Journal of Visualized Experiments

An Assay for Quantifying Protein-RNA Binding in Bacteria

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59611R2
Full Title:	An Assay for Quantifying Protein-RNA Binding in Bacteria
Keywords:	RNA binding protein (RBP), MS2, PP7, phage coat protein, binding assay, post-transcriptional regulation, translation repression, synthetic circuit, RBP binding affinity, RNA circuit, reporter gene, RBP RNA interaction
Corresponding Author:	Noa Katz Technion Israel Institute of Technology Haifa, Israel ISRAEL
Corresponding Author's Institution:	Technion Israel Institute of Technology
Corresponding Author E-Mail:	katznoa@gmail.com;katznoa@campus.technion.ac.il
Order of Authors:	Noa Katz
	Roni Cohen
	Orna Atar
	Sarah Goldberg
	Roee Amit
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Haifa, Israel

TECHNION
Israel Institute
of Technology

Biotechnology & Food Engineering

December 24th, 2018

Dear JOVE editor,

We would like to submit the enclosed manuscript entitled "In vivo binding assay for RNA binding proteins in bacteria" by Noa Katz, Roni Cohen, Orna Atar, Sarah Goldberg, and Roee Amit. The manuscript includes three figures, and three supplementary tables. The supporting material is essential for a thorough review of the paper. This paper is based on another manuscript by us entitled "An in vivo binding assay for RNA-binding proteins based on repression of a reporter gene", which was published this month in ACS Synthetic Biology.

In this manuscript, we describe a method to quantify the binding affinity of RBPs to cognate and non-cognate binding sites using a simple, live, reporter assay in bacterial cells. The assay is based on the synthetic biology approach, where the binding site is placed in the ribosomal initiation region of a fluorescent reporter gene, while we induce production of the RBP. In doing so, we create a competition between the RBP and the ribosome for binding to the mRNA molecule. This competition is reflected by declining reporter levels as increasing concentrations of inducer are added, and the production of the RBP rises.

We demonstrate the implementation of the assay on two well-known phage coat proteins, PP7 and MS2, with both native and mutated binding sites in different locations. We present quantitative results that match the predictions, as both RBPs present a high affinity for their positive controls, and a non-detectible binding affinity for the negative controls. In addition, previous studies that have used these two RBPs have seen them to be orthogonal, which is also clearly conveyed in the sample results presented.

Thus, our method is relevant for those who wish to quantify and compare between the binding affinity of different RBPs that are functional in bacteria to various known and unknown binding sites.

We look forward to your comments on our manuscript.

Sincerely,

Roee Amit
Assistant Professor
Department of Biotechnology and Food Engineering
Technion - Israel Institute of Technology
Haifa, Israel

Tel. 972-77-8871895, Fax 972-77-88718949 ; 077-8871894 פקס ,077-8871895.טל
Email: roeeamit@technion.ac.il : דואר אלקטרוני
Technion city, Haifa 3200003, Israel ; ישראל , 3200003 קרית הטכניון, חיפה

TITLE:

An Assay for Quantifying Protein-RNA Binding in Bacteria

AUTHORS AND AFFILIATIONS:

Noa Katz[1,*], Roni Cohen[1,*], Orna Atar[1], Sarah Goldberg[1], Roee Amit[1,2]

[1]Department of Biotechnology and Food Engineering, Technion-Israel Institute of Technology, Haifa, Israel

[2]Russell Berrie Nanotechnology Institute, Technion-Israel Institute of Technology, Haifa, Israel

Corresponding author:

Noa Katz (katznoa@campus.technion.ac.il)

Email addresses of co-authors:

Roni Cohen (ronico1001@campus.technion.ac.il)

Orna Atar (Atar@technion.ac.il)

Sarah Goldberg (sarah.esther.goldberg@gmail.com)

Roee Amit (roeeamit@bfe.technion.ac.il)

KEYWORDS:

RNA binding protein (RBP), MS2, PP7, phage coat protein, binding assay, post-transcriptional regulation, translation repression, synthetic circuit, RBP binding affinity, RNA circuit, reporter gene, RBP interaction

SUMMARY:

In this method, we quantify the binding affinity of RNA binding proteins (RBPs) to cognate and non-cognate binding sites using a simple, live, reporter assay in bacterial cells. The assay is based on repression of a reporter gene.

ABSTRACT:

In the initiation step of protein translation, the ribosome binds to the initiation region of the mRNA. Translation initiation can be blocked by binding of an RNA binding protein (RBP) to the initiation region of the mRNA, which interferes with ribosome binding. In the presented method, we utilize this blocking phenomenon to quantify the binding affinity of RBPs to their cognate and non-cognate binding sites. To do this, we insert a test binding site in the initiation region of a reporter mRNA and induce the expression of the test RBP. In the case of RBP-RNA binding, we observed a sigmoidal repression of the reporter expression as a function of RBP concentration. In the case of no-affinity or very low affinity between binding site and RBP, no significant repression was observed. The method is carried out in live bacterial cells, and does not require expensive or sophisticated machinery. It is useful for quantifying and comparing between the binding affinities of different RBPs that are functional in bacteria to a set of designed binding sites. This method may be inappropriate for binding sites with high structural complexity. This is due to the possibility of repression of ribosomal initiation by complex mRNA structure in the absence of RBP, which would result in lower basal reporter gene expression, and thus less-

observable reporter repression upon RBP binding.

INTRODUCTION:

RNA binding protein (RBP)-based post-transcriptional regulation, specifically characterization of the interaction between RBPs and RNA, has been studied extensively in recent decades. There are multiple examples of translational down-regulation in bacteria originating from RBPs inhibiting, or directly competing with, ribosome binding[1–3]. In the field of synthetic biology, RBP-RNA interactions are emerging as a significant tool for the design of transcription-based genetic circuits[4,5]. Therefore, there is an increase in demand for characterization of such RBP-RNA interactions in a cellular context.

The most common methods for studying protein-RNA interactions are the electrophoretic mobility shift assay (EMSA)[6], which is limited to in vitro settings, and various pull-down assays[7], including the CLIP method[8,9]. While such methods enable the discovery of de novo RNA binding sites, they suffer from drawbacks such as labor-intensive protocols and expensive deep sequencing reactions and may require a specific antibody for RBP pull-down. Due to the susceptible nature of RNA to its environment, many factors can affect RBP-RNA interactions, emphasizing the importance of interrogating RBP-RNA binding in the cellular context. For example, we and others have demonstrated significant differences between RNA structures in vivo and in vitro[10,11].

Based on the approach of a previous study[12], we recently demonstrated[10] that when placing pre-designed binding sites for the capsid RBPs from the bacteriophages GA[13], MS2[14], PP7[15], and Qβ[16] in the translation initiation region of a reporter mRNA, reporter expression is strongly repressed. We present a relatively simple and quantitative method, based on this repression phenomenon, to measure the affinity between RBPs and their corresponding RNA binding sites in vivo.

PROTOCOL:

1. System preparation

1.1. Design of binding-site plasmids

1.1.1. Design the binding site cassette as depicted in **Figure 1**. Each minigene contains the following parts (5' to 3'): EagI restriction site, ~40 bases of the 5' end of the kanamycin (Kan) resistance gene, pLac-Ara promoter, ribosome binding site (RBS), AUG of the mCherry gene, a spacer (δ), an RBP binding site, 80 bases of the 5' end of the mCherry gene, and an ApaLI restriction site.

NOTE: To increase the success rate of the assay, design three binding-site cassettes for each binding site, with spacers consisting of at least one, two, and three bases. See Representative Results section for further guidelines.

1.2. Cloning of binding site plasmids

1.2.1. Order the binding-site cassettes as double-stranded DNA (dsDNA) minigenes. Each minigene is ~500 bp long and contains an EagI restriction site and an ApaLI restriction site at the 5' and 3' ends, respectively (see step 1.1.1).

NOTE: In this experiment, mini-genes with half of the kanamycin gene were ordered to facilitate screening for positive colonies. However, Gibson assembly[17] is also suitable here, in which case the binding site can be ordered as two shorter complementary single-stranded DNA oligos.

1.2.2. Double-digest both the mini-genes and the target vector with EagI-HF and ApaLI by the restriction protocol[18], and column purify[19].

1.2.3. Ligate the digested minigenes to the binding-site backbone containing the rest of the mCherry reporter gene, terminator, and a kanamycin resistance gene[20].

1.2.4. Transform the ligation solution into *Escherichia coli* TOP10 cells[21].

1.2.5. Identify positive transformants via Sanger sequencing.

1.2.5.1. Design a primer 100 bases upstream to the region of interest (see **Table 1** for primer sequences).

1.2.5.2. Miniprep a few bacterial colonies[22].

1.2.5.3. Prepare 5 µL of a 5 mM solution of the primer and 10 µL of the DNA at 80 ng/µL concentration.

1.2.5.4. Send the two solution to a convenient facility for Sanger sequencing[23].

1.2.6. Store purified plasmids at -20 °C, and bacterial strains as glycerol stocks[24], both in the 96-well format. DNA will then be used for transformation into *E. coli* TOP10 cells containing one of four fusion-RBP plasmids (see step 1.3.5).

1.3. Design and construction of the RBP plasmid

NOTE: Amino acid and nucleotide sequences of the coat proteins used in this study are listed in **Table 2**.

1.3.1. Order the required RBP sequence lacking a stop codon as a custom-ordered dsDNA minigene lacking a stop codon with restriction sites at the ends (**Figure 1**).

1.3.2. Clone the tested RBP lacking a stop codon immediately downstream of an inducible promoter and upstream of a fluorescent protein lacking a start codon (**Figure 1**), similar to steps 1.2.2-1.2.4. Make sure that the RBP plasmid contains a different antibiotic resistance gene than

the binding-site plasmid.

1.3.3. Identify positive transformants via Sanger sequencing, similar to step 1.2.5 (see **Table 1** for primer sequences).

1.3.4. Choose one positive transformant and make it chemically-competent[25]. Store as glycerol purified plasmids at -20 °C and glycerol stocks of bacterial strains[24] at -80 °C in 96-well plates.

1.3.5. Transform the binding-site plasmids (from step 1.2.6) stored in 96-well plates into chemically-competent bacterial cells already containing an RBP-mCerulean plasmid[21]. To save time, instead of plating the cells on Petri dishes, plate them using an 8-channel pipettor on 8-lane plates containing Luria-Bertani (LB)[26] agar with relevant antibiotics (Kan and Amp). Colonies should appear in 16 h.

1.3.6. Select a single colony for each double transformant and grow overnight in LB medium with the relevant antibiotics (Kan and Amp) and store as glycerol stocks[24] at -80 °C in 96-well plates.

2. Experiment setup

NOTE: The protocol presented here was performed using a liquid-handling robotic system in combination with an incubator and a plate reader. Each measurement was carried out for 24 inducer concentrations, with two duplicates for each strain + inducer combination. Using this robotic system, data for 16 strains per day with 24 inducer concentrations was collected. However, if such a device is unavailable, or if fewer experiments are necessary, these can easily be done by hand using an 8-channel multi-pipette and adapting the protocol accordingly. For example, preliminary results for four strains per day with 12 inducer concentrations and four time-points were acquired in this manner.

2.1. Prepare, in advance, 1 L of bioassay buffer (BA) by mixing 0.5 g of tryptone, 0.3 mL of glycerol, 5.8 g of NaCl, 50 mL of 1 M $MgSO_4$, 1 mL of 10x phosphate-buffered saline (PBS) buffer pH 7.4, and 950 mL of double distilled water (DDW). Autoclave or sterile filter the BA buffer.

2.2. Grow the double-transformant strains at 37 °C and 250 rpm shaking in 1.5 mL LB with appropriate antibiotics (kanamycin at a final concentration of 25 µg/mL and ampicillin at a final concentration of 100 µg/mL), in 48-well plates, over a period of 18 h (overnight).

2.3. In the morning, make the following preparations.

2.3.1. Inducer plate. In a clean 96-well plate, prepare wells with semi-poor medium (SPM) consisting of 95% BA and 5% LB[26] in the incubator at 37 °C. The number of wells corresponds to the desired number of inducer concentrations. Add C4-HSL to the wells in the inducer plate that will contain the highest inducer concentration (218 nM).

2.3.2. Program the robot to serially dilute medium from each of the highest-concentration wells

into 23 lower concentrations ranging from 0 to 218 nM. The volume of each inducer dilution should be sufficient for all strains (including duplicates).

2.3.3. While the inducer dilutions are being prepared, warm 180 µL of SPM in the incubator at 37 °C, in 96-well plates.

2.3.4. Dilute the overnight strains from step 2.2 by a factor of 100 by serial dilutions: first dilute by a factor of 10 by mixing 100 µL of bacteria with 900 µL of SPM in 48-well plates, and then dilute again by a factor of 10 by taking 20 µL from the diluted solution into 180 µL of pre-warmed SPM, in 96-well plates suitable for fluorescent measurements.

2.3.5. Add the diluted inducer from the inducer plate to the 96-well plates with the diluted strains according to the final concentrations.

2.4. Shake the 96-well plates at 37 °C for 6 h, while taking measurements of optical density at 595 nm (OD_{595}), mCherry (560 nm/612 nm) and mCerulean (460 nm/510 nm) fluorescence via a plate reader every 30 min. For normalization purposes, measure growth of SMP with no cells added.

3. Preliminary results analysis

3.1. For each day of experiment, choose a time interval of logarithmic growth according to the measured growth curves, between the linear growth phase and the stationary (T_0, T_{final}). Take approximately 6–8 time points, while discarding the first and last measurements to avoid error derived from inaccuracy of exponential growth detection (see **Figure 2A**, top panel).

NOTE: Discard strains that show abnormal growth curves or strains where logarithmic growth phase could not be detected and repeat the experiment.

3.2. Calculate the average normalized fluorescence of mCerulean and rate of production of mCherry, from the raw data of both mCerulean and mCherry fluorescence for each inducer concentration (**Figure 2A**).

3.2.1. Calculate normalized mCerulean as follows:

$$Eq.1: Normalized\ mCerulean = \frac{mCerulean - blank(mCerulean)}{OD - blank(OD)}$$

where blank(mCerulean) is the mCerulean level [a.u.] for medium only, blank(OD) is the optical density for medium only, and mCerulean and OD are the mCerulean fluorescence and optical density values, respectively.

3.2.2. Average mCerulean over the different time points (**Figure 2B**, top two panels) as follows:

$$Eq.2: Averaged\ mCerulean = \frac{\sum_{T\_0}^{T\_final} Normalized\_mCerulean}{\#\ Time\ points}$$

where #Time points is the number of data timepoints taken into account, T_0 is the time at which the exponential growth phase begins, and T_{final} is the time at which the exponential growth phase ends.

3.2.3. Calculate mCherry rate of production (**Figure 2B**, bottom two panels) as follows:

$$Eq.3: mCherry\ production\ rate = \frac{mCherry(Tfinal) - mCherry(T0)}{\int_{T\_0}^{T\_final} ODdt}$$

where mCherry(t) is the mCherry level [a.u.] at time t, OD is the optical density value, T_0 is the time at which the exponential growth phase begins, and T_{final} is the time at which the exponential growth phase ends.

3.3. Finally, plot the mCherry rate of production as a function of mCerulean, creating dose response curves as a function of RBP-mCerulean fusion fluorescence (**Figure 2C**). Such plots represent production of the reporter gene as a function of RBP presence in the cell.

4. Dose response function fitting routine and K_{RBP} extraction

4.1. Under the assumption that the ribosome rate of translation with the RBP bound is constant, model the mCherry production rate as follows (see **Figure 2D**, green line):

$$Eq.4: mCherry\ production\ rate = \frac{k_{unbound}}{1 + \left(\frac{[x]}{k_{RBP}}\right)^n} + C$$

where [x] is the normalized average mCerulean fluorescence calculated according to Eq. 2, mCherry production rate is the value calculated according to Eq. 3, K_{RBP} is the relative binding affinity [a.u.], $K_{unbound}$ is the ribosome rate of translation with the RBP unbound, n is the cooperativity factor, and C is the base fluorescence [a.u.]. C, n, $K_{unbound}$, and K_{RBP} are found by fitting the mCherry production rate data to the model (Eq. 4).

4.2. Using data analysis software, conduct a fitting procedure on plots depicting mCherry production rate as a function of averaged mCerulean (step 3.3), and extract the fit parameters according to the formula in Eq. 4.

NOTE: Only fitting results with $R^2 > 0.6$ are taken into account. For those fits, K_{RBP} error is mostly in the range of 0.5% to 20% of K_{RBP} values, for a 0.67 confidence interval, while those with higher K_{RBP} error can be also verified by eye.

4.3. Normalize K_{RBP} values by the respective maximal value of averaged mCerulean for each dose-response function.

$$Eq.5: normalized\_k_{RBP} = \frac{k_{RBP}}{\max(averaged\ mCerulean)}$$

where K_{RBP} in [a.u.] is the value extracted from the fitting procedure in Eq. 4, and max (averaged mCerulean) is the maximal averaged mCerulean signal [a.u] observed for the current strain.

NOTE: The normalization facilitates correct comparison of the regulatory effect across strains by eliminating the dependence on the particular maximal RBP expression levels.

REPRESENTATIVE RESULTS:

The presented method utilizes the competition between an RBP and the ribosome for binding to the mRNA molecule (**Figure 1**). This competition is reflected by decreasing mCherry levels as a function of increased production of RBP-mCerulean, due to increasing concentrations of inducer. In the case of increasing mCerulean fluorescence, with no significant changes in mCherry, a lack of RBP binding is deduced. Representative results for both a positive and a negative strain are depicted in **Figure 2**. In **Figure 2A**, the OD, mCherry, and mCerulean channels are presented as a function of time and inducer over a range of four hours, with T_0 = 1 h and T_{final} = 3.5 h. In **Figure 2B**, averaged mCerulean fluorescence (top) and mCherry rate of production (bottom) are presented as a function of inducer concentration, for the two example strains. As can be seen, the results for a positive strain display a clear down-regulatory effect in the mCherry rate of production (**Figure 2B,C**), which translates into a significant non-zero value of K_{RBP} (**Figure 2D**). For the positive strain, the fitting procedure yielded the following values: K_{RBP} = 394.6 a.u., $K_{unbound}$ = 275.6, n = 2.1, C = 11.2 a.u., and R^2 = 0.93. After normalization by the maximal mCerulean fluorescence, the K_{RBP} value was 0.24. For the negative strain, a lack of distinct response was observed (**Figure 2C**), and no K_{RBP} value was extracted (**Figure 2D**).

In **Figure 3**, we present the results of this assay for two phage coat RBPs, PP7 and MS2, on several mutated binding sites, at different locations within the initiation region of the mCherry mRNA. The results are roughly classified into three kinds of responses (**Figure 3A**): strains exhibiting a down-regulatory effect at a low mCerulean level, reflecting a low K_{RBP} value (high binding affinity); strains exhibiting down-regulatory effect at either intermediate or high mCerulean levels, reflecting a high K_{RBP} value (intermediate or low affinity); and strains exhibiting no distinct response to rising levels of mCerulean, reflecting a higher K_{RBP} value than the maximum RBP concentration in the cell (no detectible binding affinity). **Figure 3B** presents the minimal K_{RBP} value computed for every RBP–binding-site combination based on all combinations of the two RBPs and ten binding-sites at different positions. The binding sites include a negative control (no binding site), non-matching binding sites, and a positive control — the native binding site for each RBP (PP7-wt for PP7 coat protein [PCP], and MS2-wt for MS2 coat protein [MCP]). The results match the predictions, as both RBPs present a high affinity for their positive controls, and a non-detectible binding affinity for the negative controls. Additionally, previous studies using these two RBPs[27,28] have observed that they are orthogonal, which is clearly conveyed in the heatmap presented: both MCP and PCP do not bind the native site of the other RBP. Furthermore, the mutated binding sites present varying results, where some binding sites displayed a similar level of affinity as that of the native site, such as PP7-mut-1, PP7-mut-2, and MS2-mut-3, while others displayed a significantly lower affinity, such as PP7-mut-3 and MS2-mut-2. Thus, the assay presented a quantitative in vivo measurement of the binding affinity of RBPs, yielding results that are comparable to those of past experiments with these RBPs.

Since the assay is based on repression of the mCherry gene, a viable mCherry signal is required. Therefore, when designing the binding site cassette, there are two design rules to keep in mind.

First, the open reading frame (ORF) of the mCherry should be kept. Since the binding-site length can vary, inserting it into the gene can cause a shift of one or two bases from the original mCherry ORF. Therefore, if needed (**Figure 4A**), insert one or two bases immediately downstream to the binding site. For example, a binding site that is 20-base long, with a δ of two bases, will yield an addition of 22 bases to the mCherry gene. To keep the ORF, we need to add two bases, for a total of 24 bases. The second design rule is to avoid insertions of stop codons into the mCherry ORF. Some binding sites, as the MS2-mut-2 (**Figure 4B**, inset), contain stop codons when positioned in one or more of the three possible ORFs. Such an example is illustrated in **Figure 4A**, where the binding site contained a stop codon that is in-frame with the mCherry ORF only when no bases are added. As can be seen in the dose-response curve for that position (**Figure 4B**), mCherry production rate was undetectable, thus the binding affinity could not be measured.

A closer look at **Figure 4B** demonstrates the effect of the spacing δ on mCherry production. For instance, for δ = 4, basal production rate was a factor of six more than those for δ = 5, ensuring a higher fold-repression effect. For δ = 14, however, the basal production levels were too low to observe a down-regulatory effect.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of system design and cloning steps. Illustration of the cassette design for the binding site plasmid (left) and RBP-mCerulean plasmid (right). The next step is consecutive transformations of both plasmids into competent *E. coli* cells, with RBP plasmids first. Double-transformants are then tested for their mCherry expression levels in increasing inducer concentrations; if the RBP binds to the binding site, mCherry levels decline as a function of mCerulean (gray bubble).

Figure 2: Analysis scheme. (**A**) Three-dimensional (3D) plots depicting raw OD levels (top), mCerulean fluorescence (middle), and mCherry fluorescence (bottom) as a function of time and inducer concentration, for a positive strain. (**B**) Top: mCerulean steady-state expression levels for each inducer concentration is computed by dividing each fluorescence level by the respective OD and averaging over all values in the 2−3 h exponential growth time window for both the positive (left) and negative (right) strains. Bottom: mCherry production rate computed according to Eq. 3 for time-points 2−3 h after induction. (**C**) mCherry production rate plotted as a function of mean mCerulean fluorescence averaged over two biological duplicates for two strains. Error bars are standard deviation of both mCherry production rate and averaged mCerulean fluorescence acquired from at least two replicates. (**D**) Fit for K_{RBP} using the fitting formula in Eq. 4 shown for the positive strain (left), exhibiting a specific binding response. For the negative strain (right), no K_{RBP} value was extracted. Data is shown in duplicate. This figure has been adapted with permission from Katz et al.[10]. Copyright 2018 American Chemical Society.

Figure 3: Representative **final results.** (**A**) Normalized dose-response curves for thirty different strains based on two RBPs and ten binding sites at different locations. Three types of responses are observed: high affinity, low affinity, and no affinity. (**B**) Quantitative K_{RBP} results for two RBPs (MCP and PCP) with five different binding site cassettes (listed). All RBP−binding-site strains were

measured in duplicate. This figure has been adapted with permission from Katz et al.[10]. Copyright 2018 American Chemical Society.

Figure 4: Example design and results for MCP with a mutant binding site. (**A**) Design illustration of the binding site cassettes in four different locations. Cassette including the ribosome binding site, start codon for the mCherry, δ spacer bases, the binding site tested, one or two bases to maintain the ORF, and the rest of the mCherry gene. Red stars indicate a stop codon. (**B**) Dose-response curves for MCP with a mutant binding site at four different locations. Inset: the sequence of the tested mutated binding site. Results presented are for duplicates of each strain.

Table 1: Binding sites and sequencing primers. Sequences for the binding sites and binding site cassettes used in this study, as well as the primers for the sequencing reactions detailed in the protocol (steps 1.2.5.1 and 1.3.3).

Table 2: RBP sequences. Amino acid and nucleotide sequences of the coat proteins used in this study.

DISCUSSION:

The method described in this article facilitates quantitative in vivo measurement of RBP-RNA binding affinity in *E. coli* cells. The protocol is relatively easy and can be conducted without the use of sophisticated machinery, and data analysis is straightforward. Moreover, the results are produced immediately, without the relatively long wait-time associated with next generation sequencing (NGS) results.

One limitation to this method is that it works only in bacterial cells. However, a previous study[12] has demonstrated a repression effect using a similar approach for the L7AE RBP in mammalian cells. An additional limitation of the method is that the insertion of the binding site in the mCherry initiation region may repress basal mCherry levels. Structural complexity or high stability of the binding site can interfere with ribosomal initiation even in the absence of RBP, resulting in decreased mCherry basal levels. If basal levels are too low, the additional repression brought on by increasing concentrations of RBP will not be observable. In such a case, it is best to design the binding site cassette with the binding site still in the initiation region, but on the verge of the transition from initiation region to elongation region (δ in the range of 12−15 bp[10,29]). We have shown that for such δ values a repression effect can still be observed. To increase the chances that the assay will work, regardless of structural complexity, we advise performing the assay on at least three different positions for a given binding site.

The main disadvantage of the method in comparison to in vitro methods, such as EMSA, is that the RBP-RNA binding affinity is not measured in absolute units of RBP concentration, but rather in terms of fusion-RBP fluorescence. This disadvantage is a direct result of the in vivo setting, which limits our ability to read out the actual concentrations of RBP. This disadvantage is offset by the benefits of measuring in the in vivo setting. For example, we have found differences in binding affinities when comparing results from our in vivo assay to previous in vitro and in situ assays. These differences may stem from discrepancies in the structure of the mRNA molecules

in vivo that emerge from their presence inside cells[10,11,30,31]. Such structural differences may lead to changes in the stability of the folded states in vivo which, in turn, either stabilize or de-stabilize RBP binding.

Since the method is relatively simple and inexpensive, we advise running multiple controls alongside the actual experiment. Running a negative control, i.e., a sequence that has no affinity to the RBP yet has similar structural features, can help avoid false positives stemming from non-specific interactions with the mRNA. In the representative results shown, the two negative controls were the mCherry gene alone (no binding site), and the native binding site of the other RBP (i.e., PP7-wt for MCP and MS2-wt for PCP). Moreover, we propose incorporating a positive control (such as an RBP and its native binding site). Such a control will help in quantifying the binding affinity by presenting a reference point, and in avoiding false-negatives stemming from low fold-repression.

Finally, for those who wish to obtain a structural perspective of RBP-RNA binding, we propose carrying out a selective 2'-hydroxyl acylation analyzed by primer extension sequencing (SHAPE-Seq)[11,32,33] experiment. SHAPE-Seq is an NGS approach combined with chemical probing of RNA, which can be used to estimate secondary structure of RNA as well as RNA interactions with other molecules, such as proteins. In our previous work we conducted a SHAPE-Seq experiment on a representative strain in both in vivo conditions[34] and in vitro with purified recombinant protein[10,35]. In our case, the results revealed that RBP-binding affected a much wider segment of RNA than previously reported for these RBPs in vitro[36].

ACKNOWLEDGMENTS:

This project received funding from the I-CORE Program of the Planning and Budgeting Committee and the Israel Science Foundation (Grant No. 152/11), Marie Curie Reintegration Grant No. PCIG11-GA- 2012-321675, and from the European Union's Horizon 2020 Research and Innovation Program under grant agreement no. 664918 - MRG-Grammar.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Cerretti, D.P., Mattheakis, L.C., Kearney, K.R., Vu, L., Nomura, M. Translational regulation of the spc operon in Escherichia coli. Identification and structural analysis of the target site for S8 repressor protein. *Journal of Molecular Biology*. **204** (2), 309–329 (1988).

2. Babitzke, P., Baker, C.S., Romeo, T. Regulation of translation initiation by RNA binding proteins. *Annual Review of Microbiology*. **63**, 27–44, doi: 10.1146/annurev.micro.091208.073514 (2009).

3. Van Assche, E., Van Puyvelde, S., Vanderleyden, J., Steenackers, H.P. RNA-binding proteins involved in post-transcriptional regulation in bacteria. *Frontiers in Microbiology*. **6**, 141, doi: 10.3389/fmicb.2015.00141 (2015).

4. Chappell, J., Watters, K.E., Takahashi, M.K., Lucks, J.B. A renaissance in RNA synthetic biology: new mechanisms, applications and tools for the future. *Current Opinion in Chemical Biology*. **28**, 47–56, doi: 10.1016/j.cbpa.2015.05.018 (2015).

5. Wagner, T.E. et al. Small-molecule-based regulation of RNA-delivered circuits in mammalian cells. *Nature Chemical Biology*. **14** (11), 1043, doi: 10.1038/s41589-018-0146-9 (2018).
6. Bendak, K. et al. A rapid method for assessing the RNA-binding potential of a protein. *Nucleic Acids Research*. **40** (14), e105, doi: 10.1093/nar/gks285 (2012).
7. Strein, C., Alleaume, A.-M., Rothbauer, U., Hentze, M.W., Castello, A. A versatile assay for RNA-binding proteins in living cells. *RNA*. **20** (5), 721–731, doi: 10.1261/rna.043562.113 (2014).
8. Ule, J., Jensen, K.B., Ruggiu, M., Mele, A., Ule, A., Darnell, R.B. CLIP identifies Nova-regulated RNA networks in the brain. *Science*. **302** (5648), 1212–1215, doi: 10.1126/science.1090095 (2003).
9. Lee, F.C.Y., Ule, J. Advances in CLIP Technologies for Studies of Protein-RNA Interactions. *Molecular Cell*. **69** (3), 354–369, doi: 10.1016/j.molcel.2018.01.005 (2018).
10. Katz, N. et al. An in Vivo Binding Assay for RNA-Binding Proteins Based on Repression of a Reporter Gene. *ACS Synthetic Biology*. **7** (12), 2765–2774, doi: 10.1021/acssynbio.8b00378 (2018).
11. Watters, K.E., Yu, A.M., Strobel, E.J., Settle, A.H., Lucks, J.B. Characterizing RNA structures in vitro and in vivo with selective 2'-hydroxyl acylation analyzed by primer extension sequencing (SHAPE-Seq). *Methods*. **103**, 34–48, doi: 10.1016/j.ymeth.2016.04.002 (2016).
12. Saito, H. et al. Synthetic translational regulation by an L7Ae–kink-turn RNP switch. *Nature Chemical Biology*. **6** (1), 71–78, doi: 10.1038/nchembio.273 (2010).
13. Gott, J.M., Wilhelm, L.J., Uhlenbeck, O.C. RNA binding properties of the coat protein from bacteriophage GA. *Nucleic Acids Research*. **19** (23), 6499–6503 (1991).
14. Peabody, D.S. The RNA binding site of bacteriophage MS2 coat protein. *The EMBO Journal*. **12** (2), 595–600 (1993).
15. Lim, F., Peabody, D.S. RNA recognition site of PP7 coat protein. *Nucleic Acids Research*. **30** (19), 4138–4144, doi: 10.1093/nar/gkf552 (2002).
16. Lim, F., Spingola, M., Peabody, D.S. The RNA-binding Site of Bacteriophage Qβ Coat Protein. *Journal of Biological Chemistry*. **271** (50), 31839–31845, doi: 10.1074/jbc.271.50.31839 (1996).
17. Gibson, D.G. et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*. **6** (5), 343–345, doi: 10.1038/nmeth.1318 (2009).
18. Optimizing Restriction Endonuclease Reactions | NEB. https://international.neb.com/tools-and-resources/usage-guidelines/optimizing-restriction-endonuclease-reactions.
19. Wizard® SV Gel and PCR Clean-Up System Protocol. https://worldwide.promega.com/resources/protocols/technical-bulletins/101/wizard-sv-gel-and-pcr-cleanup-system-protocol/.
20. Ligation Protocol with T4 DNA Ligase (M0202) | NEB. https://international.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202.
21. Routine Cloning Using Top10 Competent Cells - US. https://www.thermofisher.com/us/en/home/references/protocols/cloning/competent-cells-protocol/routine-cloning-using-top10-competent-cells.html.
22. NucleoSpin Plasmid - plasmid Miniprep kit. https://www.mn-net.com/ProductsBioanalysis/DNAandRNApurification/PlasmidDNApurificationeasyfastreliable/NucleoSpinPlasmidplasmidMiniprepkit/tabid/1379/language/en-US/Default.aspx.
23. Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., Roe, B.A. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *Journal of Molecular Biology*. **143** (2), 161–178,

doi: 10.1016/0022-2836(80)90196-5 (1980).
24. Addgene: Protocol - How to Create a Bacterial Glycerol Stock. https://www.addgene.org/protocols/create-glycerol-stock/.
25. Making your own chemically competent cells | NEB. https://international.neb.com/protocols/2012/06/21/making-your-own-chemically-competent-cells.
26. Luria-Bertani (LB) Medium Preparation · Benchling. https://benchling.com/protocols/gdD7XI0J/luria-bertani-lb-medium-preparation.
27. Delebecque, C.J., Silver, P.A., Lindner, A.B. Designing and using RNA scaffolds to assemble proteins in vivo. *Nature Protocols*. **7** (10), 1797–1807, doi: 10.1038/nprot.2012.102 (2012).
28. Hocine, S., Raymond, P., Zenklusen, D., Chao, J.A., Singer, R.H. Single-molecule analysis of gene expression using two-color RNA labeling in live yeast. *Nature Methods*. **10** (2), 119–121, doi: 10.1038/nmeth.2305 (2013).
29. Espah Borujeni, A. et al. Precise quantification of translation inhibition by mRNA structures that overlap with the ribosomal footprint in N-terminal coding sequences. *Nucleic Acids Research*. **45** (9), 5437–5448, doi: 10.1093/nar/gkx061 (2017).
30. Ding, Y. et al. In vivo genome-wide profiling of RNA secondary structure reveals novel regulatory features. *Nature*. **505**, doi: 10.1038/nature12756 (2013).
31. Rouskin, S., Zubradt, M., Washietl, S., Kellis, M., Weissman, J.S. Genome-wide probing of RNA structure reveals active unfolding of mRNA structures in vivo. *Nature*. **505** (7485), 701–705, doi: 10.1038/nature12894 (2014).
32. Lucks, J.B. et al. Multiplexed RNA structure characterization with selective 2'-hydroxyl acylation analyzed by primer extension sequencing (SHAPE-Seq). *Proceedings of the National Academy of Sciences of the United States of America*. **108** (27), 11063–11068, doi: 10.1073/pnas.1106501108 (2011).
33. Spitale, R.C. et al. Structural imprints *in vivo* decode RNA regulatory mechanisms. *Nature*. **519** (7544), 486, doi: 10.1038/nature14263 (2015).
34. Watters, K.E., Abbott, T.R., Lucks, J.B. Simultaneous characterization of cellular RNA structure and function with in-cell SHAPE-Seq. *Nucleic Acids Research*. **44** (2), e12, doi: 10.1093/nar/gkv879 (2016).
35. Flynn, R.A. et al. Transcriptome-wide interrogation of RNA secondary structure in living cells with icSHAPE. *Nature Protocols*. **11** (2), 273–290, doi: 10.1038/nprot.2016.011 (2016).
36. Bernardi, A., Spahr, P.-F. Nucleotide Sequence at the Binding Site for Coat Protein on RNA of Bacteriophage R17. *Proceedings of the National Academy of Sciences of the United States of America*. **69** (10), 3033–3037 (1972).

Figure 1

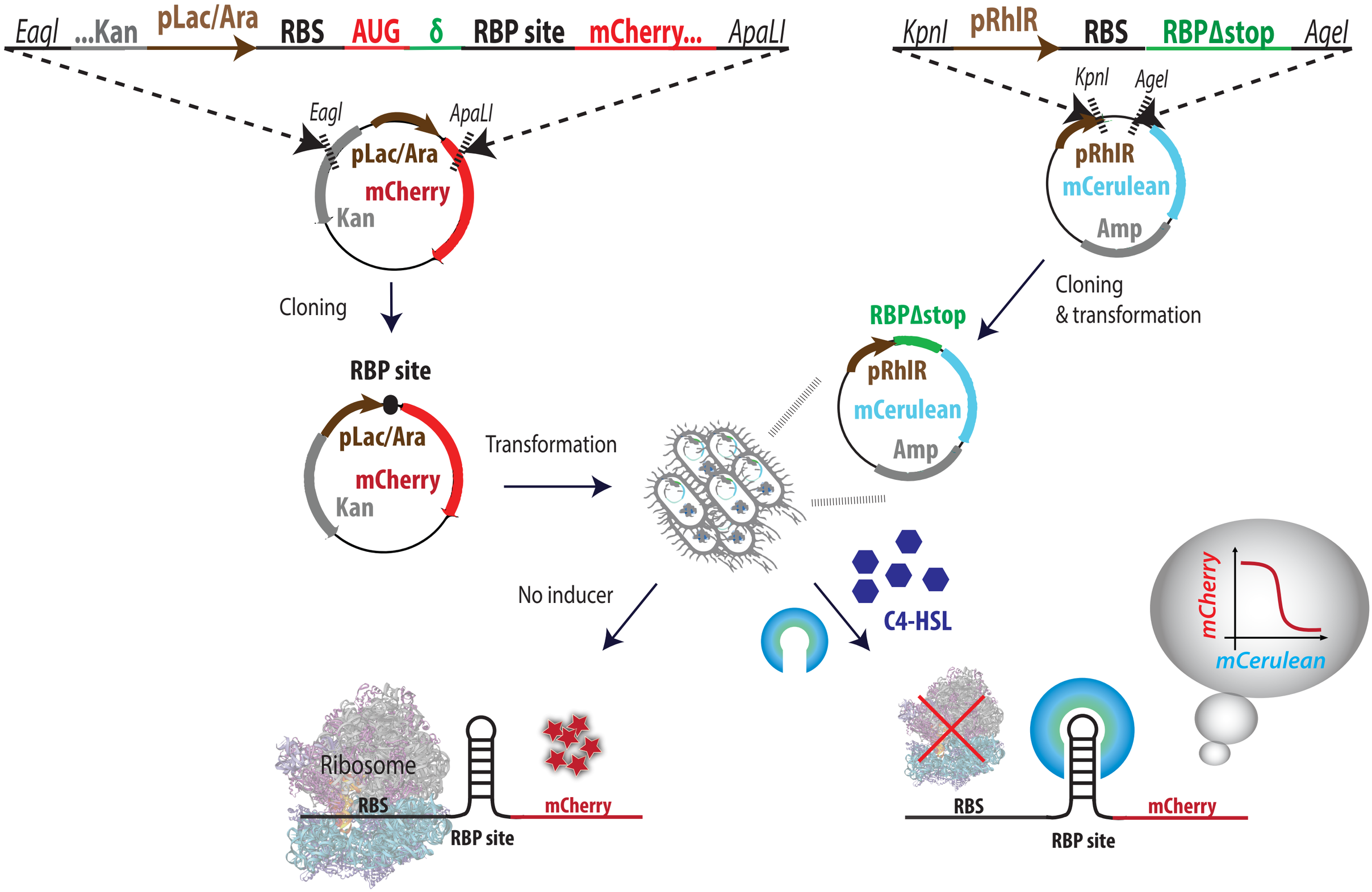

Figure 1

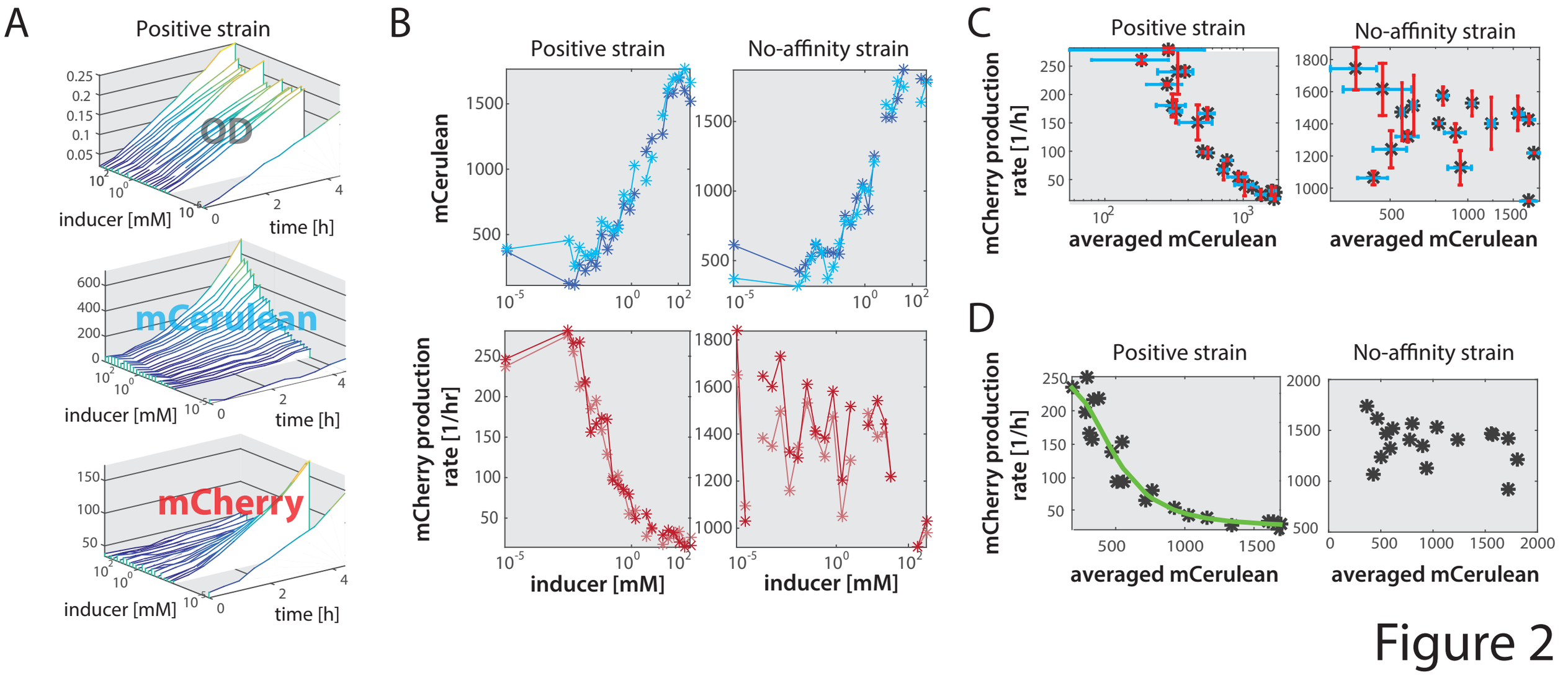

Figure 2

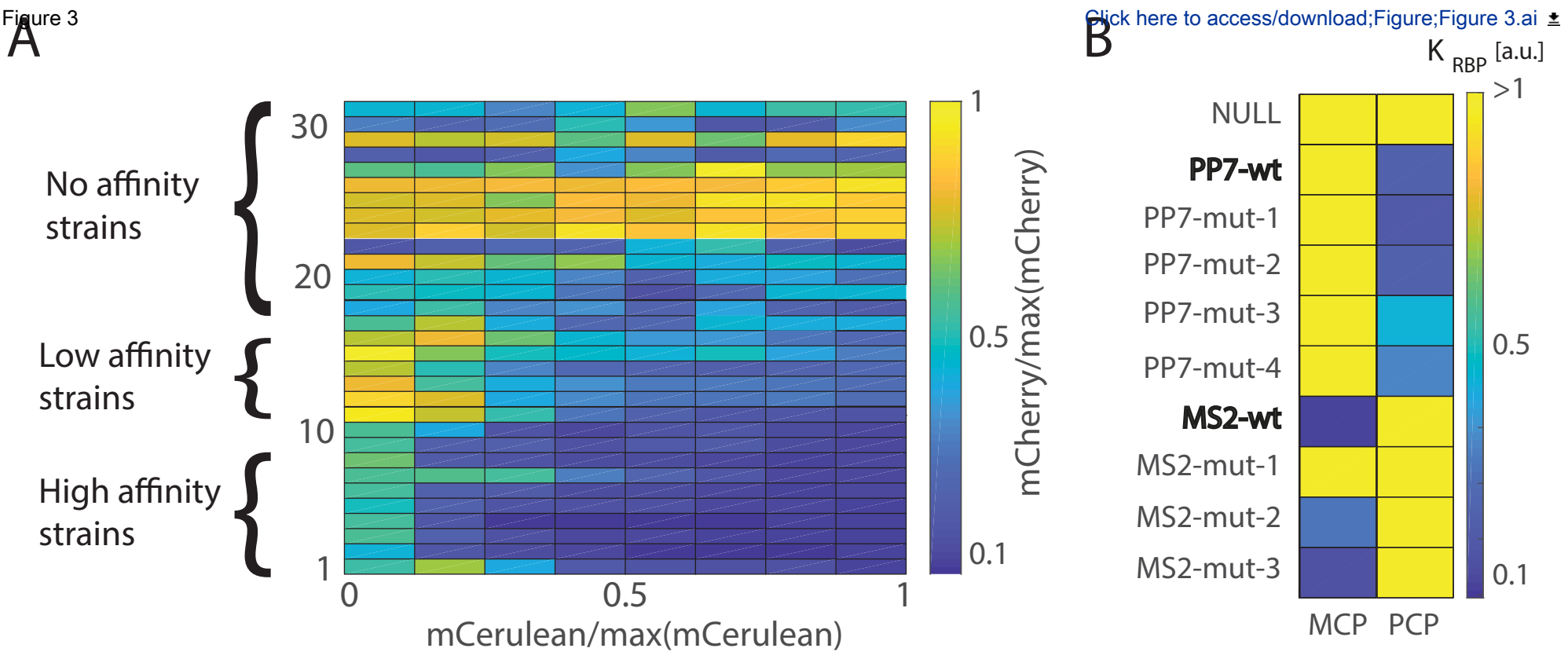

Figure 3

Figure 4

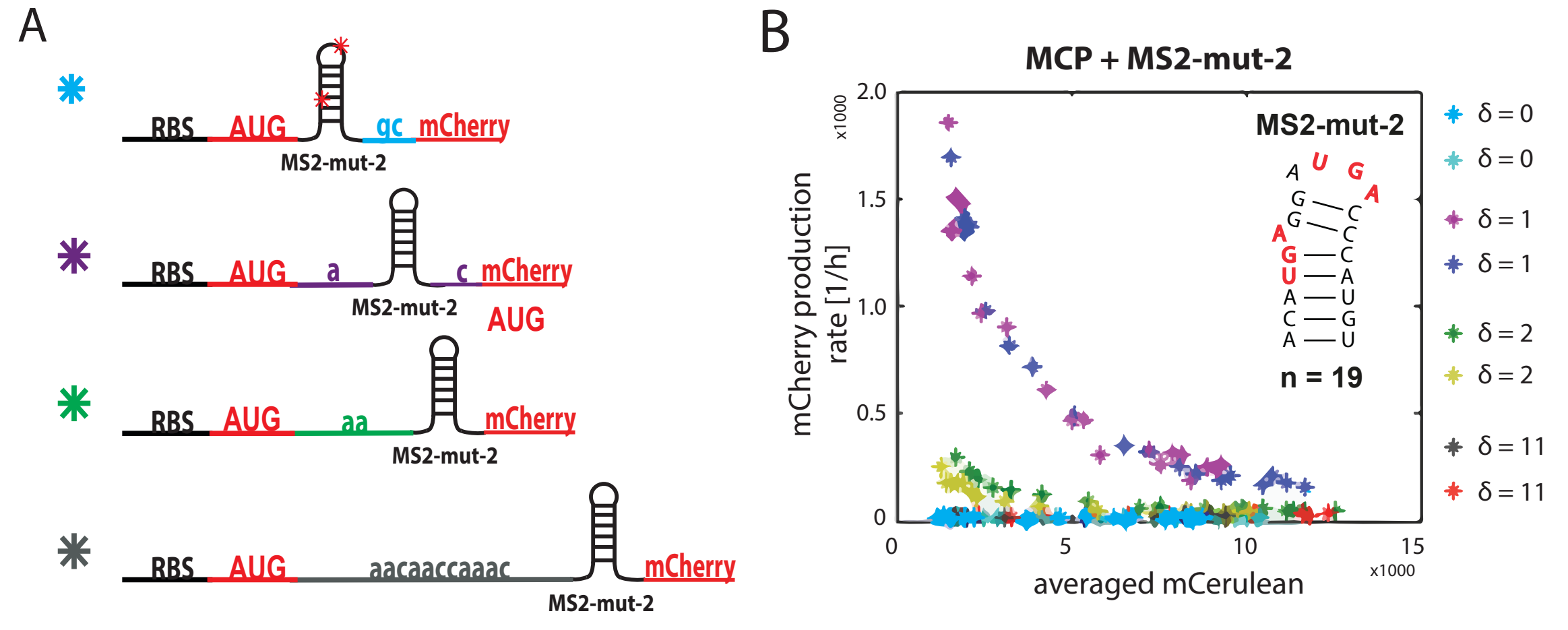

Table 1

Click here to access/download;Table;Supplementary_Table_1.xlsx

Name	Binidng site location, A in AUG = 1	Binding site sequence (RBS for controls)
MS2_wt_d5	5	acatgaggattacccatgt
MS2_wt_d6	6	acatgaggattacccatgt
MS2_wt_d8	8	acatgaggattacccatgt
MS2_wt_d9	9	acatgaggattacccatgt
MS2_U(-5)C_d8	8	acatgaggatcacccatgt
MS2_U(-5)C_d9	9	acatgaggatcacccatgt
MS2_U(-5)C_d8	8	acatgaggatgacccatgt
MS2_U(-5)G_d9	9	acatgaggatgacccatgt
MS2_struct_d9	9	cacaagaggttcacttatg
MS2_struct_d8	8	cacaagaggttcacttatg
PP7wt_d5'	5	taaggagtttatatggaaaccctta
PP7wt_d6'	6	taaggagtttatatggaaaccctta
PP7wt_d8'	8	taaggagtttatatggaaaccctta
PP7wt_d9'	9	taaggagtttatatggaaaccctta
PP7_USLSBm_d6	6	taaccgctttatatggaaagggtta
PP7_USLSBm_d15	15	taaccgctttatatggaaagggtta
PP7_nB_d5	5	taagggtttatatggaaaccctta
PP7_nB_d6	6	taagggtttatatggaaaccctta
PP7_USs_d5	5	taaggagttatatggaaccctta
PP7_USs_d6	6	taaggagttatatggaaccctta
No_BS_d1	-	-
No_BS_d4	-	-
No_BS_d10	-	-
Sequencing primer for binding site cassettes		
Sequencing primer for RBP cassettes		

Site: ATG to second mCherry codon GTG Controls: RBS to second mCherry codon GTG	Source
atgcacatgaggattacccatgtcgtg	Gen9 Inc.
atggcacatgaggattacccatgtgtg	Gen9 Inc.
atggcgcacatgaggattacccatgtcgtg	Gen9 Inc.
atggcgccacatgaggattacccatgtgtg	Gen9 Inc.
atgcacatgaggatcacccatgtggtg	Gen9 Inc.
atggcacatgaggatcacccatgtgtg	Gen9 Inc.
atgcacatgaggatgacccatgtggtg	Gen9 Inc.
atggcacatgaggatgacccatgtgtg	Gen9 Inc.
atggccacaagaggttcacttatggtg	Gen9 Inc.
atgccacaagaggttcacttatgggtg	Gen9 Inc.
atgctaaggagtttatatggaaacccttacgtg	Gen9 Inc.
atgaataaggagtttatatggaaacccttagtg	Twist Bioscience
atgaacataaggagtttatatggaaacccttacgtg	Twist Bioscience
atgaacaataaggagtttatatggaaacccttagtg	Twist Bioscience
atggctaaccgctttatatggaaagggttagtg	Gen9 Inc.
atgggcgccggcgctaaccgctttatatggaaagggttagtg	Gen9 Inc.
atgctaagggtttatatggaaacccttagcgtg	Gen9 Inc.
atggctaagggtttatatggaaacccttatgtg	Gen9 Inc.
atgctaaggagttatatggaacccttagtg	Gen9 Inc.
atggctaaggagttatatggaacccttagcgtg	Gen9 Inc.
ttaaagaggagaaaggtacccatggtg	Gen9 Inc.
ttaaagaggagaaaggtacccatgggcgtg	Gen9 Inc.
ttaaagaggagaaaggtacccatgggcgccggcgtg	Gen9 Inc.
gcatttttatccataagattagcgg	IDT
gcggcgctgggtctcatctaataa	IDT

Table 2 Click here to access/download;Table;Table 2_RE.xlsx

RBP name in this work	source organism name, protein	source organism gene	source organism refseq
MCP	Escherichia virus MS2	cp	NC_00141
PCP	Pseudomonas phage PP7	cp	NC_00162

References:

1.Peabody, D.S., Ely, K.R. Control of translational repression by protein-protein interactions. *Nucleic Acids Research* . **20** (7), 1649–1655 (1992).

2. Chao, J.A., Patskovsky, Y., Almo, S.C., Singer, R.H. Structural basis for the coevolution of a viral RNA–protein complex. *Nature Structural & Molecular Biology* . **15** (1), 103–105, doi: 10.1038/nsmb1327 (2008)

wt aa seq	changes from wt (and references)	aa seq used in this work
MASNFTQFVLVDNGGTGDVTV APSNFANGVAEWISSNSRSQA YKVTCSVRQSSAQNRKYTIKVE VPKVATQTVGGVELPVAAWRS YLNMELTIPIFATNSDCELIVKA MQGLLKDGNPIPSAIAANSGIY	delF-G [1] V29I [1] taken from addgene plasmid 27121	MASNFTQFVLVDNGG TGDVTVAPSNFANGIA EWISSNSRSQAYKVT CSVRQSSAQNRKYTI KVEVPKGAWRSYLN MELTIPIFATNSDCELI VKAMQGLLKDGNPIP SAIAANSGIY
MSKTIVLSVGEATRTLTEIQSTA DRQIFEEKVGPLVGRLRLTASL RQNGAKTAYRVNLKLDQADVV DCSTSVCGELPKVRYTQVWSH DVTIVANSTEASRKSLYDLTKSL VATSQVEDLVVNLVPLGR	delF-G [2] taken from addgene plasmid 40650	MLASKTIVLSVGEATR TLTEIQSTADRQIFEE KVGPLVGRLRLTASLR QNGAKTAYRVNLKLD QADVVDSGLPKVRYT QVWSHDVTIVANSTE ASRKSLYDLTKSLVAT SQVEDLVVNLVPLGR

nt seq used in this work

ATGGCTTCTAACTTTACTCAGTTCG
TTCTCGTCGACAATGGCGGAACTG
GCGACGTGACTGTCGCCCCAAGC
AACTTCGCTAACGGGATCGCTGAA
TGGATCAGCTCTAACTCGCGTTCA
CAGGCTTACAAAGTAACCTGTAGC
GTTCGTCAGAGCTCTGCGCAGAAT
CGCAAATACACCATCAAAGTCGAG
GTGCCTAAAGGCGCCTGGCGTTC
GTACTTAAATATGGAACTAACCATT
CCAATTTTCGCCACGAATTCCGAC
TGCGAGCTTATTGTTAAGGCAATG
CAAGGTCTCCTAAAAGATGGAAAC
CCGATTCCCTCAGCAATCGCAGCA
AACTCCGGCATCTAC
ATGCTAGCCTCCAAAACCATCGTT
CTTTCGGTCGGCGAGGCTACTCGC
ACTCTGACTGAGATCCAGTCCACC
GCAGACCGTCAGATCTTCGAAGAG
AAGGTCGGGCCTCTGGTGGGTCG
GCTGCGCCTCACGGCTTCGCTCC
GTCAAAACGGAGCCAAGACCGCGT
ATCGCGTCAACCTAAAACTGGATC
AGGCGGACGTCGTTGATTCCGGAC
TTCCGAAAGTGCGCTACACTCAGG
TATGGTCGCACGACGTGACAATCG
TTGCGAATAGCACCGAGGCCTCGC
GCAAATCGTTGTACGATTTGACCA
AGTCCCTCGTCGCGACCTCGCAG
GTCGAAGATCTTGTCGTCAACCTT
GTGCCGCTGGGCCGT

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Ampicillin sodium salt	SIGMA	A9518	
Magnesium sulfate ($MgSO_4$)	ALFA AESAR	33337	
48 plates	Axygen	P-5ML-48-C-S	
8- lane plates	Axygen	RESMW8I	
96-well plates	Axygen	P-DW-20-C	
96-well plates for plate reader	Perkin Elmer	6005029	
ApaLI	NEB	R0507	
Binding site sequences	Gen9 Inc. and Twist Bioscience		see Table 1
E. coli TOP10 cells	Invitrogen	C404006	
EagI-HF	NEB	R3505	
glycerol	BIO LAB	071205	
incubator	TECAN	liconic incubator	
Kanamycin solfate	SIGMA	K4000	
KpnI- HF	NEB	R0142	
ligase	NEB	B0202S	
liquid-handling robotic system	TECAN	EVO 100, MCA 96-channel	
Matlab analysis software	Mathworks		
multi- pipette 8 lanes	Axygen	BR703710	
N-butanoyl-L-homoserine lactone (C_4-HSL)	cayman	K40982552 019	
PBS buffer	Biological Industries	020235A	
platereader	TECAN	Infinite F200 PRO	
Q5 HotStart Polymerase	NEB	M0493	
RBP seqeunces	Addgene	27121 & 40650	see Table 2
SODIUM CHLORIDE (NaCL)	BIO LAB	190305	
SV Gel and PCR Clean-Up System	Promega	A9281	

Tryptone	BD	211705

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.8051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: An assay for quantifying RBP-RNA binding in bacteria

Author(s): Noa Katz, Roni Cohen, Orna Atar, Sarah Goldberg and Roee Amit

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked **in Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Noa Katz
Department:	Biotechnology and Food engineering
Institution:	Technion Institute of Technology
Title:	Ms
Signature:	
Date:	25.12.2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.
3. Please rewrite lines 101-114 and 133-149 to avoid overlap with previous work.
4. Please provide an email address for each author.
5. Please define all abbreviations before use.
6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Axygen, Tecan Freedom

Author response: we have edited the manuscript dramatically to make it compatible with JoVE guidelines. Additionally, the revised manuscript has been thoroughly re-read and re-written for improved grammar and flow, including the mentioned parts.

Protocol:

1. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible.
2. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Author response: we have changed all of the protocol tenses to the imperative, according with JoVE guidelines. Additionally, we have added details to the protocol, including references to published material that describe how to do relevant protocol steps.

Specific Protocol steps:

1. 1.1: Figure 1A doesn't show a binding site cassette.
2. 1.3.1: Please include more details here if this step is going to be filmed.
3. 2.6: What wavelengths are used here?

Author response: we apologize for the error in the figures, and now have added a fourth figure, new figure 1, which presents the designs and cloning strategies for the two plasmids- binding site and RBP. We have also added more details to the manuscript, including the mentioned locations.

Figures:

1. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the

editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.

Author response: together with the revision files, we have also uploaded a file with the copyright permission to use the figures from the relevant paper.

2. Figure 1A, 1C, 3B: Please use ‘h’, not ‘hr’.
3. Figure 1C: What are the error bars?
4. Figure 2B: What unit is K(RBP) in?
Author response: there errors were corrected.

References:
1. Please do not abbreviate journal titles.
Author response: there errors were corrected

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Author response: the revised table now contains all materials and equipment used in the protocol.

Reviewers' comments:
Reviewer #1:

Major Concerns:

1) Lane 71 it says that the design of the binding cassette is depicted in figure 1A. This is not the case. The author should provide clear drawings of the two basic constructs. This is the only reason why I have answered "no" for the second question : "Is there sufficient introduction for the protocol? Is there an unbiased discussion of the protocol?"

Author response: we apologize for the error in the figures, and thank the reviewer for this comment. We now have added a fourth figure, new figure 1, which presents the designs and cloning strategies for the two plasmids- binding site and RBP.

2) In several occurences of the manuscript the method is claimed to be quantitative (l 63 ...) and that a Kd can be derived (l 191,192,218,221, 222 ...). I have several issues about this:

* a Kd is a defined value of a chemical equilibrium, it is supposed to have a unit that reflects a defined concentration. This is clearly not the case here, the authors should rename their extrapolated value as a "relative binding index" or so. Calling this value a KD is clearly improper. Although I understand the drawbacks of a in vitro method and I agree with the author, but their method is not nearly as precise as an in vitro method, it should be stated somewhere, maybe in the limitationsd of the method.
*The limitation of the quantitative aspect is also apparent in the results. Figure 1B, the positive strain: looking at the point that represents roughly 10-1 mM of inducer: the cerulean fluorescence measured is at the same level that at 10-5, but the mcherry fluorescence is already down from 250 to 150 (which is in the middle of the

sigmoid). In addition in the same time the negative strain varies from 1800 to 1400.
* In summary, the "quantitative" claims should be mitigated, the realtive value obtained can not be called Kd, and in the limitations the author should in my opinion clearly state that the dynamic range is narrow (see above) and clearly narrower that what is observed with in vitro methods (this is probably inherent to the in vivo repression method used).
Author response: we thank the reviewer for this comment, and changed the term Kd to the term K_{RBP}. We also agree that the acquired resolution in-vitro is much higher- in absolute units of RBP concentration while here it is in terms of fusion-RBP fluorescence- and clearly state that in the text (see discussion). However, in our view, it does not completely contradict our quantitative claims. According to online definition (Merriam-Webster) "quantitative" means “relating to, measuring, or measured by the quantity of something rather than its quality”. Our assay quantifies the binding affinity by the fluorescence units of the RBP-FP fusion, or the scaled affinity relative to the maximal RBP-FP fluorescence *in-vivo*. It is able to provide a range of binding affinities for the different binding sites, so that a quantitative comparison can be made. We agree that we can always make the measurement more accurate, by increasing the number of inducer concentrations, measuring more replicates, etc., yet it still is a quantitative measurement.

Minor Concerns:

* I am not a native english speaker but in my opinion the manuscript would benefit from a proofreading of a native english speaker. In particular the first and the last sentence of the abstract does not sound correct and clear to me
Author response: the revised manuscript has been thoroughly re-read and re-written for improved grammar and flow, including the abstract.

This is a nice method worth reporting it can be publish after mitigating the quantitative claims.

Reviewer #2:

Manuscript Summary:
The authors here present their protocol on usage of a dual fluorescence reporter system to assay the affinity of binding of RNA-binding proteins (RBPs) to specific sequences. The system they use is based on the fact that when an RBP is bound at specific locations surrounding the ribosome binding site, translation initiation will be inhibited. Their system is set up and used in E.coli.

Major Concerns:

-The authors suggest that their method can replace CLIP-type methods, that actually reveal RBP binding sites in whole genome approaches. As it is, the method can not discover new RBP binding sites. It can only be used to assay the affinity of binding to already known sequences. One can possibly use the method to measure the affinity of the binding in E.coli, in vivo, but not for de novo discovery of binding motifs and binding sequences of RBPs on RNAs in a high-throughput manner, as one can do using CLIP assays.

Author response: we agree with the reviewer that in our method we first need to design the binding sites tested, either randomly or by rational design, as opposed to de novo discovery of binding sequences, as CLIP assays enable. This part was re-phrased (see introduction).

-The method is being used in E.coli, in vivo. As such it would be useful for people interested in E-coli RBPs and their known RNA targets. However, at their original publication the authors also use SHAPE-seq to assay the binding of the RBP to its target in more detail. In my mind, one will have to perform such analyses when using new RBP-RNA interactions (different to the ones that have been assayed here), as otherwise they will not be able to show that any inhibition of the translation initiation happens because of reduced binding and not because of a change in the secondary structure of the RNA, that alters the proximity of the binding site. And as SHAPE is not an easy method to perform and analyse, the suggested method will not be a simple method, as proposed by the authors.

Author response: we thank the reviewer for helping us clarify this point. While it is true that we conducted a SHAPE-seq assay in our original publication, it was conducted for a structural insight alone, and was not included in the analysis for binding affinity. Therefore, we do not believe it is necessary for the purpose of this method. The use of RBP-mCerulean induction ensures that any changes in the mCherry fluorescence output would be a direct effect of the RBP being expressed, as reported by mCerulean fluorescence. Otherwise, mCherry levels would be effected in a dose-independent manner. This, in turn, ensures that any changes in the structure of the RNA that occur with increasing levels of RBP is a direct result of RBP binding. In the case that RBP binding changes the structure of the mRNA when bound, so that the proximity of the binding site is also altered, it does not matter for the purpose of the method as long as mCherry is being affected. In the case that mCherry is not effected by RBP binding, which would cause a false negative result, the use of a positive control would point it out. We now relate to this possibility in the discussion. Finally, in the last paragraph of the discussion we propose the SHAPE-seq method, and say that it is a good option for those who wish to gain a structural perspective and to look into the molecular effect of binding on structure.

-More specific points:

1. Introduction, line 64. The method as presented here is not a high-throughput one.
   Author response: this error was corrected.

2. Line 71: Figure 1A does not show any design of the plasmids, as mentioned in the text. This schematic is absolutely necessary and should be detailed: show the schematic of the plasmids and the cloning procedure in a schematic.
3. Line 102: Supplemental table 2 only mentions MCP, PCP and not GCP, and QCP. Anyway, result for the last two proteins are not presented here, so they could be omitted from the sentence in line 102. However, a schematic of this cloning procedure would also be helpful/necessary.
   Author response: we apologize for the errors in the figures and text and thank the reviewer for both comments. We now have added a fourth figure, new figure 1, which presents the designs and cloning strategies for the two plasmids- binding site and RBP. In addition, we omitted the comment about GCP and QCP, as we

have decided not to add data with these proteins for this protocol-based manuscript.

Minor Concerns:

1. Introduction, line 48: The reference 1. is not relevant to the present publication. Reference 2 is quite old, whereas there are newer relevant publications, more, and also relevant reviews that could point towards the desired direction.
2. Introduction, line54: There is a large number of papers on the different CLIP methods. Rather a recent review (eg. Mol Cell. 2018 Feb 1;69(3):354-369. doi: 10.1016/j.molcel.2018.01.005. Advances in CLIP Technologies for Studies of Protein-RNA Interactions. L ee FCY, Ule J.) would be more appropriate here.
3. Line 81: "Gibson assembly" needs a reference.
4.Line 155: would be very helpful to indicate selected T0-Tend specifically here, based on the curves.
5. Lines 169-174: explanations of the symbols in the formulas are missing.
6. Lines 191-194: One can hardly follow the formula as it is. It is necessary, for a protocols publication, to refer to each value in the formula and mention where/how it is calculated. Eg. what is the value for Kun-bound? Where do we find it here?
7. In the table of materials, please add: multi-channel pipette, 8-, 96-well plates

Author response: these errors were corrected and the reference added. We thank the reviewer for these comments and the rest of the review, which helped us improve our manuscript.

Editorial comments:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.
2. Please address specific comments marked in the attached manuscript. Please turn on Track Changes to keep track of the changes you make to the manuscript.
Author response: we have used the attached version, and made the appropriate alterations while tracking changes. Editorial comments have been changed to "fixed" within the file.

3. Figure 4B: Please change the time unit “hr” to “h”.
Author response: this error was fixed.

4. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.
Author response: the table of materials is now sorted alphabetically.

5. Supplemental tables: Please include a title and a description of each table in the Figure and Table Legends section.
Author response: both titles and descriptions were added to the manuscript. We apologize for this error.

6. Supplemental Table 2: Please format the references as follows:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

Author response: the two references were formatted as requested.

RightsLink®

Home | Create Account | Help

Title: An in Vivo Binding Assay for RNA-Binding Proteins Based on Repression of a Reporter Gene

Author: Noa Katz, Roni Cohen, Oz Solomon, et al

Publication: ACS Synthetic Biology

Publisher: American Chemical Society

Date: Dec 1, 2018

LOGIN

If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials.

Already **a RightsLink user** or want to learn more?

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms & Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from (COMPLETE REFERENCE CITATION). Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your request. No additional uses are granted (such as derivative works or other editions). For any other uses, please submit a new request.

If credit is given to another source for the material you requested, permission must be obtained from that source.

BACK | CLOSE WINDOW