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ARTICLE TITLE:

RiPCA: an assay for the detection of RNA-protein interactions in live cells

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ABSTRACT: (~250 words)

Increasing interest in studying and modulating the interactions between RNAs and their RNA-binding proteins (RBPs) has borne 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, RNA interaction with Protein-mediated Complementation Assay.

Basic Protocol 1: Design and optimization of a cellular assay for the detection of RNA-protein interactions

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KEYWORDS:

RNA, RNA-binding protein, RNA-protein interaction, cellular assay

INTRODUCTION:

The human genome contains between 1,072 (Sundararaman et al., 2016) and 1,542 (Gerstberger, Hafner, & Tuschl, 2014) RNA-binding protein (RBP)-encoding genes, accounting for more than 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, RNA stability, localization, and editing, as well as 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. Yet, 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 of either an RNA- or protein-of-interest. 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).

Fluorescent resonance energy transfer (FRET)-based assays (Huranova et al., 2009; M. 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 the tagging of the RNA-of-interest with an RNA affinity tag, the MS2 hairpin, which could impact RNA structure or RBP binding and preclude it from use with small RNA species (Graindorge et al., 2019; Huranova et al., 2009; Rackham & Brown, 2004). The protocol presented here describes RiPCA, 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^{TM}-293) engineered to express the small subunit (Sm; 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 (step 1). These cells are then transiently co-transfected with a plasmid encoding the RBP-of-interest fused to the large subunit of NanoLuc (Lg; 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 cells with the NanoLuc luciferase substrate (step 5) (Fig. 1). Further engineering of SmHT by appending a nuclear localization signal (NLS) has enabled detection of RPIs in the nucleus.

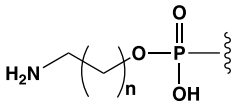
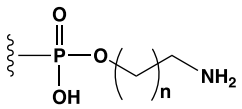
[*Insert Figure 1 near here]

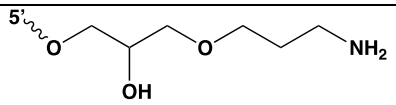
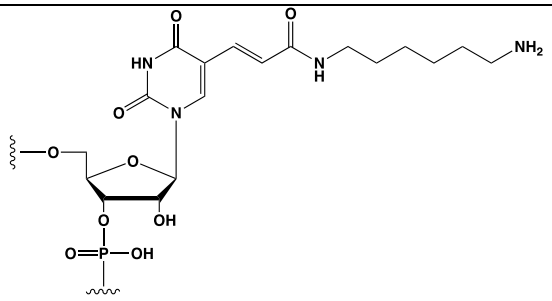
The protocol described here, originally reported in *RSC Chemical Biology* (Rosenblum, Lorenz, & Garner, 2021), provides several advantages compared to other previously reported methods of detecting RPIs. Since RiPCA uses direct chemical modification, as opposed to MS2 or similar protein-binding RNA affinity tag, this assay can be used to detect RPIs involving small or highly processed RNAs. Additionally, RiPCA leverages the weak, reversible interaction between of Sm and LgBiT (K_d of 190 μM), ensuring that the RPI drives signal generation. The low intrinsic affinity of 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 inference 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 for use in complementary methods, such as co-IP, which is not required for signal generation.

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’ Aminohexylacrylamino-uridine (5-LC-N-U)	Internal		Dharmacon
Uridine-C6-Amino Linker (U-C6-NH2)			TriLink

Additional design considerations include (1) the RNA sequence, (2) 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. Based on the validated RiPCA system, RNA probes in which the modified nucleotide was contained in an RBP binding site was well tolerated. Lastly, there are two commercially available HaloTag ligands supplied by Promega, that contain either a PEG2 or PEG4 linker separating the chloroalkane and NHS ester. Both ligands should be tested to identify optimal signal and signal-to-background (S/B).

The protocol outlined in this article (Basic Protocol 1) describes the design and optimization of RiPCA for the detection of RPIs in live cells, including the preparation of RNA probes, transfection protocol, and reading the assay.

BASIC PROTOCOL 1: RNA interaction with Protein-mediated Complementation Assay (RiPCA): an optimized assay for the detection of RNA-protein interactions.

[*Copy editor. Our format requires – please query the author to write a introduction for basic protocol 1 here. I’d ask the author the following: “Please write a summary for basic protocol 1 here. Typically this section is 1-4 sentences long telling the reader the what/how/results of the protocol is executed correctly”.]

Materials

Solutions & Reagents

Flp-In™-293 Cell Line (Invitrogen cat. no. R75007)

Flp-In™ Complete System (Thermo Fisher cat. no. K601001)

pcDNA3 vector (Or mammalian CMV promoter plasmid)
UltraPure Water (Invitrogen cat. no. 10977015)
RNase*Zap*™ (Invitrogen cat. no. AM9780)
Synthetic RNA probe (see Strategic Planning)
Phosphate buffer (PB8; see recipe)
HaloTag Succinimidyl Ester (O2) Ligand (Promega cat. no. P1691)
HaloTag Succinimidyl Ester (O4) Ligand (Promega cat. no. P6751)
Sodium acetate (see recipe)
Ethanol (200 proof)
Dimethyl sulfoxide Hybri-Max™ sterile-filtered (Sigma Aldrich cat. no. D2650-5X5ML)
DMEM (Corning cat. no. 10-017-CV)
FBS (Atlanta Biologicals S11550)
L-glutamine (Gibco cat. no. 25030081)
Hygromycin B (Gibco cat. no. 10687010)
Zeocin™ (Invitrogen cat. no. R25001)
Anti-HaloTag® Monoclonal Antibody (Promega cat. no. G9211)
Trypsin-EDTA (0.25%) (Gibco cat. no. 25300054)
Trypan Blue (Gibco cat. no. 15250061)
OptiMEM (Gibco cat. no. 31985062)
Lipofectamine RNAiMAX (Invitrogen cat. no. 13778100)
Lipofectamine LTX with PLUS reagent (Invitrogen cat. no. 15338100)
Nano-Glo Live Cell Assay (Promega cat. no. N2012)

QIAGEN Plasmid Kit or equivalent
Laminar flow hood (Labconco Purifier BSC Class II or equivalent)
Tissue culture microscope (Nikon or equivalent)
Table-top centrifuge (Eppendorf 5424 R or equivalent)
1.5 mL sterile microcentrifuge tubes
1.5 mL DNA LoBind® microcentrifuge tubes (Eppendorf cat. no. 022431021)
15 mL sterile conical tubes
50 mL sterile conical tubes
6-cm (T-25) cell culture dish
96-well white flat bottom cell culture–treated plate (Corning cat. no. 3917)
Single channel pipettes (2 µL, 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL)
Multichannel pipettes (200 µL)
Plate reader (BioTek Cytation3 or equivalent)

Preparation of stable cell lines

Note: All steps should occur in laminar flow hood using sterilized pipettes and reagents. Refer to manufacturer protocols for complete description of Flp-In™-293 stable cell line generation.

1. Clone desired SmHT construct into pcDNA5/FRT following manufacturer 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 in appropriate media (DMEM, 10% FBS, 2 mM L-glutamine) with 100 μg/mL Zeocin™.
3. Co-transfect Flp-In™-293 cells with a 9:1 ratio of pOG44:SmHT-encoding pcDNA5/FRT plasmid using Lipofectamine LTX with PLUS reagent according to manufacturer protocol.
4. 24 hours after transfection, wash and add fresh media to cells.
5. 48 hours after transfection, split cells into culture media with 100 mg/mL hygromycin B.

   Split cells to low density (25% confluent) to ensure proper antibiotic selection.
6. Expand cells and change media every 2-3 days until the plate is confluent following manufacturer protocol.
7. Split cells once and prepare stocks using standard cell culture protocols.
8. Confirm SmHT expression by performing a Western Blot with the anti-HaloTag antibody.

Preparation of DNA for RiPCA

1. Clone the RBP-of-interest into a pcDNA3 vector containing LgBiT.

   It is advisable to clone both an N- and C-terminally tagged RBP as the location of the tag could influence assay results.
2. Extract the pcDNA3/RBP-Lg plasmid using either mini or midi-prep kits (QIAGEN) and elute the DNA or dissolve the final pellet in 50 μL water. Incubate DNA on ice.
3. Perform a follow-up ethanol precipitation by sequentially adding 10 μL ice-cold sodium acetate (3 M, pH 5.2) and 500 μL ice-cold ethanol (200 proof) to the DNA and mixing well. Incubate on ice (or at -20°C) for at least 30 minutes. Collect the precipitated DNA by centrifugation (45 minutes at 15,000 rpm, 4°C).

   Performing this ethanol precipitation step typically increases consistency of results.
4. Carefully remove supernatant and clean the pellet with 500 μL 70% ethanol. Re-collect the pellet by centrifugation (5-10 minutes at 15,000 rpm, 4°C).
5. Dry the pellet and dissolve in water.
6. Prepare a diluted stock of DNA (3.9 ng/μL) in a LoBind 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 of 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 probes

Note: All steps should occur in an RNase-free environment with sterilized pipettes and reagents. Surfaces and pipettes can be additionally cleaned with RNaseZap™.

1. Prepare a 1 mM stock of unlabeled RNA probe in PB8 (100 mM phosphate buffer, pH 8.0; see recipe).
2. In a fresh tube, mix equal volumes 1mM unlabeled RNA probe and 10 mM of either O2 or O4 HaloTag ligand (dissolved in DMSO) and incubate at room temperature for 2-3 hours.

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

3. 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. Collect the precipitated RNA by centrifugation (45 minutes at 15,000 rpm, 4°C).

   Volumes listed are sufficient for reaction volumes of 10 μL or less.

4. Dry the pellet and dissolve to ~1 mM in PB8.
5. Dilute to 50 μM in PB8 to be used in the 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 Lin28

Note: All steps should occur in laminar flow hood using sterilized pipettes and reagents.

1. Culture Flp-In™-293 cells expressing SmHT in appropriate media (DMEM, 10% FBS, 2 mM L-glutamine, 100 mg/mL hygromycin B).

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

2. 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 performed.

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

3. Prepare n number of solution B tubes, one for each transfection condition, by mixing 50 μL OptiMEM, 2.5 μL pcDNA3/Lin28A-Lg (3.9 ng/μL), and 0.3 mL RNA probe (50 μM) for a final concentration 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 9-37 pg/μL DNA and 0.1-0.6 μM RNA.

4. Add 50 μL of solution A to each solution B tube and mix well by pipetting up and down and let the mixture incubate for at least 15 minutes at room temperature.

5. During this incubation, harvest and count the Flp-In™-293 SmHT cells using standard cell culture methods.

6. Prepare one solution C tube for each transfection condition by diluting 400 μL of cells to a density of 1 x 10^5 cells/mL.

7. Following the 15-minute incubation, add 50 μL of solution A+B to solution C and mix well by pipetting up and down.

8. Plate 100 μL in 4 wells of a 96-well plate for each transfection condition.

9. Incubate the plate for 24 hours at 37°C in 5% CO_2.

Measure chemiluminescence and analyze data

Note this can be done outside of a laminar flow hood/on the bench.

1. Following the 24-hour incubation, aspirate and replace the media with 100 μL Opti-MEM.

2. Add 25 μL of Nano-Glo Live Cell Reagent (prepared as a 1:20 dilution of Nano-Glo Live Cell Reagent: Nano-Glo LCS Dilution Buffer).

3. After addition of Nano-Glo Live Cell Reagent, measure chemiluminescence using BioTek Cytation3 or equivalent 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.

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

[*Insert figure 3 near here]

REAGENTS AND SOLUTIONS

Use DNAse, RNAse free water in all recipes and protocol steps.

Phosphate buffer (PB8)

100 mM sodium phosphate (mix monobasic and dibasic phosphate in appropriate ratio for pH 8)
Adjust to pH 8 with NaOH or HCl

Sodium acetate

3 M sodium acetate
Adjust to pH 5.2 with HCl

COMMENTARY:

Background Information:

Previously, our group developed a plate-based, antibody-free biochemical assay, 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 reduced risk of compound interference (Garner & Janda, 2010; D. A. Lorenz & Garner, 2016; D. A. Lorenz, Kaur, et al., 2018; D. A. Lorenz, Song, & Garner, 2015; D. A. 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. The use of MS2 hairpins to recruit a MS2 coat protein fused to a split fluorescent reporter (e.g. split fluorophore or split fluorescent protein) enables detection of the RPI with a protein-of-interest that is labeled with the complementary reporter molecule (Huranova et al., 2009; M. Lorenz, 2009). While this strategy is attractive since 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 chemiluminescent-producing reporter. RiPCA utilizes NanoLuc, a split luciferase reporter composed of a small (SmBiT) and large (LgBiT) subunit (Dixon et al., 2016). Due to the low intrinsic affinity of Sm and LgBiTs, 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 which 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. 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 reaction 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 measurement for the DNA and RNA elements are within the accepted limits (~1.8 and ~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, N-hydroxysuccinimydl ester (NHS ester), is readily hydrolyzed in the presence of water or in higher than optimal pH (>8.5). It is best practice to ensure the use of dry DMSO to dissolve the HaloTag ligand and immediately make single-use aliquots to avoid reduction in coupling efficiency.

Troubleshooting:

Table 2. Troubleshooting Guide for RiPCA

Problem	Possible Cause	Solution
Low signal or signal/background	Poor binding of RBP and RNA probe or too little RNA probe	Re-visit 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

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 signal-to-background (S/B) as well as the consistency of the S/B. The average signal generated by the non-binding control, pre-miR-21 in the included example, is used as the denominator

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

Time Considerations

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

CONFLICT OF INTEREST STATEMENT:

There are no conflicts to declare.

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FIGURE LEGENDS:

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.

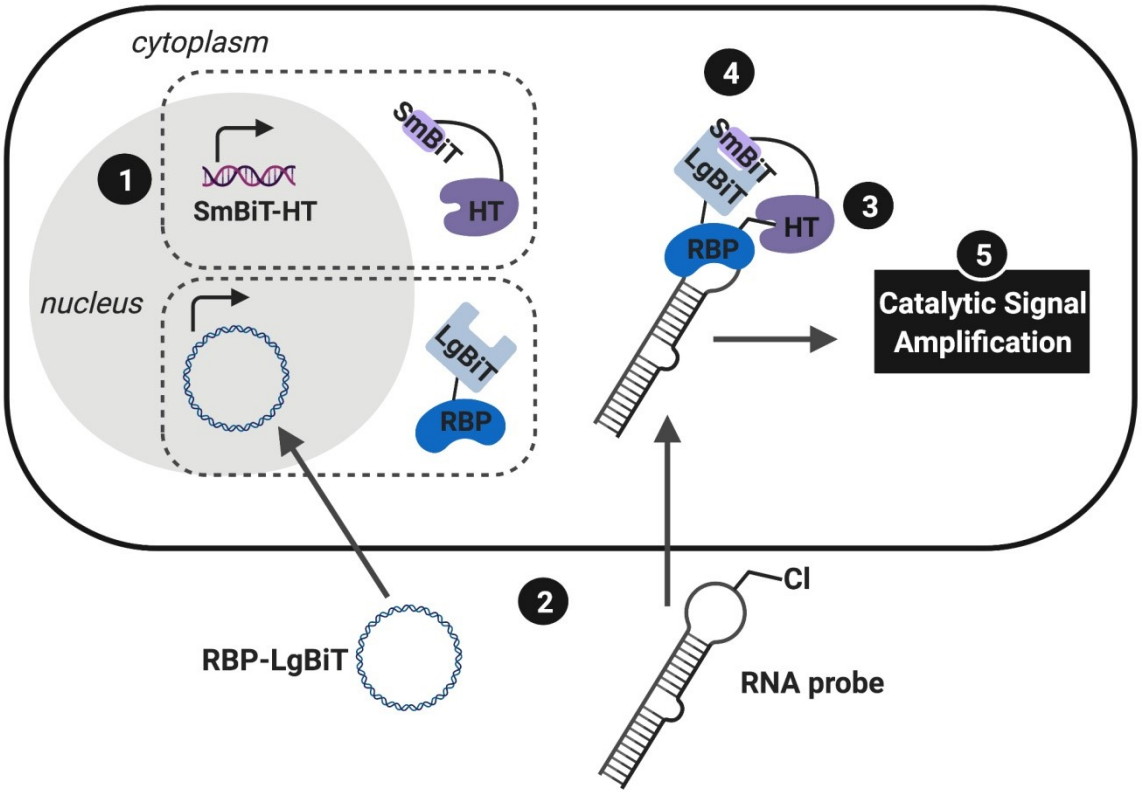

Figure 2. RiPCA transfection workflow. Solution of A is prepared, mixed with solution B, and incubated prior to its addition to solution C. Solution A+B+C is plated in 96-well plate. Reproduced from (Rosenblum et al., 2021) with permission from the Royal Society of Chemistry.

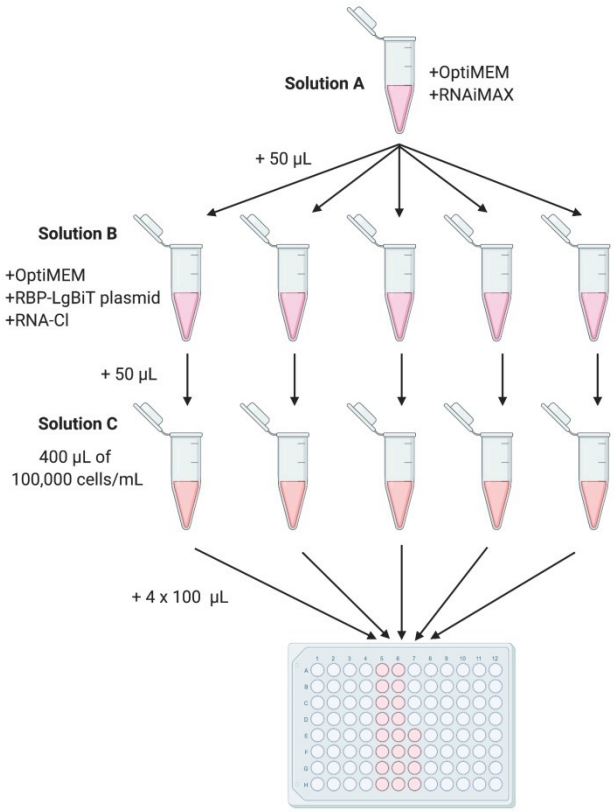

Figure 3. Example of RiPCA data characterizing the interaction between pre-let-7d and its RBP, Lin28A. Tabulated chemiluminescent data generated by RiPCA performed with Lin28A-LgBiT or LgBiT-Lin28A and pre-miR-21 and pre-let-7d in either the cytoplasm (left panel) or nucleus (right panel). The average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

	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