Chapter 9

Fluorescence-Based Methods for Characterizing RNA Interactions In Vivo

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Abstract

Fluorescence-based tools that measure RNA-RNA and RNA-protein interactions in vivo offer useful experimental approaches to probe the complex and dynamic physiological behavior of bacterial RNAs. Here we document the step-by-step design and application of two fluorescence-based methods for studying the regulatory interactions RNAs perform in vivo: (i) the in vivo RNA Structural Sensing System (iRS^3) for measuring RNA accessibility and (ii) the trifluorescence complementation (TriFC) assay for measuring RNA-protein interactions.

Key words RNA-RNA interaction, RNA-protein interaction, In vivo fluorescence assay, Hybridization efficacy, RNA accessibility, Complementation assay, RNA regulator, Protein regulator, Target network

1 Introduction

Recent years have been marked by identification and characterization of noncoding RNAs (ncRNAs), such as small RNAs (sRNAs) [1], in all types of bacteria, ranging from model strains such as *E. coli* to extremophiles like *D. radiodurans* [2] and other biotechnologically relevant organisms [3, 4]. The number of tools to probe RNA structure has grown in response to a deeper understanding of ncRNA roles in gene expression, regulatory cascades, and control of critical metabolic and cellular processes [5–7]. Nucleotide-specific chemical modification-based techniques like in vitro and in vivo SHAPE and DMS footprinting have uncovered structure-function paradigms such as sRNA-protein binding footprints [8], riboswitch conformation changes [9], and principles for designing synthetic RNA regulator parts [10]. However, these methods are limited because they do not represent true collective-nucleotide behavior of interactions and thus may overlook

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weak or low-frequency interactions [5]. The two techniques detailed in this work pose an advantage in this area by (i) reproducing RNA-RNA hybridization to capture regional intermolecular interaction preferences and (ii) directly measuring interactions between RNAs and proteins to elucidate physiological reasons for inaccessibility, i.e., structure versus protein occlusion.

This chapter details the design, preparation, and execution of two fluorescence-based methods for monitoring RNA interactions in vivo. The first measures the propensity of unique antisense RNAs (asRNAs) to interact with distinct regions within a target RNA of interest to offer insights into dynamic molecular behavior (Fig. 1a, b) [5]. Specifically, by regionally probing a target RNA in continuous 9–16 nucleotide-long segments, a profile of the RNA's hybridization landscape can be built. This technique, called the in vivo RNA Structural Sensing System (iRS^3) has been used to investigate hybridization landscapes of many RNA types, including *E. coli* sRNAs, mRNAs, and tRNAs as well as non-native RNAs known to host complex interactions. In this way, the iRS^3 captures structure-function information. Particularly, it has demonstrated sensitivity to known RNA-RNA and RNA-protein interactions [11]. The second technique probes RNA-protein interactions via a trifluorescence complementation assay (Fig. 1c, d). Making use of

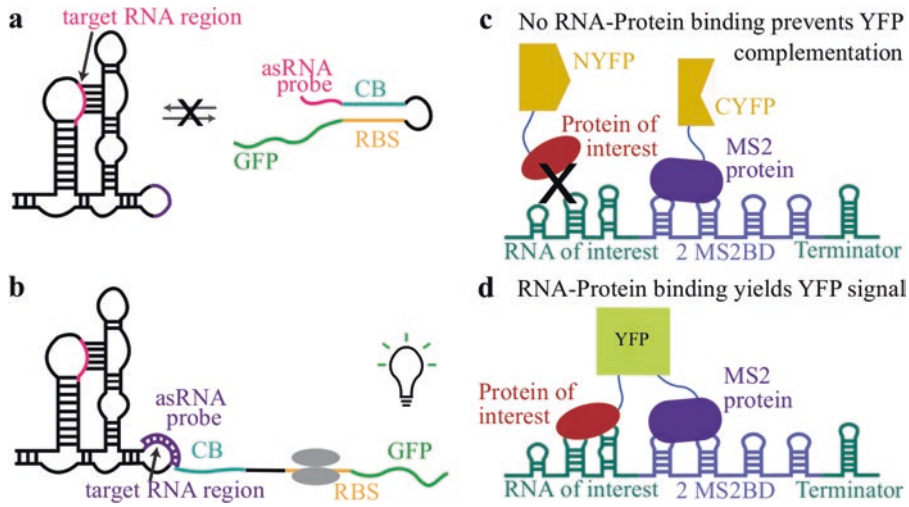

Fig. 1 Schematic of RNA hybridization (**a**, **b**) and RNA-protein probing (**c**, **d**) methods. (**a**, **b**) The iRS^3 assay evaluates the likelihood of an RNA region to engage in RNA-RNA interactions with its asRNA. If the region is inaccessible to the asRNA probe within the iRS^3 transcript, the ribosomal binding site (RBS) remains sequestered by the cis-blocking (CB) region (**a**). If the asRNA interacts with its target region, the hairpin loop of the iRS^3 is disrupted, and the ribosomal binding site is available for GFP translation initiation (**b**). (**c**, **d**) The trifluorescence complementation (TriFC) assay probes RNA-protein interactions by making use of three fusion constructs: the RNA of interest fused to a MS2 binding domain (MS2BD), the protein of interest linked to NYFP, and the MS2 protein linked to CYFP. Only in the presence of a RNA-protein interaction do all three components interact and allow for complementation of NYFP and CYFP to produce yellow fluorescence (**d**)

the high-affinity MS2 protein-MS2 binding domain interaction [12] and the well-established NYFP-CYFP split yellow fluorescent protein system [13], this assay has been used to quantify direct sRNA-protein and mRNA-protein interactions in vivo. In particular, the TriFC assay has quantified regulatory sRNA-protein interactions, capturing differential effects of protein active site mutations on the extent of YFP complementation [14]. Additionally, a dual-fluorescence variation of this assay has been used to test upward of 75 possible mRNA targets for protein binding [15].

We foresee these techniques having broad impact on our understanding of the in vivo functionality of sRNAs. For instance, the iRS^3 approach can be used to map and infer the in vivo functionality of an sRNA's alternative structures and track their relevance to regulatory function under various environmental conditions. Given that many sRNAs are known to confer bacterial virulence [16], this could provide a strong basis for targeted antimicrobial design. The TriFC assay could be applied to screen sRNAs for association with Hfq or any other regulatory proteins. Given the challenges of predicting RNA-protein interactions [17], methods amenable to large-scale screening should prove useful.

The iRS^3 method is capable of measuring regional hybridization efficacy of RNAs at basal levels; however, the TriFC assay requires overexpression of the RNA and protein involved. As with any overexpression system, questions of relevance remain, but with proper negative and positive controls, strong, physiologically relevant conclusions can be made.

2 Materials

2.1 iRS^3 General Materials and Reagents

1. Disposable pipette tips.
2. PCR tubes.
3. Nuclease-free water.
4. 1.7 mL polypropylene microtubes.
5. Thermocycler.
6. Vortex.
7. Microcentrifuge.
8. Luria Broth.
9. Agar.
10. Petri dishes.
11. 2.5–1000 μL pipette set.
12. 25 mL culture tubes.
13. Kanamycin stock solution: 10 or 100 mg/mL in nuclease-free water.

14. UV spectrophotometer.
15. Incubator, set to 37 °C with shaking capability.

2.2 iRS³ Target RNA Insertion

1. Gibson Primers Designed using NEBuilder Assembly Tool.
2. pO-iRS³GG or pN-iRS³GG vector (Addgene plasmids 98589 and 98858, respectively).
3. Deoxynucleotide Solution Mix (NEB).
4. Phusion High-Fidelity (HF) DNA polymerase (2000 U/mL, NEB).
5. 5× Phusion HF buffer (NEB).
6. DpnI (20,000 U/mL, NEB).
7. PCR DNA Purification Kit.
8. Gibson Assembly master mix (NEB or made according to [18]).
9. Electrocompetent *E. coli* cells for cloning (Turbo High Efficiency competent *E. coli*).
10. Plasmid miniprep kit.

2.3 iRS³ Probe asRNA Insertion

1. 50 ng pO-iRS³GG or pN-iRS³GG vector.
2. Linker buffer: 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA.
3. Primers diluted in linker buffer to 100 μM.
4. 10× T4 DNA ligase buffer (NEB).
5. T4 DNA ligase (400,000 U/mL, NEB).
6. Nuclease-free water.
7. Bsmb1/Esp3I (10 U/μL, NEB).
8. 0.025 μm nitrocellulose membrane filters.
9. Electrocompetent *E. coli* cells for cloning (Turbo High Efficiency competent *E. coli*).
10. 4-Chloro-DL-phenylalanine.

2.4 iRS³ Experiment

1. K-12 MG1655 *E. coli* or another strain of choice to conduct the assay.
2. Sterile 250 mL Erlenmeyer flasks OR Sterile 200 μL 96-well black clear-bottom plates.
3. Arabinose stock solution: 20% w/v, filter sterilized through 0.22 μm filter.
4. Anhydrotetracycline (aTc) stock solution: 100 μg/mL, filter sterilized through 0.22 μm filter.
5. 1× phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, sterilized through 0.22 μm filter.

6. 20 mL syringes with Leur lock tip.
7. Syringe-tip filters (0.22 μm PVDF 30 mm diameter).
8. 5 mL polystyrene round-bottomed tubes.
9. BD FACSFlow sheath fluid.
10. BD FACSCalibur Flow cytometer and BD CellQuest Pro software.

2.5 TriFC General Materials and Reagents

1. Disposable pipette tips.
2. PCR tubes.
3. 1.7 mL polypropylene microtubes.
4. Luria Broth.
5. Agar.
6. Petri dishes.
7. Kanamycin stock solution: 10 or 100 mg/mL in nuclease-free water.
8. Carbenicillin stock solution: 50 mg/mL in 1:1 nuclease-free water to ethanol.
9. IPTG stock solution (optional): 100 mM in nuclease-free water.
10. 2.5–1000 μL pipette set.
11. Sterile 25 mL culture tubes.
12. Sterile 250 mL Erlenmeyer flasks.
13. Vortex.
14. Microcentrifuge.
15. Thermocycler.
16. Incubator, set to 37 °C with 200 rpm shaking capability.

2.6 TriFC Cloning Supplies

1. Deoxynucleotide Solution Mix (NEB).
2. Nuclease-free water.
3. Phusion High-Fidelity (HF) DNA Polymerase (2000 U/mL, NEB).
4. 5× Phusion HF buffer (NEB).
5. Taq Polymerase (5000 U/mL, NEB).
6. 10× ThermoPol Buffer (NEB).
7. pTriFC (or pTriFC-mStrawberry) and pMS2-CYFP vectors (Addgene plasmids 98584 or 98848 and 98587, respectively).
8. Control pTriFC and pMS2-CYFP vectors (Addgene plasmids 98586, 98585, and 98588).
9. DpnI restriction enzyme (20,000 U/mL, NEB).
10. Gibson Assembly master mix (NEB or made according to [18]).

11. Electrocompetent *E. coli* cells for cloning (NEB 5-alpha or Turbo High Efficiency competent *E. coli*).
12. 0.025 μM nitrocellulose membrane filters.
13. Genomic DNA purification kit.
14. Miniprep kit.
15. PCR DNA purification and/or gel extraction kit.
16. UV spectrophotometer.

2.7 TriFC Experiment

1. K-12 MG1655 *E. coli* or another strain of choice to conduct the assay.
2. 1× phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, sterilized through 0.22 μm filter.
3. Nanopure water.
4. 20 mL syringes with Leur lock tip.
5. Syringe-tip filters (0.22 μm PVDF 30 mm diameter).
6. Biotek Cytation 3 Imaging Reader with Gen5 software.
7. Black 96-well clear flat bottom assay plates.
8. BD FACSCalibur Flow cytometer with BD CellQuest Pro software.
9. BD FACSFlow sheath fluid.
10. 5 mL polystyrene round-bottomed tubes (Note that **items 6** and **7** or **8–10** are required).

3 iRS³ Method

The iRS³ plasmid-based system can be used to investigate the propensity of a synthetic asRNA to interact with its complementary target region on distinct heterologously expressed RNA (pO-iRS³GG plasmid, Fig. 2a) or native RNA (pN-iRS³GG plasmid, Fig. 2b) in vivo, a measurement termed hybridization efficacy [11]. Specifically, the iRS³ system consists of a target region-specific asRNA probe upstream of a hairpin-forming loop and green fluorescent protein (GFP) reporter (Fig. 1a). This asRNA probe is specifically designed to be complementary to a target RNA region of interest. If, upon iRS³ expression, the synthetic asRNA probe is able to bind to its corresponding target RNA region, disruption of the hairpin loop causes exposure of the GFP ribosomal binding site and consequent GFP translation (Fig. 1b). This phenomenon allows the hybridization likelihood of a target RNA region to be quantified by fluorescence shift as measured using flow cytometry [5]. As the system directly mimics in vivo RNA-RNA interactions, results are believed to speak to the structural conformation and availability of RNA regions for regulatory purposes.

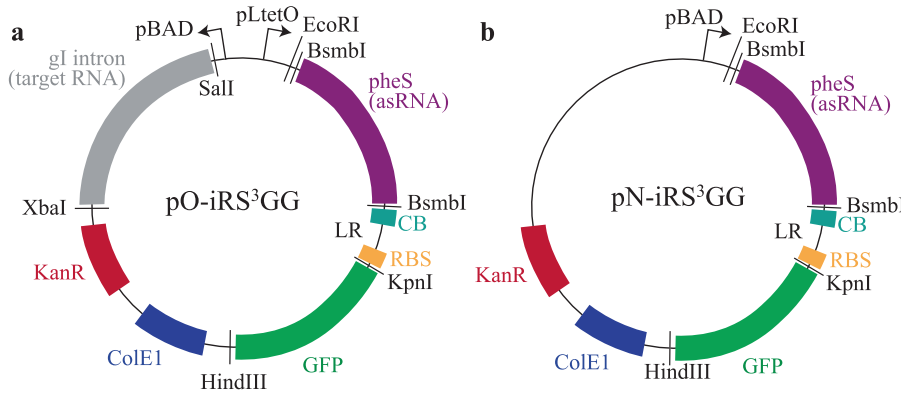

Fig. 2 Overexpression (pO-iRS^{3}GG) or and native (pN-iRS^{3}GG) iRS^3 assay plasmids. (**a**) The pO-iRS^{3}GG plasmid contains the gI intron (default target RNA) expressed by a pBAD promoter. The iRS^3 transcript, consisting of the pheS cassette (to be replaced by an asRNA), cis blocking region (CB), ribosomal binding site (RBS), then GFP, is under pLtetO control. (**b**) The pN-iRS^{3}GG plasmid differs from the O-iRS^3 in that (i) it lacks target RNA overexpression capabilities and (ii) the iRS^3 transcript expression is under pBAD promoter control

As illustrated in Fig. 3, the iRS^3 parent vectors (Fig. 2) must be modified for each unique RNA region being targeted. Depending on whether the RNA expression levels are heterologous or basal, necessary cloning will differ. In the case of heterologous target RNA expression, (i) the target RNA is cloned into the plasmid following the pBAD promoter to replace the default target RNA in pO-iRS^{3}GG (*Tetrahymena* group I, gI, intron) via Gibson Assembly (Fig. 3a). Depending on plasmid chosen, (ii) the asRNA probe is cloned in to follow either the pLtetO (pO-iRS^{3}GG) or pBAD (pN-iRS^{3}GG) promoter via a high-throughput Golden Gate cloning protocol in which the asRNA sequence replaces a 4-chloro-DL-phenylalanine negative selection cassette (pheS) (Fig. 3b). Once the plasmids have been (iii) transformed into the strain of interest (Fig. 3c), (iv) strains are cultured. During early log growth (OD ~ 0.2–0.4), (v) strains are separated into "uninduced" and "induced" samples, the latter in which expression of target RNA and iRS^3 system (pO-iRS^{3}GG) or iRS^3 system only (pN-iRS^{3}GG) is stimulated (Fig. 3d). At the environmental conditions of interest (Fig. 3e), (vi) fluorescence shift between uninduced and induced samples is evaluated via flow cytometry (Fig. 3f) and analyzed (Fig. 3g).

3.1 Rational Experimental Design

RNA regions with considerable overlap have been shown to exhibit significant differences in hybridization efficacy [11]. This is not surprising, considering that binding between single-stranded loops or linear segments of RNA has been implicated in RNA-RNA strand displacement [19]. These observations further support the notion that this hybridization-based system adequately mimics regulatory in vivo antisense-based RNA-RNA interactions, despite

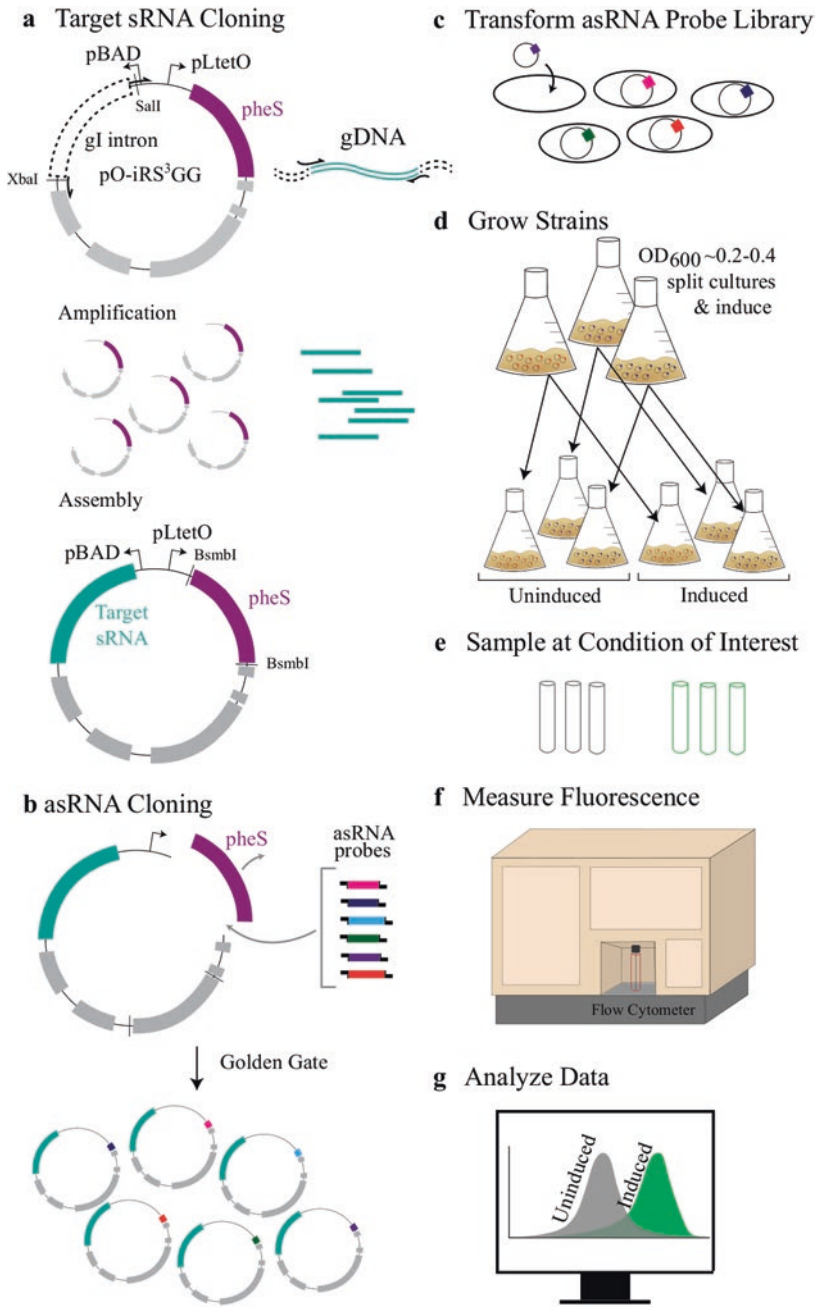

Fig. 3 Work flow for the iRS^3 assay. (**a**) If using pO-iRS^{3}GG, standard two-fragment Gibson cloning is used to amplify and insert the target RNA of interest to replace the default target RNA (gI intron). (**b**) For either pO-iRS^{3}GG or pN-iRS^{3}GG, the pheS selection cassette is replaced by desired asRNAs via Golden Gate cloning via BsmbI restriction digest to create a library of iRS^3 transcripts. (**c**–**g**) Experimental work flow. (**c**) Plasmids are transformed into an *E. coli* strain of choice and grown in triplicate. (**d**) Cultures volumes are split into 2 containers per replicate and the expression of iRS3 transcripts (pO-iRS^{3}GG and pN-iRS^{3}GG) and target RNAs (pO-iRS^{3}GG only) are induced for each representative strain. (**e**) At conditions of interest, cultures are sampled and (**f**) assayed via flow cytometry for fluorescence. (**g**) Finally, induced and uninduced fluorescence data are analyzed compared to an appropriate negative control

the bulkiness of the iRS^3 transcript. Thus, selection of target regions, especially in an uncharacterized RNA, is critical to obtaining valuable hybridization efficacy insights. To this end, we offer loose guidelines. Generally, target RNA regions under investigation should (i) be 9–16 nt long to mimic lengths of seed sequences and known RNA-RNA interactions [20, 21]. Additionally, target regions should be chosen to ensure cognate probe asRNAs (ii) show low complementarity to the genome (besides the target of interest) (i.e., top BLASTn [22] search hits with Expect (E) value ≥1) and (iii) contribute to iRS^3 transcript folding in a previously determined optimal free energy range ($-19.3 < \Delta G < -17.8$ kcal/mol) [11].

Many target region selection schemes will offer valuable insights to the hybridization landscape of an RNA molecule. A few are listed below.

1. Structural Prediction Based Design: Secondary structure predictions may be used to inform probe design. Specifically, we have designed experiments in which the standard deviation of the base pairing probability (as evaluated via Nupack [23]) of each target RNA region is minimized within our length constraint (9–16 nt).
2. Blind Design: If characterization information is sparse, it may be valuable to walk the RNA with overlapping probes, as probes with considerable overlap (≥6 nt) have shown vastly different propensity for hybridization [11]. This design method has been used in previously published work to blindly create a pool of potential asRNA probes [11]. Specifically, random-length regions (within our length constraint) were selected in a manner to represent all possible overlaps and cover the entire length of the RNA of interest. These regions were then filtered based on iRS^3 transcript folding energy (*see* iii under Rational Experimental Design above). A simpler alternative to this blind design would be the exclusion of overlap.

3.2 Target RNA Insertion

Overexpression of the target RNA may be useful if (i) evaluating the functional structure of non-native sRNAs or (ii) titratable control of RNA expression is desired. When using pO-iRS^{3}GG, Gibson Assembly is recommended for insertion of the DNA encoding for the desired target RNA.

1. We recommend designing primers for two-fragment Gibson Assembly of the designated target RNA and pO-iRS^{3}GG using NEBuilder Assembly Tool. Specifically, preferences may be set to Product/Kit = E2611 Gibson Assembly Master Mix, No. of Fragments = 2–3, Total Construct Length = less than 10 kb, Min. Overhang Length (nt) = 20, PCR Product Group = Phusion, PCR Product—Phusion High-Fidelity DNA Polymerase (HF Buffer), PCR Primer Conc. (nM) = 500

(standard), Min. Primer Length (nt) = 18. An example cloning scheme for the replacement of default *Tetrahymena* gI intron with LtrB gII intron [11] is depicted in Fig. 4 and corresponding primer sequences listed in Table 1.

2. Assemble PCR amplification reaction for DNA encoding the target RNA of interest from *E. coli* genomic DNA, previously prepared plasmids, or synthesized DNA fragments using forward and reverse insert primers, with overhang complementary to the vector backbone (*see* example in Fig. 4). Add 50 ng genomic DNA, 10 μM forward and reverse insert primers (2.5 μL each, diluted in nuclease-free water), 10 mM dNTPs (1 μL), 5× Phusion High-Fidelity (HF) buffer (10 μL), Phusion HF DNA polymerase (0.5 μL), and nuclease-free water to 50 μL. Genomic DNA can be obtained with a genomic DNA purification kit or by boiling a colony (single colony, diluted in 50 μL of nuclease-free water, heated at 96 °C for 5 min); 2 μL of this reaction can be used above as genomic DNA template.
3. Assemble PCR amplification reaction for pO-iRS^{3}GG backbone with forward and reverse backbone primers (*see* example in Fig. 4) designed above. Add 50 ng of parent vector (pO-iRS^{3}GG), paired forward and reverse backbone primers (2.5 μL of each, diluted in nuclease-free water), 10 mM dNTPs (1 μL), 5× Phusion High-Fidelity (HF) buffer (10 μL), Phusion HF DNA polymerase (0.5 μL), and nuclease-free water to 50 μL.
4. Cycle all PCR reactions as follows: (i) 98 °C for 30 s (ii) 25 cycles of 98 °C for 10 s, 3° above lowest melting temperature of primer pairs (excluding overhang region) for 30 s, 72 °C for 2.5 min (vector backbone) or 30 s/kb (iii) final extension 72 °C for 10 min (iv) hold at 4 °C (*see* **Note 1**).
5. Digest methylated DNA by adding 1.5 μL DpnI directly to PCR products, incubating at 37 °C for 90 min, then heat deactivating at 80 °C for 20 min.
6. Agarose gel electrophoresis is recommended to confirm the size and amplification of backbone and insert. Using approximately 5.0 μL of each reaction (with EZ Vision or loading dye and ethidium bromide), check for proper band size and amplification specificity (pO-iRS^{3}GG backbone at ~4.5 Kb).
7. Clean-up PCR reactions per PCR DNA purification kit directions. Measure DNA concentrations via spectrophotometry.
8. Insert DNA of target RNA via Gibson fragment assembly. Add 50–100 ng amplified vector backbone, 2× amplified target RNA insert (5× if insert is smaller than nucleotides), and Gibson Assembly Master Mix (10 μL), and nuclease-free water to 20 μL. Additionally, perform the assembly with a negative control omitting the insert: 50–100 ng of amplified vector, Gibson Assembly Master Mix (10 μL), and nuclease-free water to 20 μL. Incubate samples at 50 °C for 45 min.

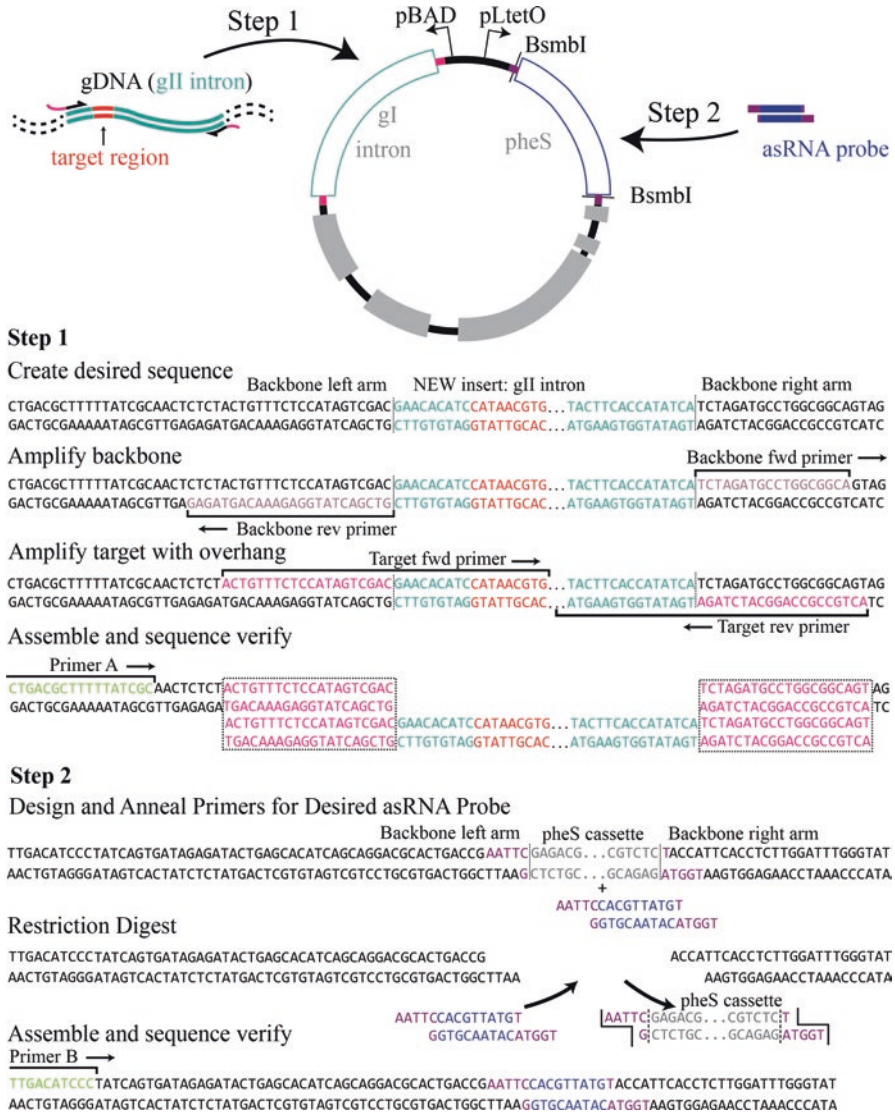

Fig. 4 Example cloning schemes for insertion of target RNA and asRNA probe into pO-iRS^{3}GG. Cloning of target RNA (**step 1**) and a representative cognate asRNA probe (**step 2**) into pO-iRS^{3}GG. Necessary overhangs for target RNA cloning via Gibson and asRNA probe cloning via Golden Gate are shown in pink and purple, respectively. The target region within the DNA of target RNA sequence (turquoise) is shown in orange. DNA corresponding to the asRNA probe, targeting desired region (orange), is pictured in blue. Target RNA sequences and primers corresponding to the cloning scheme example are listed in Table 1

9. Dilute products fourfold in nuclease-free water and use 1 μL for *E. coli* electroporation using standard protocols. After recovery period has elapsed, plate on LB agar with 50 μg/mL kanamycin and let grow overnight at 37 °C (Day 1).
10. Continue to sequencing if significantly greater number of colonies is observed on sample plate than on negative control plate (Day 2). Grow one to two colonies to saturation

Table 1
Example RNA sequences, corresponding Gibson Assembly primers, and recommended sequence confirmation primers for pO-iRS^{3}GG and pN-iRS^{3}GG

Sequence or primer name	Sequence (5′ overhangs in lower case)
Target RNA	GAACACATCCATAACGTG...TACTTCACCATATCA
Target RNA region of interest	CATAACGTG
asRNA probe sequence	CACGTTATG
Backbone Rev. primer	GTCGACTATGGAGAAACAGTAGAG
Backbone Fwd primer	TCTAGATGCCTGGCGGCA
Insert (Target RNA) Fwd primer	actgtttctccatagtcgacGAACACATCCATAACGTG
Insert (Target RNA) Rev. primer	actgccgccaggcatctagaTGATATGGTGAAGTAGGGAG
Primer A	CCATAAGATTAGCGGATCCTACCTGACGCTTTTTATCGC
Primer B	CGAGTCCCTATCAGTGATAGAGATTGACATCCC

(overnight, 37 °C) in separate 5 mL tubes of LB supplemented with 50 μg/mL kanamycin per unique target RNA insert (Day 2).

11. The following day, extract plasmid DNA of 2–3 mL saturated culture using plasmid miniprep kit. Measure purified DNA concentration via spectrophotometry. Prepare and submit sequencing samples as prescribed by local facility (Day 3). A forward pBAD primer (Primer A) is recommended for sequencing inserted target RNAs (binding location and sequence shown in Fig. 4 and Table 1).

3.3 asRNA Probe Insertion

Both plasmid systems that can be used for experimentation (pO-iRS^{3}GG and pN-iRS^{3}GG) are Golden Gate cloning compatible. As previously described [24], the cloning design supports elimination of strain sensitivity to 4-chloro-DL-phenylalanine once the pheS selection cassette is replaced by sequences complementary to target RNA regions of interest, referred to as "asRNA probes" [11]. Cloning schemes for the insertion of an example asRNA probe targeting a region in the heterologous and "native" gII intron are shown in Figs. 4 and 5, respectively, and corresponding primer sequences listed in Table 1.

1. asRNA probe primers must be designed to contain the full forward and reverse complement of the target RNA region flanked by BsmbI-compatible sites. Specifically, (i) the forward asRNA Probe Primer should include AATTC(reverse complement of target region sequence)T and (ii) the Reverse asRNA Probe Primer should include TGGTA(target region sequence)G.

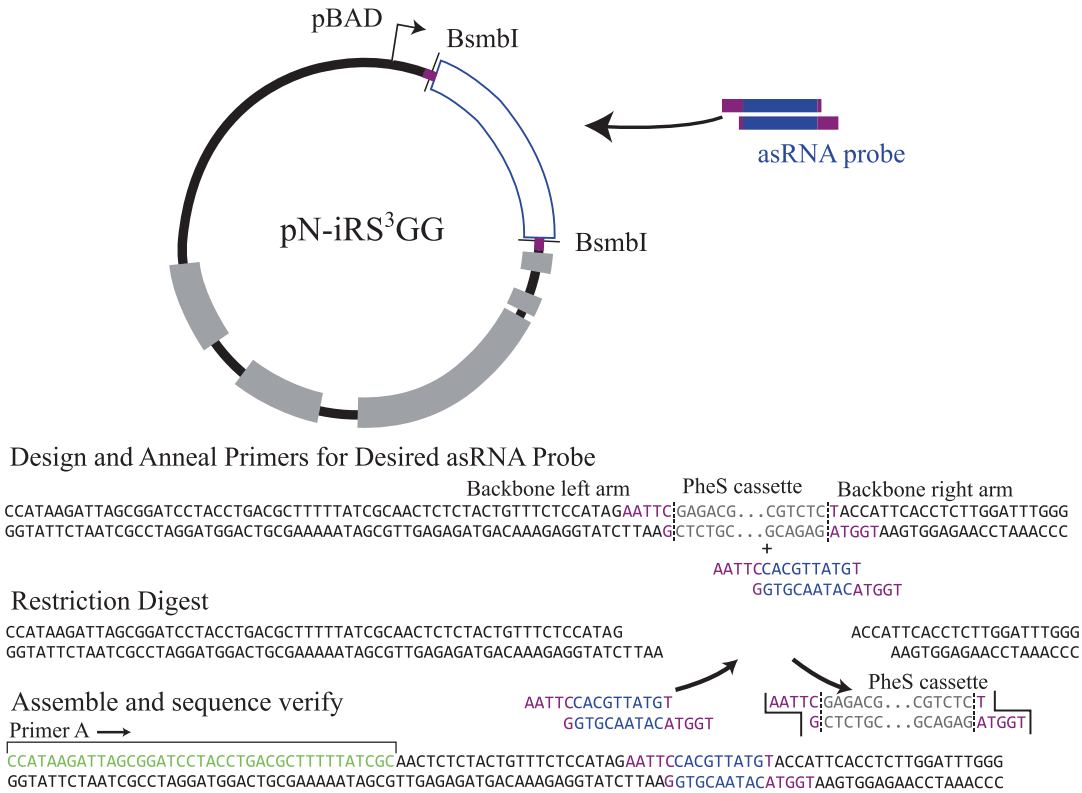

Fig. 5 Example cloning schemes for insertion of asRNA probe into pN-iRS³GG. Cloning of an asRNA probe targeting a region within a natively expressed target RNA into pN-iRS³GG. Necessary restriction sites and DNA corresponding to the asRNA probe are pictured in purple and blue, respectively. Necessary Golden Gate overhang and consecutive cloning steps are pictured. Target RNA sequences and primers corresponding to the cloning scheme example are listed in Table 1

2. In the case that (i) all samples needed for an experiment are split over multiple days or (ii) samples represent multiple unique environmental conditions, control iRS³ transcripts should be used to account for instrument shift or environment-related fluorescence changes, respectively. Specifically, the controls should represent the largest possible fluorescence range. The low-fluorescence "scramble" iRS³ transcript should (i) contain a probe which represents the average length of the target regions within the experimental set and (ii) contain a random sequence, i.e., a "scramble probe," with limited sequence similarity to the genome (i.e., top BLASTn [22] search hits with E value ≥1). The high-fluorescence "open RBS" iRS³ transcript should: (i) contain mutations in the cis-blocking (CB) region of the iRS³ transcript to render the RBS (seen in Fig. 1a) consistently accessible and (ii) contain a "scramble probe" asRNA (*see* low range iRS³, above). Sequences for control iRS³ transcripts previously used can be found in Table 2.

Table 2
Standard asRNA probe and CB region sequences for iRS³ controls

Control name	asRNA sequence	CB sequence
Scramble	CAGCGACAATATCGT	TACCATTCACCTCTTGGAT
Open RBS	CAGCGACAATATCGT	GCATAAATTAGGGAGTCAA

3. Anneal primers. First, dilute primers to 100 μM in linker buffer: 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA. Combine 10 μL of forward and reverse primers in unique PCR tubes for each asRNA probe to yield 20 μL total. Thermocycle as follows: (i) 95 °C for 2 min, (ii) 52 °C for 10 min, (iii) hold at 4 °C. Meanwhile, dilute pO-iRS³GG or pN-iRS³GG to 50 ng/μL in nuclease-free water.
4. Insert asRNA probe via Golden Gate cloning. In PCR tubes, add diluted (50 ng/μL) vector (1 μL), annealed primers (2 μL of 100 μM solution), 10× T4 DNA Ligase Buffer (NEB) (1 μL), nuclease-free water (3 μL), 400 U T4 DNA Ligase (NEB) (1 μL), 20 U BsmbI (NEB) (2 μL) (*see* **Note 2**). Incubate at 37° for 45 min.
5. Transform Golden Gate reaction. First, desalt reaction for 20 min on 0.025 μm nitrocellulose membrane filters. Electroporate approximately 5 μL into *E. coli* using standard protocols. Upon outgrowth, plate onto LB agar plates supplemented with 50 μg/mL kanamycin and 2 g/L 4-chloro-DL-phenylalanine. These selection markers will not facilitate growth of bacteria lacking kanamycin resistance or maintaining the pheS cassette, respectively. Let grow overnight at 37 °C (Day 1) (*see* **Note 3**).
6. On the following day, confirm insertion of the asRNA probe. Grow two colonies *for each unique asRNA cloned* to saturation (overnight, 37 °C) in separate 5 mL tubes of LB supplemented with 50 μg/mL kanamycin (Day 2).The next day, extract plasmid DNA of 2–3 mL saturated culture using a plasmid miniprep kit. Measure purified DNA via spectrophotometry. Prepare and submit sequencing samples as prescribed by local facility (Day 3). Forward primers corresponding to iRS³ asRNA promoters (pBAD for pN-iRS3GG (Primer A) and pLtetO for pO-iRS3GG (Primer B)) are recommended (binding locations shown in Figs. 4 and 5, respectively, and sequences listed in Table 1).

3.4 iRS³ Experiment

1. Select an experimental strain. The iRS³ system was developed and exclusively utilized in *E. coli* K-12 MG1655; however, no predetermined hindrances to using this system in other *E. coli* strains exist (*see* **Note 4**). We foresee value in performing iRS³

experiments in genomic sRNA-knockout strains to allow titratable control of sRNA expression that may have an impact on the hybridization landscape. Each strain containing a piRS^{3}GG (O- or N-) that targets a unique RNA region should have at least biological triplicate representation. If splitting samples into multiple experiments, fluorescence shift of strains containing control iRS^3 plasmids should be evaluated at every sampling instance in order to account for instrument shift (scramble and open RBS).

2. When the experimental strain has been selected, transform all confirmed iRS^3 plasmids via standard $CaCl_2$ transformation protocols (or electroporation if desired). Plate each strain on unique kanamycin-containing (50 μg/mL) LB agar plates. Grow at 37 °C overnight.
3. Two distinct cell culture schemes have been successfully used for iRS^3 experimentation and will influence the preparation of overnights with experimental strains. Specifically, cultures supplemented with 50 μg/mL kanamycin can be grown in (i) 40 mL volumes in 250 mL shake flasks (split into 20 mL at induction) [5] or (ii) 200 μL volumes in 200 μL 96-well black clear-bottom plates (split into 100 μL at time of induction) [11] (*see* **Note 5**). Plates are recommended when more than 16 unique strains will be sampled at once, to limit required incubator space and maximize efficiency of seeding and inducing (as it supports use of multiwell pipettes) (*see* **Notes 6** and **7**). Once culturing method has been selected, overnight culturing volumes can be adjusted according to experimental culturing (i.e., 5 mL for flasks and 100 μL for plates). Grow 3 unique colonies from each strain to saturation at 37 °C, 200 rpm overnight (Day 1).
4. Prepare all necessary materials for iRS^3 experiment (Day 1). Specifically, prepare in advance (i) 20% w/v arabinose, (ii) 100 μg/mL aTc, and (iii) LB supplemented with 50 μg/mL kanamycin (*see* **Notes 8** and **9**).
5. On Day 2, inoculate flasks (40 mL LB) or plates (200 μL LB) with saturated culture by adding 1% of total container volume. Shake-incubate at 37 °C and 200 rpm.
6. After approximately 2 h, in early exponential phase (OD ~ 0.2–0.4), split each culture volume into two equal volumes—to serve as uninduced and induced samples. Induce expression of iRS^3 transcript and, if applicable, the target RNA (pO-iRS^{3}GG only) for *designated induced* samples only. For pN-iRS^{3}GG, induce with 20% arabinose for 0.8% final concentration in culture; for pO-iRS^{3}GG, induce with 20% arabinose for 0.8% final concentration in culture and 100 μg/mL aTc for 100 ng/mL final concentration in culture. Continue to shake-incubate at 37 °C and 200 rpm (*see* **Note 10**).

7. At desired sampling OD (at least ~30 min post-induction and with OD < ~2), begin sampling for flow cytometry. Resuspend a small volume of each culture (~1–20 μL, depending on OD at sampling time) in 1 mL of 1× PBS (in 5 mL polystyrene round-bottomed tubes) to achieve a concentration of ~10^7 cells/mL. If testing the strain fluorescence at multiple ODs, return samples to shake-incubator. Evaluate green fluorescence of samples using a flow cytometer (530/30 nm band pass filter on the Benton Dickinson FACSCalibur). Collect fluorescence data of sample until representation of >150,000 active cells has been achieved (*see* **Note 11**).

3.5 Data Analysis

As fluorescence results are expected have a non-normal distribution, median fluorescence is assumed representative of the population. The hybridization efficacy of a target RNA region is defined as the average logarithm of the ratio of the induced to uninduced (background) fluorescence. Because each unique induced sample has a corresponding uninduced sample originating from the same biological replicate, statistics are performed on the ratio (as opposed to each uninduced and induced fluorescence individually) and uncertainty is propagated through to the logarithm of the ratio. Two normalization schemes may be useful for comparing hybridization efficacies to draw conclusions about structure-function relationships (i) within the same molecule when probed under unique growth/environmental conditions which are likely to influence target RNA abundance or (ii) between unique molecules under equivalent growth/environmental conditions.

3.5.1 Intra-RNA Normalization

When evaluating the hybridization efficacy changes of an RNA under various conditions, it may be difficult to separate abundance effects from structure-function effects. This is particularly relevant to sRNAs because of their aptitude to differentially express between conditions associated with their function [25]. One approach for decoupling structure-function insights from abundance effects normalizes each sRNA dataset between the regions exhibiting the highest and lowest observed hybridization efficacies. Specifically, for each sRNA in each unique condition, hybridization data corresponding to the region exhibiting the highest and lowest observed fluorescence ratios will be transformed to 1 and 0, respectively. Uncertainty is propagated to account for errors in the minimum/maximum.

3.5.2 Inter-RNA Normalization

To compare absolute hybridization efficacies between RNAs within unique environmental conditions (i.e., experiments were performed on separate days), normalization to low-end fluorescence (scramble probe) and high-end fluorescence (open RBS) controls is critical. This normalization scheme accounts for instrument shift between days and facilitates comparison of absolute hybridization landscape differences between molecules.

4 TriFC Method

The TriFC assay detects direct RNA-protein binding interactions in vivo by simultaneously expressing three distinct fusions: the RNA of interest with a MS2 RNA binding domain (MS2BD) sequence, the protein of interest with NYFP, and the viral MS2 RNA binding protein with CYFP (Fig. 1c, d). The well-characterized MS2-MS2BD binding interaction is strong (K_d = 3 nM [12]) and renders a direct RNA-protein binding interaction responsible for NYFP-CYFP complementation and thus fluorescent signal generation. Several steps are required to apply the TriFC assay to any RNA-protein interaction of interest: (i) the RNA-MS2BD and protein-NYFP fusions are designed and cloned into the appropriate plasmid by Gibson Assembly, (ii) the two plasmid system is sequentially transformed into a desired *E. coli* strain, (iii) strains are seeded and grown for 18–48 h prior to (iv) fluorescence measurements by plate reader or flow cytometry and (v) data analysis. Construct design, recommended cloning procedures, experimental execution, and data analysis are documented for the TriFC assay below.

A variation of the TriFC assay has also been demonstrated; it includes a third fluorescent protein, mStrawberry, fused to the 3′ end of the RNA fusion construct, to monitor function of RNAs containing Shine-Dalgarno and translation start site sequences. In this way, the TriFC assay can be made particularly useful for measuring interactions between regulatory elements of mRNAs, like 5′ untranslated regions (UTRs), and suspected protein interaction partners. Outside of construct design, this dual-fluorescence variation of the TriFC assay differs from the original assay only in fluorescence measurement and data analysis procedures, which are documented in Subheading 5.

4.1 Construct Design

The TriFC RNA-protein interaction assay uses a two-plasmid system to express three fusion constructs: (i) protein-linker-NYFP, (ii) RNA-MS2BD, and (iii) MS2-linker-CYFP (Fig. 1c, d). The first two components are expressed on the same plasmid, pTriFC, under control of separate pLacO promoters (Fig. 6a). The third is expressed under pLacO control on a second plasmid, pMS2-CYFP (Fig. 6b). Importantly, this design pairs one universal plasmid with one modular plasmid that can be adapted to any RNA-protein interaction of interest. Based on previous successes, we do not anticipate modification the MS2-CYFP fusion being necessary for testing most RNA-protein interactions [14, 15, 26].

When adapting pTriFC for a specific protein-RNA interaction, tag location is a critical consideration as both the RNA and protein of interest need to retain biological function in their respective fused states. An excellent starting place for design of the protein-NFYP fusion is previously published tag placements for the protein

of interest. For instance, in design of a CsrA protein-NYFP fusion [14], the C-terminus of CsrA was linked to NYFP because this arrangement had been shown successful for HIS_6 tagged constructs (*see* **Note 12**) [27, 28]. Regarding linker selection, general guidelines for constructing protein fusions should be considered [29]. Three repeats of a glycine-serine linker (GGGGS) have been successfully used for the protein-NYFP fusion [14, 15]; this linker composition and length imparts sufficient flexibility to the construct (*see* **Note 13**). We foresee that this linker composition and length will be amenable to other protein-NYFP fusions as well (*see* **Note 14**).

Similarly, design of the RNA-MS2BD fusion requires consideration of where to place the MS2BD sequence relative to the RNA of interest and how many repeats of MS2BD to include. Two locations of MS2BD insertion have been demonstrated. In the first instance, the MS2BD sequence was placed in the 3′ portion of an sRNA of interest, between the last predicted non-terminator RNA structure and the terminator hairpin (Fig. 1c, d and Fig. 6a). This selection was made in light of known sRNA co-transcriptional folding patterns (*see* **Note 15**) [14]. Alternatively, a 5′ placement of the MS2BD sequence before an sRNA sequence can be considered [15], particularly if functional 3′ degradation products of the sRNA are known or suspected (*see* **Note 16**). Additionally, two repeats of the MS2BD sequence should be included for either placement, as this maximized RNA-protein interaction signal in the proof-of-concept study [14]. As such, we recommend placing two repeats of the MS2BD sequence (noted as 2MS2BD) flanking the 5′ end of the sRNA of interest if 3′ degradation products are anticipated. Similarly, we suggest inserting the 2MS2BD sequence 3′ of the sRNA of interest, just before the sRNA's terminator, if critical and complex co-transcriptional folding the sRNA is anticipated.

Once designs of the fusion constructs are established, the following series of control constructs should be included to ensure fluorescent signal is from true RNA-protein interactions: (i) pTriFC lacking the protein of interest (NYFP + RNA-2MS2BD present), (ii) pTriFC lacking the RNA of interest (protein-NYFP + 2MS2BD present), and (iii) pMS2-CYFP lacking the MS2 protein. The pTriFC and pMS2-CYFP plasmids, as well as the no-protein pTriFC, no-RNA pTriFC, and no-MS2 pMS2-CYFP control plasmids, are available from Addgene.

4.2 Cloning Procedures

The pTriFC plasmid is amenable to restriction digest and Gibson Assembly cloning approaches. The Gibson Assembly strategy is the focus of this section as it is the most amenable to the large-scale cloning required for screening applications. Cloning procedures are described in reference to Fig. 7, which illustrates an example cloning scheme for adding the CsrA protein and the CsrB

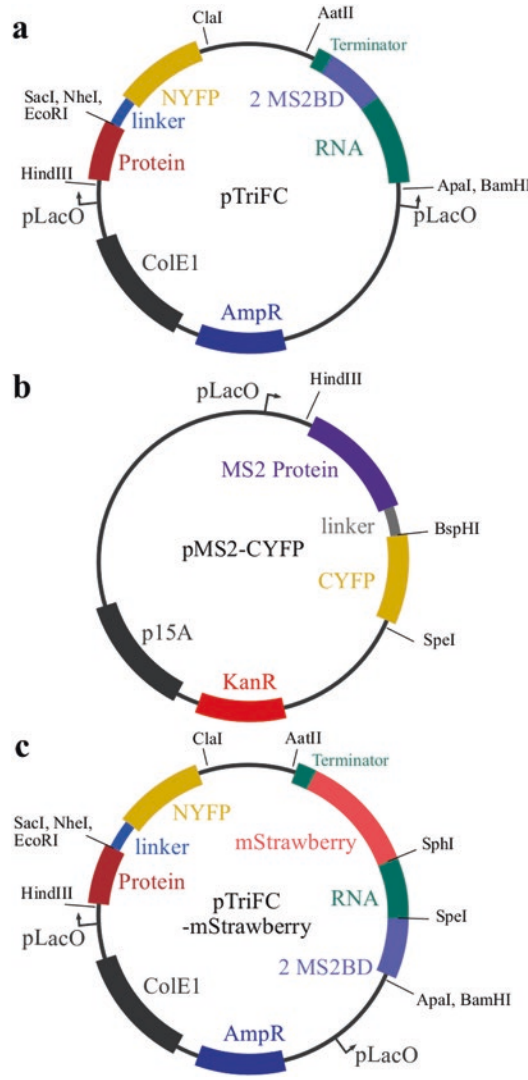

Fig. 6 Plasmids involved in TriFC assay. (**a**) The pTriFC plasmid expresses the protein of interest fused by a linker to NYFP from a pLacO promoter and the RNA fusion from a distinct pLacO promoter. Two repeats of the MS2BD sequence are inserted at the 3′ end of the RNA of interest, just before the RNA's terminator sequence. (**b**) The pMS2-CYFP plasmid contains the MS2-linker-CYFP fusion expressed by a pLacO promoter. (**c**) The pTriFC-mStrawberry plasmid is used in the dual fluorescence version of the complementation assay. It differs from pTriFC in design of the RNA fusion; two repeats of the MS2BD sequence are fused upstream of the RNA of interest and an mStrawberry sequence is fused just downstream of the RNA of interest to confirm functional expression

sRNA to pTriFC. We recommend designing primers for separate two-fragment Gibson Assembly reactions (using the NEBuilder Assembly Tool) to independently insert the protein of interest (Fig. 7, **step 1**) and the RNA of interest (Fig. 7, **step 2**) into pTriFC.

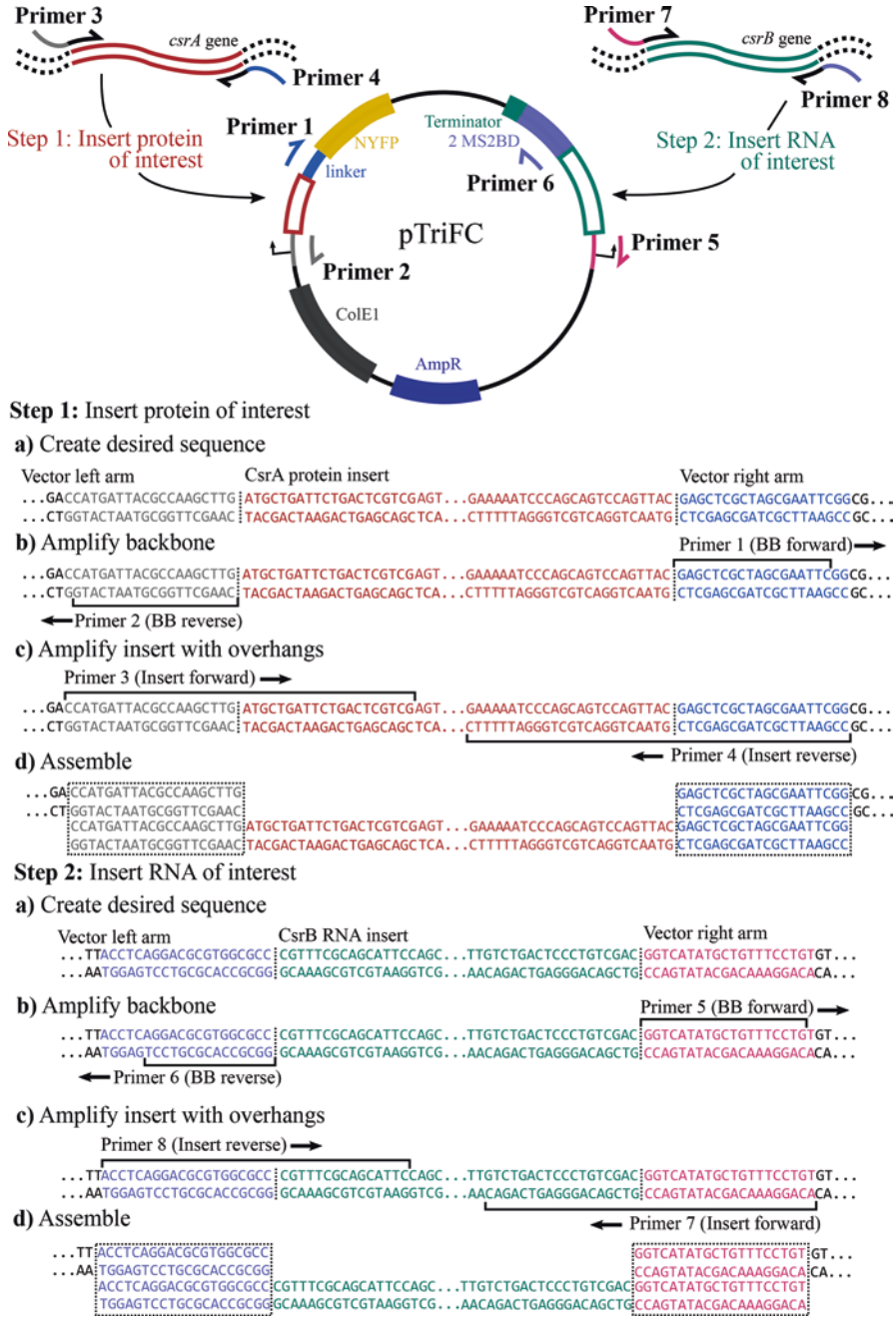

Fig. 7 Example cloning scheme for pTriFC. The TriFC assay can be adapted to investigate an RNA-protein interaction of interest via Gibson cloning; an example cloning scheme for adding the CsrA protein and the CsrB sRNA is illustrated in two steps. (**Step 1a**) The desired composite sequence, left (5′) vector arm + *csrA* gene sequence + right (3′) vector arm, is determined. (**Step 1b**) Primers 1 and 2 are designed (NEBuilder Assembly tool) and used to amplify the vector backbone forward upstream and reverse downstream of the protein insertion site. (**Step 1c**) Primers 3 and 4 are designed to amplify the *csrA* gene with overhangs homologous to the ends of the amplified vector backbone (NEBuilder Assembly tool); they are used to amplify the *csrA* gene (with homologous overhangs) from genomic DNA. (**Step 1d**) Lastly, the amplified vector fragment and the amplified

Table 3
Example primers for Gibson Assembly of RNA and protein of interest with pTriFC ("BB," vector backbone)

Cloning objective	Primer name	Sequence (5′ overhangs in lower case)
pTriFC, protein of interest	Primer 1 (BB forward)	GAGCTCGCTAGCGAATTC
	Primer 2 (BB reverse)	CAAGCTTGGCGTAATCATG
	Primer 3 (insert forward)	ccatgattacgccaagcttgATGCTGATTCTGACTCGTCG
	Primer 4 (insert reverse)	ccgaattcgctagcgagctcGTAACTGGACTGCTGGGATTTTTC
pTriFC, RNA of interest	Primer 5 (BB forward)	GGTCATATGCTGTTTCCTG
	Primer 6 (BB reverse)	GGCGCCACGCGTCCT
	Primer 7 (insert forward)	acaggaaacagcatatgaccGTCGACAGGGAGTCAGAC
	Primer 8 (insert reverse)	acctcaggacgcgtggcgccCGTTTCGCAGCATTC

1. To add the protein of interest to pTriFC, build a composite sequence containing the 5′ arm of the pTriFC vector (sequence just upstream of the protein site), the gene sequence of the protein of interest, and the 3′ arm of the pTriFC vector (sequence just downstream of the protein site) (Fig. 7, **step 1a**).
2. Design Primers 1 and 2 (Table 3) to bind to the pTriFC vector backbone and amplify around the protein site (Fig. 7, **step 1b**).
3. Design Primers 3 and 4 (Table 3) to amplify the gene sequence of the protein of interest from genomic DNA (Fig. 7, **step 1c**). Include overhang sequence homologous to the appropriate arm of the pTriFC vector on the 5′ ends of Primers 3 and 4. Specifically, Primer 3 should contain 20 nucleotides homologous to the 5′ pTriFC vector arm and Primer 4 should contain 20 nucleotides homologous to the 3′ pTriFC vector arm.

Fig. 7 (continued) *csrA* gene (with homologous overhangs) are combined in a standard Gibson Assembly reaction. (**Step 2a**) The desired composite sequence, left (5′) vector arm + *csrB* gene sequence + right (3′) vector arm, is determined. (**Step 2b**) Primers 5 and 6 are designed (NEBuilder Assembly tool) and used to amplify the vector backbone forward upstream and reverse downstream of the RNA insertion site. (**Step 2c**) Primers 7 and 8 are designed to amplify the *csrB* gene with overhangs homologous to the ends of the amplified vector backbone (NEBuilder Assembly tool); they are used to amplify the *csrB* gene (with homologous overhangs) from genomic DNA. (**Step 2d**) Lastly, the amplified vector fragment and the amplified *csrB* gene (with homologous overhangs) are combined in a standard Gibson Assembly reaction

Including homologous overhang regions on just the insert-amplifying primers (Primers 3 and 4), rather than the backbone-amplifying primers (Primer 1 and 2) maximizes amenability of the cloning scheme toward large-scale screening endeavors.

4. To add the RNA of interest to pTriFC, build a composite sequence containing the 5′ arm of the pTriFC vector (sequence just upstream of the RNA site), the gene sequence of the RNA of interest, and the 3′ arm of the pTriFC vector (sequence just downstream of the RNA site) (Fig. 7, **step 2a**). Keep in mind that the RNA fusion is expressed on the reverse strand, relative to expression of the protein-NYFP fusion. **Step 2** illustrates 5′ to 3′ the forward strand of pTriFC, the same orientation as the illustration of **step 1**. Thus, the gene sequence of the RNA of interest is encoded 5′ to 3′ on the reverse strand.
5. Design Primers 5 and 6 (Table 3) to bind to the pTriFC vector backbone and amplify around the RNA site (Fig. 7, **step 2b**).
6. Design Primers 7 and 8 (Table 3) to amplify the gene sequence of the RNA of interest from genomic DNA (Fig. 7, **step 2c**). Include overhang sequence homologous to the appropriate arm of the pTriFC vector on the 5′ end of Primers 7 and 8. Specifically, Primer 7, the forward primer for the RNA, should contain 20 nucleotides homologous to the 3′ pTriFC vector arm and Primer 8, the reverse primer for the RNA, should contain 20 nucleotides homologous to the 5′ pTriFC vector arm.

Fig. 7 depicts 3′ placement of the 2MS2BD sequence relative to the RNA of interest. Note that the RNA terminator sequence annotated in Fig. 7 (and Fig. 6a) is that of CsrB. While this element of the RNA-2MS2BD construct does not have to be altered (*see* **Note 17**), it can be exchanged for the terminator sequence of the RNA of interest by single-fragment Gibson Assembly if desired (*see* **Note 18**). The same primer design steps hold for 5′ placement of the 2MS2BD sequence relative to the RNA of interest; the composite sequence determined in **step 2** A will have different 5′ and 3′ vector arms, thus changing the sequence of the appropriate backbone-amplifying primers (Primers 5 and 6) and the homologous overhang sequences on the insert-amplifying primers (Primers 7 and 8). Lastly, one-fragment Gibson Assembly can be used to build the three control constructs listed above; in this instance, only backbone-amplifying primers that include homologous overhangs to exclude the element to be deleted are needed (for an example, *see* manual for Gibson Assembly Site-Directed Mutagenesis Kit from Synthetic Genomics).

7. PCR amplify the protein and/or RNA of interest from previously prepared genomic DNA with insert primers designed above. Insert amplification reactions should be composed as follows: (i) 10.0 μL of 5× Phusion HF buffer (final concentra-

tion 1×), (ii) 1.0 μL of 10 mM DNTPs (final concentration 200 μM), (iii) 2.5 μL of 10 μM forward primer (Primer 3 or Primer 7, final concentration 0.5 μM), (iv) 2.5 μL of 10 μM reverse primer (Primer 4 or Primer 8, final concentration 0.5 μM), (v) 100 ng of genomic DNA, (vi) 0.5 μL of Phusion polymerase, and (vii) nuclease-free water, up to 50.0 μL to reaction volume. Genomic DNA can be obtained with a genomic DNA purification kit or by boiling a colony (single colony, diluted in 50 μL of nuclease-free water, heated at 96 °C for 5 min); 2 μL of this reaction can be used above as genomic DNA template.

8. Amplify pTriFC with backbone primers designed above. Backbone amplification reactions should be composed as above, except with Primers 1 and 2 or Primers 3 and 5 and approximately 5 ng of purified parent pTriFC DNA rather than 100 ng genomic DNA.
9. Both insert and backbone amplification reactions should be cycled as follows: (i) 98 °C for 30 s, (ii) 98 °C for 10 s, (iii) proper annealing temperature for 30s, with 0.5 °C/s ramp rate, (iv) 72 °C for 30 s/kb, (v) Repeat **steps ii–iv** 34 times, and (vi) 72 °C for 5 min, where proper annealing temperature is 3 °C above the lowest primer initial melting temperature (i.e., initial binding region that excludes 5′ overhangs). We have had success with 4 min and 45 s extension times for backbone and insert (150–300 bp) amplification reactions, respectively.
10. Run approximately 5.0 μL of each reaction on an agarose gel (with EZ Vision or loading dye and ethidium bromide) to check for proper band size and amplification specificity (backbone at ~4.3 Kb, insert at ~100–300 bp, depending on RNA or protein of interest).
11. Add 1.0 μL of DpnI to each reaction and digest at 37 °C for 1–3 h to remove parent plasmid (or digest overnight).
12. PCR clean up according to PCR Purification Kit instructions. Determine concentration of purified linear DNA fragments by UV spectrophotometry (*see* **Note 19**).
13. Compose Gibson Assembly reaction of amplified and purified backbone and insert DNA (illustrated as **step 1d** for protein insert and **step 2d** for RNA insert in Fig. 7) as follows: (i) 15 μL 1.33× (or 10 μL 2×) Gibson master mix, (ii) 50 ng amplified purified backbone DNA, (iii) amplified purified insert at 5–10× molar excess of the backbone, such that the total amount of DNA is less than 0.5 pmol, and (iv) nuclease-free water up to 20 μL. Incubate reaction at 50 °C for 60 min using a thermocycler. For assembly of control constructs, 50 ng of the single fragment (i.e., as backbone) is used without

an insert and more nuclease-free water is added to reach a total volume of 20 μL.

14. Desalt 10 μL of the Gibson Assembly reaction on a 0.025 μM nitrocellulose membrane filter floating on nanopure water for 25–50 min. Recover as much as possible (typically 7–8 μL), and transform into electrocompetent *E. coli* cells by standard protocols. After recovery, plate on LB agar with 100 μg/mL carbenicillin as the selective agent and let grow overnight at 37 °C.
15. Colony PCR, restriction digest, and sequencing can all be used to confirm proper assembly of the insert and backbone fragments. We recommend sequencing for final confirmation and colony PCR for pre-sequencing screening in large-scale cloning endeavors. A sample workflow is detailed in **steps 16–20**.
16. Pick 4–10 colonies from each plated Gibson Assembly reaction and dilute into 50 μL of nuclease-free water. Save each colony by streaking onto a new carbenicillin (100 μg/mL) LB agar plate after dilution (and growing over night at 37 °C). Heat diluted colonies at 96 °C for 5 min.
17. Compose an amplification reaction that is specific to the intended pTriFC using an insert-specific primer and a backbone-specific primer: (i) when adding an RNA to pTriFC with 5′ 2MS2BD placement, use Primer 8 (Table 3) and Primer 18 (Table 4), (ii) when adding an RNA to pTriFC with 3′ 2MS2BD placement, use Primer 7 (Table 3) and Primer 17 (Table 4), (iii) when adding a protein to pTriFC, use Primer 3 (Table 3) and Primer 19 (Table 4). The reaction can be set up

Table 4
Example primers for sequencing pTriFC and pTriFC-mStrawberry

Primer name	Description	Sequence
Primer 17	Binds MS2BD reverse to sequence RNA of interest on pTriFC (with 3′ placement of 2MS2BD to RNA)	CCTTAGGATCCATATATAGGGCCC
Primer 18	Binds MS2BD forward to sequence RNA of interest on pTriFC-mStrawberry (or on pTriFC with 5′ placement of 2MS2BD to RNA)	GGGTTCATTAGATCTGCGCGCG
Primer 19	Binds NYFP reverse to sequence protein of interest on pTriFC and pTriFC-mstrawberry	CCGTTTACGTCGCCGTCCAGCTCGACCAGG
Primer 20	Binds CYFP reverse to sequence MS2 binding protein on pMS2-CYFP	GTTATATCGATTTACAGATCTTCTTCGC

as follows: (i) 2.5 μL 10× ThermoPol buffer, (ii) 0.5 μL 10 mM DNTPs (final concentration 200 μM), (iii) 0.5 μL 100 μM forward primer (final concentration 2 μM), (iv) 0.5 μL 100 μM reverse primer (final concentration 2 μM), (v) 2.0 μL boiled colony, (vi) 0.25 μL Taq polymerase, and (vii) nuclease-free water up to 20.25 μL. Reaction should be cycled as follows: (i) 95 °C for 30s, (ii) 95 °C for 30s, (iii) proper annealing temperature for 60s, (iv) 68 °C for 60s/kb, (v) repeat **steps ii–iv** 29 times, and (vi) 68 °C for 5 min, where proper annealing temperature is 5 °C lower than the lowest primer melting temperature.

18. Run approximately 10.0 μL of each colony PCR reaction on an agarose gel (with EZ Vision or loading dye and ethidium bromide) to check for the band size and specificity. Reactions with only the correct band present are promising colonies to sequence.
19. Retrieve the plate on which colonies picked for colony PCR were saved. Grow each promising colony overnight in liquid culture (5 mL LB in 25 mL test tubes with 100 μg/mL carbenicillin).
20. Miniprep saturated cultures the next day, according to kit directions. Measure the concentration of purified plasmid DNA and prepare sequencing reactions as prescribed by your local sequencing facility. An MS2BD-binding primer (Table 4, Primer 17 or 18) is recommended for sequencing inserted RNAs and an NYFP-binding primer (Table 4, Primer 19) for sequencing inserted proteins.

4.3 Fusion Confirmation

Expression and proper biological function of the RNA and protein of interest in the new RNA-2MS2BD and protein-NFYP fusions should be confirmed. Conventional Western and Northern blotting approaches can be used to confirm protein-NYFP and RNA-2MS2BD expression, respectively. Methods to confirm proper protein and RNA function in their respective fusions vary depending on the molecule. For instance, detecting dimerization in a western blot of a protein-NYFP fusion where the protein is known to dimerize in vivo suggests biological function was retained. Similarly, observation of a phenotype characteristic of overexpressing the RNA of interest when the RNA-2MS2BD fusion is overexpressed suggests biological function was retained.

4.4 TriFC Experiment

1. Select a strain for performing the TriFC experiment. The TriFC assay was developed and tested in a derivative of *E. coli* K-12 MG1655 (*see* **Note 20**); however, we foresee that the assay could be successfully adapted for different bacterial species. If high stoichiometries between the RNA or protein of interest are expected or if high endogenous expression of either

component has been established, the TriFC signal may be diluted by the RNA or protein fusion constructs interacting with endogenous versions of each other, rather than the fused versions. This renders a genomic deletion strain of one or both of the components necessary. While the TriFC assay was developed in a strain without lacIq, we expect that inducible control of expression of the RNA and protein fusion constructs will enhance optimization of assay conditions and impart greater flexibility for probing RNA-protein interactions dynamically (*see* **Note 21** and Subheading 4.4, **step 3**).

2. Sequentially transform the strain of choice with pMS2-CYFP and pTriFC (Fig. 8a) by standard $CaCl_2$ transformation protocols. The pMS2-CYFP plasmid (or version lacking MS2) is transformed first and selected for on kanamycin-containing (50 μg/mL) LB agar plates. The strain harboring pMS2-CYFP is next made competent, transformed with pTriFC, and selected for on kanamycin- and carbenicillin-containing (50 and 100 μg/mL, respectively) LB agar plates.
3. Select triplicate colonies of the *E. coli* strain + pMS2-CYFP + pTriFC and grow in LB cultures (supplemented with 50 μg/mL kanamycin and 100 μg/mL carbenicillin) for 18–48 h prior to measuring fluorescence (Fig. 8b). IPTG induction is not required to express the fusions if the strain is not enhanced with lacIq. However, inducible control of the fusion constructs, though not previously demonstrated, proffers flexibilities and ease for optimizing assay conditions. Two distinct cell culture schemes have been successfully demonstrated for the assay: (i) 40 mL cultures in 250 mL shake flasks, grown at 25 °C with 200 rpm shaking for 24–48 h [14] and (ii) 5 mL cultures in 25 mL test tubes, grown at 37 °C with 200 rpm shaking for 18 h [15]. The first scheme was optimized for a single sRNA-protein interaction; the second scheme was optimized for screening multiple mRNA-protein interactions with the dual-fluorescence variation of the TriFC assay (*see* Subheading 5). The first workflow, i.e., growth at lower temperatures, is a standard approach for optimizing proper protein folding [30]; however, we recommend the second work flow as a starting point for optimizing future interaction-specific cell culture schemes due to experimental convenience of shorter growth times.
4. Fluorescence can be measured by flow cytometry (Fig. 8c) or with a plate reader. For plate reader-based measurements, collect 1 mL of each saturated culture and pellet in a 1.7 mL microtube.
5. Decant the LB supernatant and resuspend the pellet in 1 mL of 1× PBS.

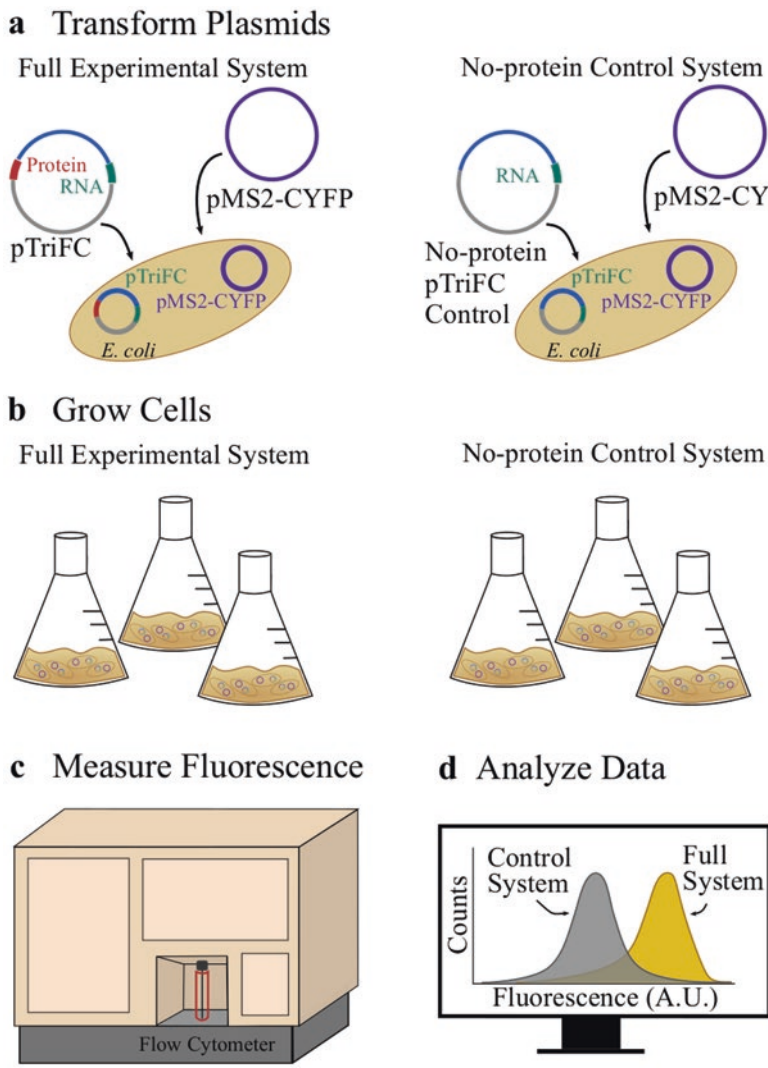

Fig. 8 Work flow for TriFC assay. (**a**) The pTriFC and pMS2-CYFP plasmids are sequentially transformed into an *E. coli* strain of choice to constitute the full experimental system. Similarly, the control systems are transformed into the strain of choice (no-protein, no-RNA, and no-MS2); the no-protein control system is illustrated as an example. (**b**) Cells are grown in triplicate, (**c**) and fluorescence measured by flow cytometry or by plate reader (not shown). (**d**) Finally, fluorescence data are analyzed and the full experimental system is compared to the control system to determine RNA-protein binding

6. Pellet the samples second time and resuspend in a final 1 mL volume of 1× PBS.
7. Transfer 200 uL of each to a 96 well plate (black with clear, flat bottom) for fluorescence and optical density measurements. Be sure to include at least duplicate blank measurements for normalization.
8. Measure yellow fluorescence at the conventional 514/527 nm excitation/emission (ex/em) wavelengths and optical density at 600 nm.

9. Normalize samples' yellow fluorescence measurements by subtracting the average blank reading and dividing by their optical density for further analysis.
10. Alternatively, for flow cytometry, dilute 1–3 μL of the saturated culture into 1 mL of 1× PBS in 5 mL round bottom polystyrene tubes (final concentration ~10^7 cells/mL) and analyze fluorescence by flow cytometry for at least 25,000–50,000 active cells.
11. It is not usually possible to isolate the fluorescent population from non-fluorescent background noise using the forward and side scatter values. Thus, measure fluorescence on both green and yellow channels (530/30 nm and 585/42 nm band pass filter channels, respectively, for the BD FACSCalibur) and track events on a dot plot of the 530 nm channel fluorescence versus the 585 nm channel fluorescence.
12. Distinguish the YFP fluorescent signal from background noise via the 530 nm channel measurement and apply a minimum threshold value to isolate the fluorescent population. The isolated population's 585 nm channel fluorescence measurements are then used for further analysis.

4.5 Data Analysis

Fluorescence measurements of the control constructs, described in Subheading 4.1, are critical for determining whether the recorded fluorescence of a given RNA-protein interaction constitutes true RNA-protein binding (Fig. 8d). We recommend pairing the control and experimental constructs as follows to form three negative control experiments, each in triplicate: (i) pTriFC lacking the protein of interest (NYFP + RNA-2MS2BD present) with pMS2-CYFP, (ii) pTriFC lacking the RNA of interest (protein-NYFP + 2MS2BD present) with pMS2-CYFP, and (iii) pTriFC with pMS2-CFYP lacking the MS2 protein. Additionally, a scramble or known nontarget RNA sequence can be paired with the protein of interest as a negative control [15]. If fluorescence is measured by flow cytometry, geometric means (of the isolated population) of triplicate measurements of the *E. coli* strain + pMS2-CYFP + pTriFC can be compared to those of the control experiments by one-tailed heteroscedastic Student's T-tests. If the full system's average geometric mean fluorescence is significantly greater than that of the control(s), a true RNA-protein interaction is determined to have occurred. Similarly, if fluorescence is measured by plate reader, simply use the normalized yellow fluorescence measurements to compare triplicate samples of the *E. coli* strain + pMS2-CYFP + pTriFC to those of the control experiments by one-tailed heteroscedastic Student's T-tests. If the full system's average normalized yellow fluorescence is significantly greater than that of the control(s), a true RNA-protein interaction is determined to have occurred.

5 Dual-Fluorescence TriFC

The dual-fluorescence variation of the TriFC assay was developed to assess the interaction of mRNAs, particularly anticipated regulatory 5′ UTRs, with a protein. The critical difference between the dual-fluorescence TriFC assay and the original is the addition of a red fluorescent protein, mStrawberry, to the RNA fusion construct (Fig. 6c). Conceivably, the dual-fluorescence TriFC assay could also be applied to any suspected regulatory element of an mRNA, not necessarily just the 5′ UTR, as long as a ribosome binding site and translation start site are included. Tracking red fluorescence along with yellow fluorescence offers (i) confirmation of functional expression of the RNA fusion construct and (ii) potential for tracking the effect of protein-5′ UTR binding on 5′ UTR-controlled red fluorescence. The following details the specifics of the dual-fluorescence TriFC assay where it differs from the original assay.

5.1 Construct Design

In the dual-fluorescence TriFC assay, pTriFC is modified to include an mStrawberry sequence on the 3′ end of the RNA fusion construct and is renamed "pTriFC-mStrawberry" (Fig. 6c). Two repeats of the MS2BD sequence are recommended and, in this instance, required to be placed on the upstream of the RNA of interest as to not interrupt translation of mStrawberry. Due to the usual length of mRNAs (average length of coding sequence of a K-12 *E. coli* gene is 950 base pairs [31]), only a portion of the mRNA sequence can be probed at a time by TriFC. If the protein binding site within the mRNA is known or suspected, this knowledge can guide selection of the RNA sequence to be probed. Otherwise, the 5′ UTR and initial portion of coding sequence of an mRNA are typically of interest. We recommend probing the 5′ UTR sequence, defined by the gene's closest annotated promoter, plus the first 100 nucleotides of coding sequence of the mRNA of interest. RegulonDB can be used to identify annotated promoters for a given *E. coli* gene [32]. In this way, any promoter elements that could affect expression, and thus fluorescence, of the 2MS2BD-RNA-mStrawberry construct are excluded. The SphI restriction site, located between the RNA of interest and mStrawberry in pTriFC-mStrawberry (Fig. 6c), is designed with two spacer nucleotides preceding it so that the 100 nucleotide coding sequence contained in the RNA of interest will be in frame with mStrawberry. For many *E. coli* genes, this definition would yield RNA sequences of interest of about 150 to 350 nucleotides in length. If the mRNA in question does not have a defined promoter sequence or is in the interior of an operon, probing the 100 nucleotides just upstream of the translation start site plus the first 100 nucleotides of coding sequence has been shown reasonable [15].

Recommended control constructs include: (i) pTriFC-mStrawberry lacking the protein of interest (NYFP + 2MS2BD-RNA-mStrawberry present), (ii) pTriFC-mStrawberry with a scramble or known nontarget RNA of interest (protein-NYFP + 2MS2BD-scramble RNA-mStrawberry present), and (iii) pMS2-CFYP lacking the MS2 protein. Using a scramble or known nontarget RNA instead of a no RNA control allows the second control system to retain red fluorescence. The first control construct offers opportunity to compare the effect of RNA-protein binding on red fluorescence controlled by the RNA of interest.

5.2 Cloning Procedures

Cloning procedures are the same as for the original TriFC assay, described in Subheading 4.2. Fig. 9 illustrates an example cloning scheme for adding the CsrA protein and the *glgC* 5′ UTR to pTriFC-mStrawberry. Table 5 documents the sequences of Primers 9–16 shown in Fig. 9. Primers for sequence confirmation of the RNA or protein of interest are given in Table 4.

5.3 Fusion Confirmation

Expression and function of the 2MS2BD-RNA-mStrawberry construct can be confirmed by measuring red fluorescence of an *E. coli* strain harboring pTriFC-mStrawberry and pMS2-CYFP (by plate reader at ex/em 570/590 or 570/600 nm).

5.4 Dual-Fluorescent TriFC Experiment

The experiment is performed according to the protocol of the original TriFC assay (*see* Subheading 4.4 and Fig. 8) with minor modifications. Yellow fluorescence should be measured by plate reader at ex/em 430/510 nm wavelengths and optical density measured at 900 nm to minimize conflict of the YFP-RFP signals. Conceivably, fluorescence can also be measured by flow cytometry, though separation of the YFP and RFP signals by gating has not been attempted.

5.5 Data Analysis

Data analysis proceeds as for the original assay, except that red fluorescence must be present (pass-fail test) to indicate proper construct expression prior to analysis of yellow fluorescence.

Fig. 9 (continued) (**Step 1d**) Lastly, the amplified vector fragment and the amplified *csrA* gene (with homologous overhangs) are combined in a standard Gibson Assembly reaction. (**Step 2a**) The desired composite sequence, left (5′) vector arm + 5′ UTR sequence of the *glgC* gene + right (3′) vector arm, is determined. (**Step 2b**) Primers 13 and 14 are designed (NEBuilder Assembly tool) and used to amplify the vector backbone forward upstream and reverse downstream of the RNA insertion site. (**Step 2c**) Primers 15 and 16 are designed to amplify the *glgC* 5′ UTR sequence with overhangs homologous to the ends of the amplified vector backbone (NEBuilder Assembly tool); they are used to amplify the *glgC* 5′ UTR sequence (with homologous overhangs) from genomic DNA. (**Step 2d**) Lastly, the amplified vector fragment and the amplified *glgC* 5′ UTR (with homologous overhangs) are combined in a standard Gibson Assembly reaction

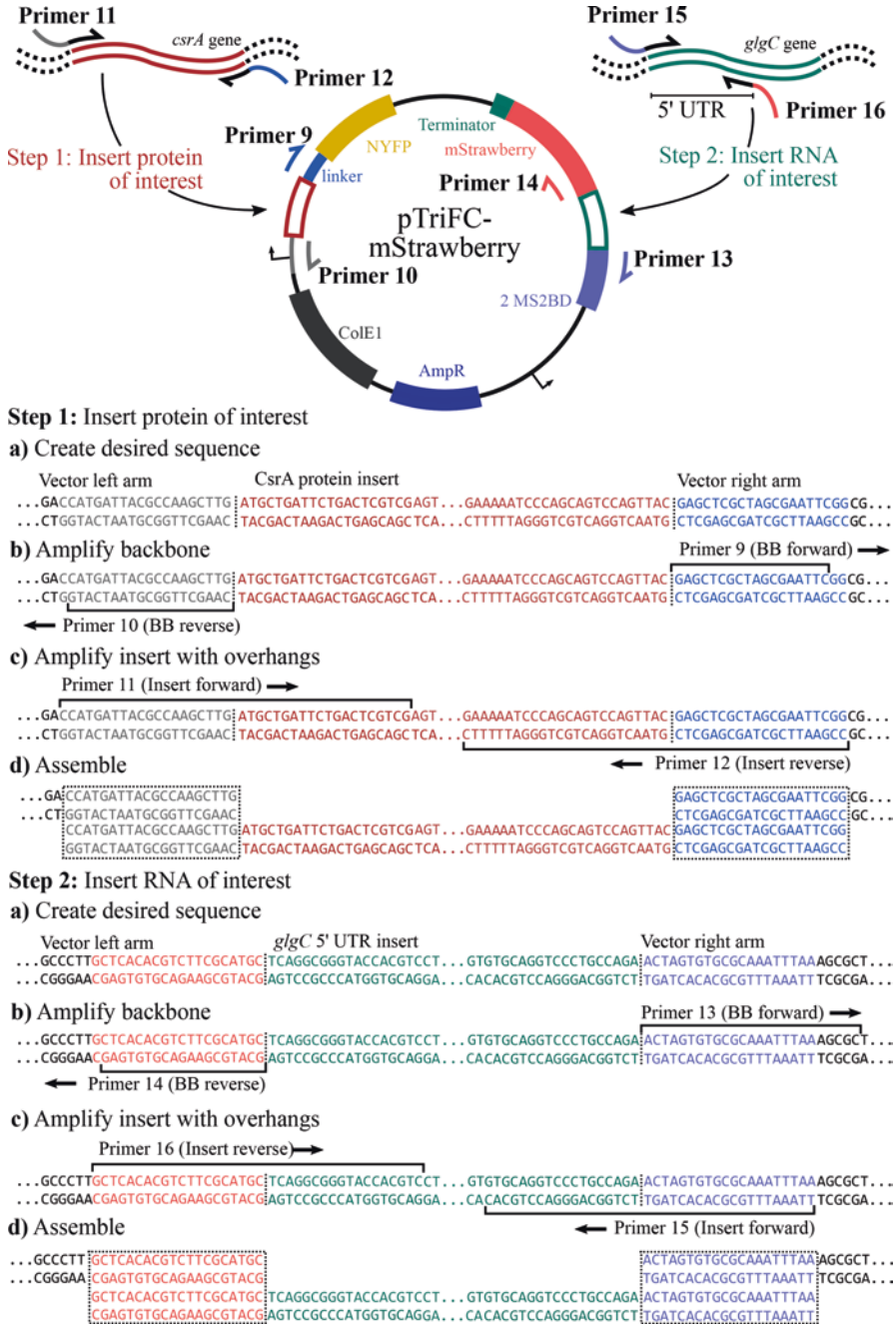

Fig. 9 Example cloning scheme for pTriFC-mStrawberry. The Dual-fluorescence TriFC assay can be adapted to investigate an mRNA-protein interaction of interest via Gibson cloning; an example cloning scheme for adding the CsrA protein and the 5′ UTR of the *glgC* gene is illustrated in two steps. (**Step 1a**) The desired composite sequence, left (5′) vector arm + *csrA* gene sequence + right (3′) vector arm, is determined. (**Step 1b**) Primers 9 and 10 are designed (NEBuilder Assembly tool) and used to amplify the vector backbone forward downstream and reverse upstream of the protein insertion site. (**Step 1c**) Primers 11 and 12 are designed to amplify the *csrA* gene with overhangs homologous to the ends of the amplified vector backbone (NEBuilder Assembly tool); they are used to amplify the *csrA* gene (with homologous overhangs) from genomic DNA.

Table 5
Example primers for Gibson Assembly of RNA and protein of interest with pTriFC-mStrawberry ("BB," vector backbone)

Cloning objective	Primer name	Sequence (5′ overhangs in lower case)
pTriFC-mStrawberry, protein of interest	Primer 9 (BB forward)	GAGCTCGCTAGCGAATTC
	Primer 10 (BB reverse)	CAAGCTTGGCGTAATCATG
	Primer 11 (insert forward)	ccatgattacgccaagcttgATGCTGATTCTGACTCGTCG
	Primer 12 (insert reverse)	ccgaattcgctagcgagctcGTAACTGGACTGCTGGGATTTTTC
pTriFC-mStrawberry, RNA of interest	Primer 13 (BB forward)	ACTAGTGTGCGCAAATTTAAAGCGC
	Primer 14 (BB reverse)	GCATGCGAAGACGTGTGAG
	Primer 15 (insert forward)	ttaaatttgcgcacactagtTCTGGCAGGGACCTGCAC
	Primer 16 (insert reverse)	gctcacacgtcttcgcatgcTCAGGCGGGTACCACGTC

6 Notes

1. For inserts difficult to amplify, ramp temperature at 0.5–2 °C/s during entrance into and exit from annealing stage of cycle.
2. Up to 10 pairs of annealed primers can be used in one reaction if desired. Simply substitute 2 μL of single annealed primer pair for 2 μL of solution made up of equal volumes of the desired annealed primers (each unique annealed primer pair represented at 10 μM).
3. Autoclaving 4-chloro-DL-phenylalanine within LB agar solution promotes dissolution.
4. The most important strain criterion is the detection of a significant fluorescence range between a negative control (scramble asRNA construct) and a positive control (open RBS construct).
5. Although these are the unique methods used to perform iRS[3] assays in our lab, we foresee no issues using other culturing volumes given that (i) the protocol is consistent across strains and (ii) there is ample culture volume at sampling OD.

6. If growing cultures in plates, it is recommended to perform an initial growth curve analysis to gauge time required till desired induction and/or sampling ODs are reached.
7. A higher likelihood of culture contamination is present when growing cultures in plates. If possible, leave blank LB wells that can serve as a qualitative measure of whether or not culturing and splitting protocols are causing well contamination.
8. 2× quantity of flasks or plate wells are required due to splitting of culture at time of induction
9. If using plates, create a map which divides prospective uninduced and induced samples for ease of experimental execution on Day 2.
10. If growing culture in plates, pre-mix arabinose and aTc prior to inducing, as required aTc volumes (per well) will be small.
11. Samples corresponding to the same strain should be run on cytometer in close temporal proximity to avoid data skews due to instrument shift over duration of fluorescence measurements.
12. In general, C-terminal attachment of NYFP to the protein of interest is preferred in light of possible co-translational folding of the protein of interest. If the protein of interest has no previously published tag designs, we recommend first attempting C-terminal attachment of NYFP to the protein of interest. Alternatively, one can use a structure of the protein to predict whether critical N- or C-terminal geometry exists and select the other terminus for linking the protein of interest to NYFP.
13. Flexibility in the protein-NYFP construct is particularly important when working with complex, high-stoichiometry RNA-protein interactions, such as the CsrB sRNA-CsrA protein system, where CsrB binds CsrA with a stoichiometry of ~18 proteins to a single sRNA molecule [33]. A shorter, generic linker derived from a residual multiple cloning site was unsuccessfully attempted for the CsrA-NYFP fusion before the $(GGGGS)_3$ linker was shown to allow expected protein-RNA complementation [14].
14. It should be noted that the optimal linker length for the protein-NYFP fusion is dependent on both the geometry of the protein of interest and the concentration of the fusion expressed from the plasmid. Linker length may need to be altered in conjunction with experimental assay conditions to optimize detection of a RNA-protein interaction of interest.
15. CsrB is a 369 nucleotide-long sRNA that folds into 15 repeating stem-loop structures, most of which are putative binding sites for its target protein, CsrA. Placement of the 2MS2BD

sequence on the 5′ end of CsrB could have disrupted important 5′ to 3′ folding that occurs during CsrB transcription.

16. Importantly, placement of the MS2BD sequence 5′ of the RNA of interest is more amenable to screening applications because the MS2BD sequence can be included as part of the pTriFC backbone, rather than as an insertion before the terminator in each RNA. This arrangement is advantageous for large-scale cloning endeavors that are required for screening applications [15].
17. Given that 3′ placement of the 2MS2BD sequence separates the 3′ portion RNA of interest from its terminator by 200 nucleotides, any contribution of the terminator to RNA function (other than ending transcription) is likely abolished. As such, we do not foresee altering the terminator sequence of the RNA-2MS2BD fusion to match that of a new RNA of interest as necessary.
18. Single-fragment Gibson Assembly can be used to change the terminator sequence of the RNA-2MS2BD fusion in pTriFC from that of CsrB to that of a new RNA of interest. Backbone-amplifying primers are designed to exclude the CsrB terminator and contain overhangs homologous to each other, i.e., the terminator sequence of the new RNA of interest (for an example, *see* manual for Gibson Assembly Site-Directed Mutagenesis Kit from Synthetic Genomics).
19. Alternatively, the full digest could be run in Subheading 4.2, **step 10**, Subheading 4.2, **step 11** skipped, and a full gel extraction of the amplified bands performed in Subheading 4.2, **step 12**. However, we have had most success with DpnI digestion and PCR cleanup rather than gel extraction.
20. Specifically, endogenous expression of CsrB was greater than the pLacO-driven expression of the CsrB fusion construct. Since CsrA binds to CsrB with a stoichiometry of ~18:1 [33], an *E. coli* K-12 MG1655 strain with a genomic deletion of CsrB was used to maximize detectable CsrB-CsrA interactions [14].
21. Expression of pTriFC in a strain without lacIq led to a substantial growth defect. Notably, the defect was only present with the full system; the three control pTriFC constructs that have either the protein, RNA, or MS2 protein missing grew unencumbered. In light of this, we attributed the growth defect to insoluble protein formation due to NYFP-CYFP refolding. We expect that performing the TriFC assay in a strain that contains lacIq and an appropriate genomic deletion will allow for dose-dependent expression of the fusion constructs and maximize the information the TriFC assay can provide.

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