferase, NanoLuc, fused to HT, an engineered dehalogenase that covalently binds to chloroalkane-containing ligands. The SmBiT-HT fusion protein, herein referred to as SmHT, is stably expressed in either the cytoplasm or the nucleus via a nuclear localization signal (NLS) (Fig. 1, step 1). These cells are then transiently co-transfected with a plasmid encoding the RBP of interest fused to the large subunit of NanoLuc (LgBiT) and a chloroalkane-modified RNA probe (step 2), which allows covalent conjugation to SmHT (step 3). Subsequent interaction between the RBP and RNA drives reconstitution of NanoLuc (step 4), generating chemiluminescence upon treatment of the cells with the NanoLuc luciferase

substrate (step 5). Further engineering of SmHT by appending an NLS has enabled detection of RPIs in the nucleus.

The protocol described here, originally reported in *RSC Chemical Biology* (Rosenblum, Lorenz, & Garner, 2021), provides several advantages compared to other previously reported methods of detecting RPIs. Given that RiPCA uses direct chemical modification, as opposed to MS2 or similar protein-binding RNA affinity tags, this assay can be used to detect RPIs involving small or highly processed RNAs. Additionally, RiPCA leverages the weak, reversible interaction between SmBiT and LgBiT (K_d of 190 μM), ensuring that the RPI drives signal generation. The low intrinsic affinity engineered in the NanoBiT system allows for the accurate detection of biomolecular interactions with a $K_d < 10$ μM (Dixon et al., 2016). This feature may also facilitate the monitoring of RPI dynamics, allowing for the study of interaction modulation by cellular stimuli or inhibitors. By using a NanoLuc-based chemiluminescent readout, RiPCA avoids issues related to fluorescent interference and promotes favorable assay statistics. Finally, as a live-cell assay, RiPCA enables the study of RPIs under physiological conditions, unlike biochemical methods.

Strategic Planning

Thoughtful design of RNA probes is essential for successful adaptation of RiPCA to any RPI. Synthetic RiPCA RNA probes must contain a free amine chemical handle to enable conjugation of the RNA and *N*-hydroxysuccinimidyl (NHS) ester–containing HaloTag ligand. RiPCA probes also contain a biotin handle, which is not required for signal generation, for use in complementary methods, such as co-IP.

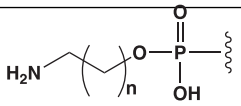
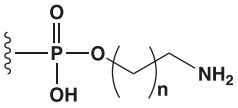
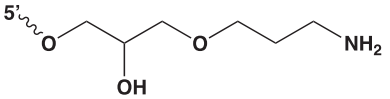
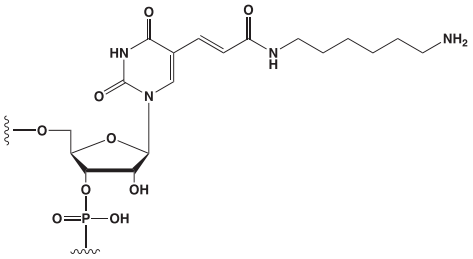
Additional design considerations include (1) the RNA sequence, (2) the position of the modified nucleotide, and (3) the HaloTag ligand. When designing the probe's RNA sequence, it is prudent to include known or theoretically structured regions of the RBP binding motif to enhance the stability of the probe. As a measure of background signal and to ensure specificity of signal in RiPCA, prepare a control RNA probe containing a non-binding sequence. Several companies, including Dharmacon/Horizon Discovery, Integrated DNA Technologies (IDT), and TriLink BioTechnologies, are capable of synthesizing custom RNA oligonucleotides with a variety of amine-containing modified bases, making it possible to append the HaloTag ligand to an RNA probe internally, via modified uridine, or to either the 5′ or 3′ terminus (Table 1). The modified nucleotide should be located proximal to the putative RBP binding site, but it is advisable to test a set of RNA probes with the modified base at varying locations. In the validated RiPCA system, RNA probes in which the modified nucleotide was contained in an RBP binding site were well tolerated. Lastly, there are two commercially available HaloTag ligands supplied by Promega that contain either a PEG2 or a PEG4 linker separating the chloroalkane and NHS ester. Both ligands should be tested to identify the optimal signal and signal-to-background ratio (S/B).

The protocol outlined in this article (Basic Protocol) describes the design and optimization of RiPCA for the detection of RPIs in live cells, including the preparation of RNA probes, the transfection protocol, and reading the assay.

Materials

SmHT construct
Flp-In™ Complete System (Thermo Fisher, cat. no. K601001), including pcDNA5/FRT vector and pOG44 plasmid vector
Flp-In™-293 cell line (Invitrogen, cat. no. R75007)
Complete DMEM (see recipe) with 100 μg/ml Zeocin™ (Invitrogen, cat. no. R25001), 37°C
Lipofectamine LTX with PLUS reagent (Invitrogen, cat. no. 15338100)
Complete DMEM (see recipe) with 100 mg/ml hygromycin B (Gibco, cat. no. 10687010), 37°C

Table 1 Commercially Available Amino Modifiers Compatible with HaloTag Ligand Conjugation to RNA Probes

Modification	Position	Structure	Source
5′ Amino modifier	5′ terminus		Dharmacon (n = 2, 4, 5, 11) IDT, TriLink (n = 5, 11)
3′ Amino modifier	3′ terminus		Dharmacon (n = 2, 5, 11) TriLink (n = 2, 5, 6)
3′ Amino modifier	3′ terminus		IDT
5′ Aminohexy lacrylamino-uridine (5-LC-N-U)	Internal		Dharmacon
Uridine-C6-amino linker (U-C6-NH2)			TriLink

Anti-HaloTag monoclonal antibody (Promega, cat. no. G9211)
RBP of interest
pcDNA3 vector (or mammalian CMV promoter plasmid) containing LgBiT
Mini-prep or midi-prep kit (Qiagen plasmid kit or equivalent)
3 M sodium acetate, pH 5.2 (adjusted with HCl), 4°C
200-proof ethanol, 4°C
70% (v/v) ethanol
100 mM phosphate buffer, pH 8.0 (PB8; see recipe)
Unlabeled synthetic RNA probe (see Strategic Planning)
10 mM O2 or O4 HaloTag ligand [HaloTag Succinimidyl Ester (O2) Ligand, Promega, cat. no. P1691, or HaloTag Succinimidyl Ester (O4) Ligand, Promega, cat. no. P6751] dissolved in dimethyl sulfoxide (DMSO; Hybri-Max^{TM}, sterile-filtered, Sigma-Aldrich, cat. no. D2650-5 × 5ML)
OptiMEM (Gibco, cat. no. 31985062)
Lipofectamine RNAiMAX (Invitrogen, cat. no. 13778100)
Diluted Nano-Glo Live Cell Reagent (Promega, cat. no. N2012; prepared as 1:20 dilution of Nano-Glo Live Cell Reagent in Nano-Glo LCS Dilution Buffer)
0.25% (w/v) trypsin-EDTA (Gibco, cat. no. 25300054)
Trypan blue (Gibco, cat. no. 15250061)

6-cm (T-25) cell culture dishes
Vortex (optional)
Microcentrifuge (Eppendorf 5424 R or equivalent), 4°C
1.5-ml DNA LoBind® microcentrifuge tubes (Eppendorf, cat. no. 022431021)
1.5-ml microcentrifuge tubes
15-ml conical tubes
Tissue culture microscope (Nikon or equivalent)
96-well plate (white, flat bottom, cell culture treated; Corning, cat. no. 3917)
Chemiluminescence-enabled Plate reader (BioTek Cytation3 or equivalent)

Additional reagents and equipment for cloning and transfection (see manufacturers' instructions), preparing cell stocks and harvesting and counting cells (see Current Protocols article; Phelan & May, 2015), SDS gel electrophoresis (see Current Protocols article; Gallagher, 2012), and western blot (see Current Protocols article; Gallagher, Winston, Fuller, & Hurrell, 2011)

NOTE: All steps involving cells should occur in a laminar flow hood (Labconco Purifier BSC Class II or equivalent) while using sterilized pipets and reagents. Refer to the manufacturer's protocol for complete description of Flp-In™-293 stable cell line generation.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All RNA-related steps should occur in an RNase-free environment with sterilized pipets and reagents. Surfaces and pipets can be additionally cleaned with RNaseZap™ (Invitrogen, cat. no. AM9780).

NOTE: Use DNase- and RNase-free water (e.g., UltraPure Water, Invitrogen, cat. no. 10977015) in all protocol steps and recipes.

Preparation of stable cell line

1. Clone desired SmHT construct into the pcDNA5/FRT vector (from Flp-In™ Complete System) following the manufacturer's recommendations.

   Expression of SmHT can be directed to specific cellular compartments by appending localization tags (e.g., SV90 NLS for the nucleus).

2. Culture Flp-In™-293 cell line in 3 ml complete DMEM with 100 μg/ml Zeocin™ in 6-cm (T-25) cell culture dishes.
3. Co-transfect Flp-In™-293 cells with a 9:1 ratio of pOG44 plasmid vector (from Flp-In™ Complete System) to SmHT-encoding pcDNA5/FRT plasmid (see step 1) using Lipofectamine LTX with PLUS reagent according to the manufacturer's protocol.
4. Twenty-four hours after transfection, wash cells with medium and add fresh medium to cells.
5. Forty-eight hours after transfection, split cells into culture medium with 100 mg/ml hygromycin B.

   Split cells to low density (25% confluent) to ensure proper antibiotic selection.

6. Expand cells and change medium every 2 to 3 days following the cell line manufacturer's protocol until the plate is confluent.
7. Split cells once and prepare stocks using standard cell culture protocols.
8. Confirm SmHT expression by performing SDS gel electrophoresis and a western blot with anti-HaloTag monoclonal antibody.

Preparation of DNA for RiPCA

9. Clone RBP of interest into pcDNA3 vector containing LgBiT following the manufacturer's recommendations.

   It is advisable to clone both N- and C-terminally tagged RBPs, as the location of the tag could influence assay results.

10. Extract pcDNA3/RBP-Lg plasmid (e.g., pcDNA3/Lin28A-Lg) using either a mini-prep or a midi-prep kit and elute DNA or dissolve final pellet in 50 μl water. Incubate DNA on ice.
11. Perform a follow-up ethanol precipitation by sequentially adding 10 μl ice-cold 3 M sodium acetate (pH 5.2) and 500 μl ice-cold 200-proof ethanol to the DNA

and mixing well by pipetting or gentle vortexing. Incubate on ice (or at –20°C) for ≥30 min. Collect precipitated DNA by centrifugation (45 min at 15,000 rpm, 4°C).

Performing this ethanol precipitation step typically increases consistency of results.

12. Carefully remove supernatant and clean pellet with 500 µl room-temperature 70% ethanol. Re-collect pellet by centrifugation (5 to 10 min at 15,000 rpm, 4°C).
13. Air-dry pellet.
14. Prepare a diluted stock of DNA (3.9 ng/µl) in water in a 1.5-ml DNA LoBind® microcentrifuge tube and store at –80°C.

    The concentration of the diluted stock can be adjusted to minimize the volume added in later steps if necessary. Carefully measure the concentration and purity of the DNA using standard nucleic acid quantification methods and ensure that the 260/280 measurement is ∼1.8.

Conjugation of HaloTag ligand to RNA probe

15. Prepare a 1 mM stock of unlabeled synthetic RNA probe in PB8.
16. In a fresh 1.5-ml microcentrifuge tube, mix equal volumes of 1 mM unlabeled synthetic RNA probe and 10 mM O2 or O4 HaloTag ligand dissolved in DMSO and incubate at room temperature for 2 to 3 hr.

    It is desirable to perform this reaction in small volumes (not exceeding 10 µl). Be sure to aliquot single-use stocks of 100 mM HaloTag ligand and store these at –80°C to avoid hydrolysis of the NHS ester.

17. Ethanol-precipitate conjugated RNA from excess HT ligand and DMSO by adding 1.1 µl ice-cold sodium acetate (3 M, pH 5.2; see recipe) and 40 µl ice-cold ethanol (200 proof) to the reaction and mixing well by pipetting or gentle vortexing. Collect the precipitated RNA by centrifugation (45 min at 15,000 rpm, 4°C).

    Volumes listed are sufficient for reaction volumes of ≤10 µl.

18. Air-dry pellet and dissolve to ∼1 mM in PB8.
19. Dilute to 50 µM in PB8 to be used in assay and store at –80°C.

    Carefully measure of the concentration and purity of the RNA probe using standard nucleic acid quantification methods and ensure that the 260/280 measurement is ∼2.

RiPCA for RBP

20. Culture Flp-In™-293 cells expressing SmHT from step 7 in 3 ml complete DMEM containing 100 mg/ml hygromycin B in 6-cm (T-25) cell culture dishes.

    Typically, a confluent 6-cm culture dish will have enough cells to perform RiPCA for ∼10 conditions.

21. In a 1.5-ml microcentrifuge tube, prepare solution A for transfection by mixing (n + 1) × 50 µl OptiMEM and (n + 1) × 2.4 µl Lipofectamine RNAiMAX (where n is equal to the number of transfections to be performí).

    Point of optimization: Change the amount of transfection reagent used per condition.

    A graphical representation of steps 21 to 27 can be found in Figure 2.

22. In another 1.5-ml microcentrifuge tube, prepare n number of solution B tubes, one for each transfection condition, by mixing 50 µl OptiMEM, 2.5 µl pcDNA3/RBP-Lg (3.9 ng/µl; see step 14), and 0.3 ml RNA probe (50 µM; see step 19) for final concentrations of 18.5 pg/µl DNA and 0.28 µM RNA.

    Point of optimization: Change the amount of DNA and/or RNA transfected. It is recommended to initially test several final concentrations of both DNA and RNA, ranging from 9 to 37 pg/µl DNA and 0.1 to 0.6 µM RNA.

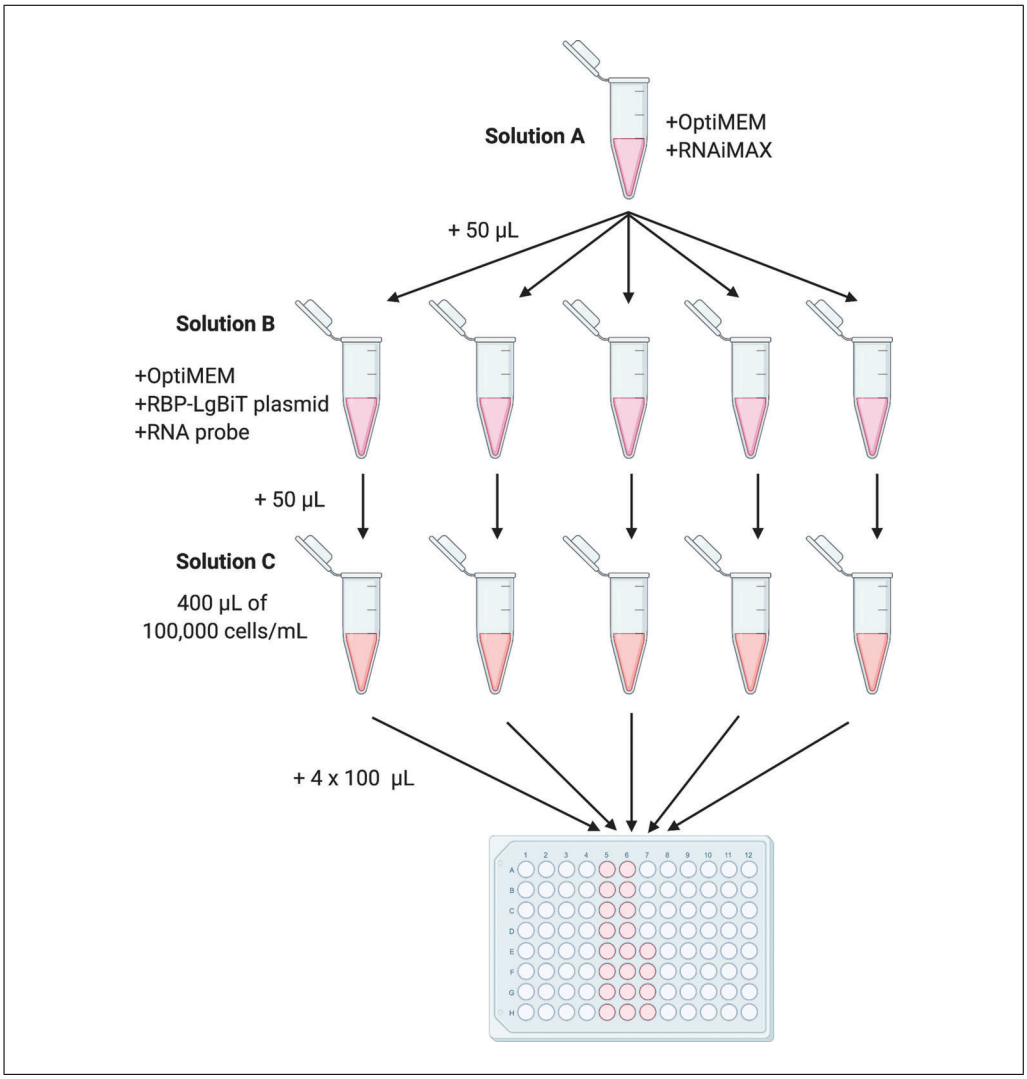


Figure 2 RiPCA transfection workflow. Solution A is prepared and mixed with solution B, and solution A+B is incubated prior to its addition to solution C. Solution A+B+C is plated in a 96-well plate. Created with BioRender.com. Reproduced, with slight modification, from Rosenblum et al. (2021) with permission from the Royal Society of Chemistry.

23. Add 50 µl solution A to each solution B tube and mix well by pipetting up and down. Let mixture incubate for ≥15 min at room temperature.

24. During this incubation, harvest and count Flp-In™-293 SmHT cells from step 20 in a 15-ml conical tube using standard cell culture methods, including trypsinization with 0.25% trypsin-EDTA, staining with trypan blue, and visualization under a tissue culture microscope.

25. Prepare one 1.5-ml tube of solution C for each transfection condition by diluting cells to a density of 1×10^5 cells/ml in 400 µl complete DMEM containing 100 mg/ml hygromycin B.

26. Following the 15-min incubation in step 23, add 50 µl solution A+B mixture to solution C and mix well by pipetting up and down.

27. Plate 100 µl in each of four wells of a 96-well plate for each transfection condition.

28. Incubate plate for 24 hr at 37°C in 5% CO_2.

	CYTOPLASM			
	Lin28A-LgBiT		LgBiT-Lin28A	
Well	pre-miR-21	pre-let-7d	pre-miR-21	pre-let-7d
1	6647	75160	3973	70962
2	7627	100900	5371	48601
3	6997	68590	7852	66258
4	6701	100340	6741	59468
Average	*6993*	*86247.5*	*5984.25*	*61322.25*
	S/B			
Well				
1	0.95	10.75	0.66	11.86
2	1.09	14.43	0.90	8.12
3	1.00	9.81	1.31	11.07
4	0.96	14.35	1.13	9.94

	NUCLEUS			
	Lin28A-LgBiT		LgBiT-Lin28A	
Well	pre-miR-21	pre-let-7d	pre-miR-21	pre-let-7d
1	7533	30383	6553	25749
2	8465	27276	9541	38006
3	7128	36184	10636	34966
4	4417	39788	12504	40005
Average	*6993*	*86247.5*	*5984.25*	*61322.25*
	S/B			
Well				
1	1.09	4.41	0.67	2.63
2	1.23	3.96	0.97	3.87
3	1.04	5.25	1.08	3.56
4	0.64	5.78	1.27	4.08

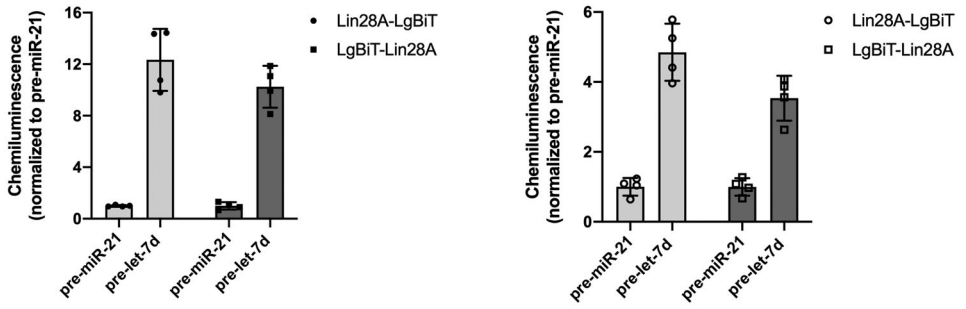


Figure 3 Example of RiPCA data characterizing the interaction between pre-let-7d and its RBP, Lin28A. Tabulated chemiluminescence data generated by RiPCA performed with Lin28A-LgBiT or LgBiT-Lin28A and pre-miR-21 and pre-let-7d in either the cytoplasm (left panel) or the nucleus (right panel). The average of the signal generated by the four wells transfected with pre-miR-21 (bold box) is used as the denominator to yield S/B. Individual S/B data points are graphed (bottom).

Measure chemiluminescence and analyze data

29. Following the 24-hr incubation, aspirate medium and replace with 100 µl OptiMEM.

    Steps 29 to 32 can be performed outside of a laminar flow hood/on the bench.

30. Add 25 µl diluted Nano-Glo Live Cell Reagent.

31. After addition of Nano-Glo Live Cell Reagent, measure chemiluminescence using a chemiluminescence-enabled plate reader.

    It is advised to read the plate at several time points (e.g., 0, 5, and 10 min) to determine the optimal protocol for each system.

32. Export data and analyze in terms of S/B, which is the ratio of signal generated by the binding versus non-binding RNA probes. To analyze the S/B of individual wells, use average of background wells as the denominator for the S/B of each signal well (Fig. 3).

REAGENTS AND SOLUTIONS

Complete DMEM

Dulbeccos modified Eagles medium (DMEM; Corning, cat. no. 10–017–CV)
10% (v/v) fetal bovine serum (FBS; Atlanta Biologicals, cat. no. S11550)
2 mM L–glutamine (Gibco, cat. no. 25030081)
Store ≤2 months at 4°C

Phosphate buffer (pH 8.0), 100 mM (PB8)

100 mM sodium phosphate (mix monobasic and dibasic phosphate at appropriate ratio for pH 8.0)
Adjust pH to 8 with NaOH or HCl
Store ≤6 months at room temperature

COMMENTARY

Background Information

Previously, our group developed a plate-based, antibody-free biochemical assay, the catalytic Enzyme-Linked Click Chemistry Assay (cat-ELCCA), capable of detecting and measuring the inhibition of RPIs. In cat-ELCCA, a biotinylated biomolecule, which can either contain a click-chemistry handle or be capable of accepting one through an enzymatic reaction or biomolecular interaction, is immobilized on a streptavidin-coated plate. The RPI is then detected upon reaction with horseradish peroxidase (HRP) functionalized with a complementary click-chemistry handle. cat-ELCCA is an appealing in vitro platform due to catalytic signal amplification generated by HRP, yielding increased sensitivity; its adaptability to high-throughput format; and the reduced risk of compound interference (Garner & Janda, 2010; Lorenz & Garner, 2016; Lorenz et al., 2018; Lorenz, Song, & Garner, 2015; Lorenz, Vander Roest, Larsen, & Garner, 2018).

RiPCA was designed to address the key limitations of previously reported cell-based assays (e.g., dependence on fluorescent readout and the use of MS2 tagging) while incorporating the advantages of cat-ELCCA. Use of MS2 hairpins to recruit an MS2 coat protein fused to a split fluorescent reporter (e.g., split fluorophore or split fluorescent protein) enables detection of an RPI with a protein of interest that is labeled with the complementary reporter molecule (Huranova et al., 2009; Lorenz, 2009). Although this strategy is attractive because MS2-tagged RNAs can be generated directly in cells, compared to the need for chemically modified RNAs in RiPCA, the addition of hairpins to the RNA of interest could affect the RPI under investigation.

In contrast, RiPCA utilizes Promega's NanoBiT and HaloTag technology to covalently label the RNA with a chemiluminescence-producing reporter. RiPCA uses NanoLuc, a split-luciferase reporter composed of small (SmBiT) and large (LgBiT) subunits (Dixon et al., 2016). Due to the low intrinsic affinity of SmBiT and LgBiT, not only is signal generation driven by the interaction between the biomolecules fused to the BiT, but it also allows for detection of interaction dynamics. Instead of using MS2 hairpins to label RNAs with a reporter, RiPCA takes advantage of HaloTag, an engineered haloalkane dehalogenase that covalently binds to biomolecules modified with a chloroalkane, by fusing it to SmBiT and thus enabling covalent labeling of RNA with SmBiT via a chloroalkane appended to the RNA via a 5′ aminohexylacrylamino uridine included in the synthetic sequence. By directly labeling the RNA of interest, RiPCA enables more site-selective detection of RPIs as well as the detection of RPIs involving small or highly processed RNAs. Accordingly, the strategic design of RiPCA provides a useful platform for detecting cellular RPIs.

Critical Parameters

Reagent purity

Given the sensitivity of RiPCA to the amount of DNA and RNA transfected, it is important that each of these elements is pure and accurately quantified. The best results are seen if the DNA isolated using a spin or flow column kit is additionally purified by ethanol precipitation. Similarly, after the coupling of the synthetic RNA to the HaloTag ligand, the resulting RNA probe must be carefully purified via ethanol extraction. Careful measurement of the concentration is also essential for optimal results. Ensure that the 260/280 measurements for the DNA and RNA elements are within the accepted limits ($\sim$1.8 and $\sim$2, respectively). It is prudent to store the DNA and RNA in LoBind® tubes to avoid changes in concentration due to nucleic acid binding to plasticware.

Storage of HaloTag ligand

The chemical handle of the HaloTag ligand that enables labeling of the amine in the RNA probe, NHS ester, is readily hydrolyzed in the presence of water or at higher-than-optimal pH ($>$8.5). It is best practice to ensure that dry DMSO is used to dissolve the HaloTag ligand and immediately make single-use aliquots to avoid reduction of coupling efficiency.

Troubleshooting

Please see Table 2 for a troubleshooting guide.

Understanding Results

Successful RPI detection in RiPCA is determined by the magnitude of the difference between signal generated by the binding vs. non-binding RNA probes, or S/B, as well as the consistency of the S/B. The average signal generated by the non-binding control, premiR-21 in the included example, is used as the denominator to calculate S/B for each

Table 2 Troubleshooting Guide for RiPCA

Problem	Possible cause	Solution
Low signal or signal-to-background	Poor binding of RBP and RNA probe or too little RNA probe	Revisit RNA probe design or increase the amount of RNA transfected
High background	Too much background expression of RBP-LgBiT	Decrease the amount of DNA transfected
Large variation in signal	Poor expression of SmHT in stable cells	Ensure proper maintenance of cell lines; if passaged to a substantially low density, results are less consistent

data point. RiPCA data can be visualized in bar graphs showing the individual points, as shown in Figure 3. Acceptable values for S/B will vary for each RPI and could differ based on the stable cell line utilized, as evidenced in Figure 3, in which S/B is lower in the nucleus than the cytoplasm for the same RPI.

Time Considerations

Preparation of the DNA from cloning to stock preparation could be completed within 5 to 7 days. Preparation of the RNA takes 4 to 5 hr. The transfection protocol takes 1 to 2 hr. After cells are plated, they are incubated for 24 hr. Reading the plate takes 30 min to 1 hr. Thorough optimization for a new RPI requires several iterations of the assay and could take several weeks to several months.

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Author Contributions

Sydney L. Rosenblum: Conceptualization, Data curation, Formal analysis, Methodology, Writing—original draft, Writing—review and editing; **Amanda L. Garner**: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed.

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