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Quantitative characterization of translational riboregulators using an in vitro transcription-translation system

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Abstract

Riboregulators are short RNA sequences that, upon binding to a ligand, change their secondary structure and influence the expression rate of a downstream gene. They constitute an attractive alternative to transcription factors for building synthetic gene regulatory networks because they can be engineered de novo. However, riboregulators are generally designed in silico and tested in vivo, which provides little quantitative information about their performances, thus hindering the improvement of design algorithms. Here we show that a cell-free transcription-translation (TX-TL) system provides valuable information about the performances of in silico designed riboregulators. We first propose a simple model that provides a quantitative definition of the dynamic range of a riboregulator. We further characterize two types of translational riboregulators composed of a cis-repressed (cr) and a trans-activating (ta) strand. At the DNA level we demonstrate that high concentrations of taDNA poisoned the activator until total shut off, in agreement with our model, and that relative dynamic ranges of riboregulators determined in vitro are in agreement with published in vivo data. At the RNA level, we show that this approach provides a fast and simple way to measure dissociation constants of functional riboregulators, in contrast to standard mobility-shift assays. Our method opens the route for using cell-free TX-TL systems for the quantitative characterization of functional riboregulators in order to improve their design in silico.

Keywords

in vitro synthetic biology, RNA translational riboregulator, cell-free protein synthesis

24 During the early wave of synthetic biology,^{1,2} known transcription factors were wired
25 to their corresponding promoter sequences to control the expression of other transcription
26 factors or effector proteins. While this approach has been very successful in engineering
27 gene regulatory networks (GRNs)³ with few nodes, the number of different elements in the
28 majority of synthetic GRNs has stagnated at 5-6,^{4,5} although a remarkable example contains
29 11 elements.⁶ Two arguments may explain this limit. First, protein-DNA interactions are
30 very difficult to design, although very promising computational methods are arising,⁷ and
31 the engineer must thus choose well-known transcription factor-promoter pairs. Second, the
32 expression of these transcription factors imposes a metabolic burden to the cells.⁸

33 Implementing regulatory circuits at the RNA level may help solving these issues essen-
34 tially because RNA-RNA interactions can be predicted from the sequence.⁹⁻¹¹ Moreover, in
35 the case of transcriptional regulators, protein expression is not needed for regulation, which
36 lowers the metabolic burden.¹² The principal component of an RNA-regulated GRN is the
37 riboregulator: an RNA sequence in the 5' untranslated region (UTR) of a gene of interest
38 that has an effect on its expression rate. Since they were first used in synthetic biology more
39 than a decade ago,¹³ several riboregulators have been designed and implemented *in vivo*,
40 both in prokaryotic¹⁴⁻¹⁸ and eukaryotic cells.¹⁹ However, their design remains more difficult
41 than expected and many implementations do not work *in vivo*.¹² One reason to this is that
42 structure-prediction tools do not yet precisely capture the complexity involved in the folding
43 of RNA species several hundreds of nucleotides long. Furthermore, *in silico* design relies on a
44 structural model of riboregulation, which needs to be transformed into predictable features
45 in order to generate optimized sequences. Another reason is that it is hard to control and
46 tune the copy number of plasmids or genes *in vivo* and thus testing new parts *in vivo*^{20,21}
47 often provides information that is difficult to correlate with thermodynamic parameters used
48 *in silico*.

49 Including a phase of *in vitro* testing in the workflow of engineering riboregulators could
50 potentially solve these problems. Structural characterization of riboregulators^{22,23} helps as-

51 sessing the correctness of the designed structures and has been recently combined with func-
52 tional information²⁴ but does not provide quantitative kinetic and thermodynamic data.
53 To overcome these difficulties and accelerate the improvement of in silico designs, cell-free
54 transcription-translation (TX-TL) platforms are an attractive tool for testing genetic regu-
55 latory modules in synthetic biology.^{5,25-27} Currently, there are two types of TX-TL systems
56 available, cell-extract-based and PURE (Protein synthesis Using Recombinant Elements)
57 systems. The first one is obtained by recovering the protein fraction from *E. coli*²⁶ while
58 the PURE is just composed of individually-purified recombinant elements necessary for ex-
59 pression in vitro.^{28,29} TX-TL in vitro testing can be used to qualitatively evaluate the per-
60 formances of new designs in a faster manner^{5,27,30} or to provide quantitative data such as
61 thermodynamic and kinetic rates³¹ that are of great value to improve in silico methods.

62 Here we used a PURE TX-TL platform to illustrate the second approach. Briefly, the
63 PURE system includes T7 RNA polymerase (RNAP), an energy-coupling module for NTP
64 regeneration, transfer RNAs, ribosomes and translation initiation, elongation and release
65 factors in a suitable buffer.^{29,32} Its composition is well-controlled and it contains low levels
66 of ribonucleases. The PURE system has already been used to characterize transcription-
67 translation dynamics^{33,34} and GRNs³⁵ but has not yet been used to characterize riboreg-
68 ulators. Cell-extract TX-TL systems have been used to study transcriptional^{30,31} but not
69 translational riboregulators. To the best of our knowledge, TX-TL systems have so far in-
70 vestigated GRNs that mix both transcriptional and translational dynamics. In this work,
71 we characterize the dynamics of translational riboregulators at the DNA and RNA level,
72 which allows to independently study transcription and translation and clearly pinpoint de-
73 sign shortcomings. The simplicity of the PURE system allows us to propose an analytical
74 model of riboregulation that fits our data. The proposed model, together with the in vitro
75 experiments, show that: i) TX-TL linearly amplifies the concentration of active RNA and
76 quadratically amplifies the concentration of coding DNA, ii) we can provide a quantitative
77 definition of the dynamic range of a riboregulator, $\rho_{ON/OFF}$, iii) $\rho_{ON/OFF}$ is a bell-shaped

78 function of the concentration of regulatory DNA, iv) the relative values of $\rho_{ON/OFF}$ measured
79 in vitro coincide with published data in vivo for four riboregulators out of five and v) we can
80 reliably use TX-TL to measure the dissociation constant, K_d , between the two RNA species.
81 Although these results were facilitated by the simplicity of the PURE system, they could be
82 extended to other cell-free TX-TL systems and possibly in vivo. These quantitative insights
83 on translational riboregulators may also help improving in silico design routines.

84 Results and discussion

85 Translation rate vs. structure as the optimization goal for a ri- 86 boregulator

87 Our study focuses on translational riboregulators, which are composed of two RNA strands
88 (Figure 1A). One of them, called cis repressed RNA, noted R_{cr} , about 800 nucleotides (nt)
89 long, codes for a gene but bears a hairpin in its 5'-untranslated region (5'-UTR) that pre-
90 vents the ribosome to start translating the downstream gene. The other one, a small trans-
91 activating RNA, about 100 nt long, noted R_{ta} , hybridizes to the 5'-UTR of R_{cr} , opens up
92 the hairpin and forms an active complex, R_{act} , increasing the translation rate.

93 Ultimately, the riboregulator engineer is interested in controlling the rate of translation
94 for R_{cr} and R_{act} , noted respectively r_{tl}^{cr} and r_{tl}^{act} , and seek the objective $r_{tl}^{act} \gg r_{tl}^{cr} \approx 0$ for
95 an activator (Figure 1B). For convenience we assign a species name to an RNA sequence,
96 but one must bear in mind that a given RNA sequence, for instance R_{cr} , may fold in an
97 ensemble of different structures $\{R_{cr}^i\}$, with different translation rates $\{r_{tl}^{cr,i}\}$. Current in
98 silico design methods^{11,36} compute the ensemble of secondary structures $\{R_{cr}^i, R_{ta}^j, R_{act}^k\}$ that
99 minimizes free energy. However, the structure-to-function relationship that associates an
100 RNA conformation with its translation rate is hard to establish. Thus, a set of heuristic
101 rules attributes low values of translation rates $r_{tl}^{cr,j}$ to structures where the RBS or the
102 start codon are buried in a hairpin (Figure 1), and high values of $r_{tl}^{act,k}$, to structures where

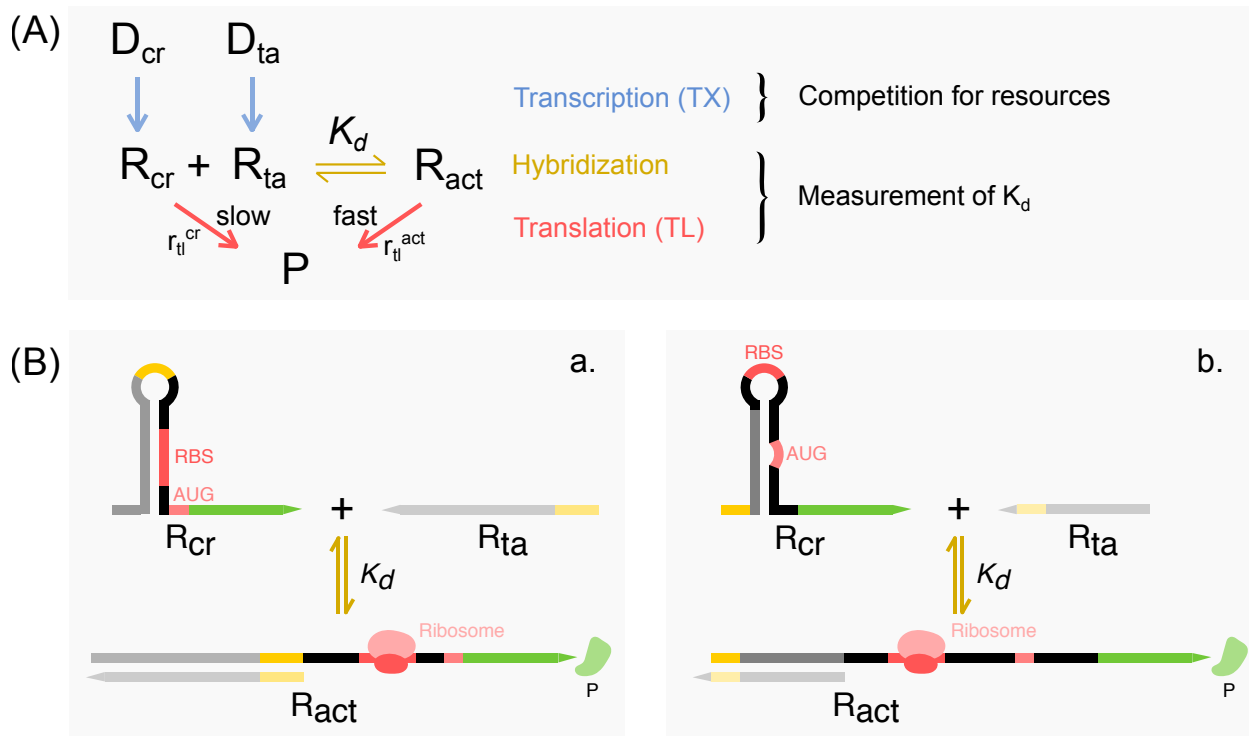


Figure 1: Principle of a translational riboregulator and of its characterization using a cell-free transcription-translation system (TX-TL). (A) Mechanism of transcription, riboregulation through RNA hybridization and translation used in this work. DNA sequences D_{cr} and D_{ta} are transcribed into a cis-repressed, R_{cr} , and a trans-activator, R_{ta} , RNA strands. R_{cr} may be slowly translated into protein P or hybridize with R_{ta} to form R_{act} that is translated more rapidly into P. Measuring the dynamics of fluorescence production by a fluorescent protein P provides information about resource competition when evaluating the system at the DNA level and quantitative values of dissociation constants K_d when RNA concentration is fixed. (B) Sketches of the two operation modes of translational riboregulators functioning as an activator. The 5'-UTR of R_{cr} RNA, forms a hairpin that hides either the ribosome binding site (RBS, a.) or the start codon (AUG, b.) away from the ribosome. R_{ta} hybridizes with R_{cr} , unwinding the hairpin and liberating the RBS and/or the AUG promoting translation.

103 these are accessible. However, these heuristic rules often fail. Moreover, minimizing the free
104 energy of the RNA structures implies that the hypothesis of thermodynamic equilibrium
105 holds, which is far from being true in vivo in the presence of co-transcriptional folding and
106 RNA chaperones.^{37,38} To shed light into this problem we measured translation and expression
107 (transcription and translation) dynamics of recently in silico designed riboregulators^{20,21} in
108 the PURE system.²⁸

109 **The TX-TL system linearly amplifies the concentration of RNA** 110 **and quadratically amplifies the concentration of DNA**

111 We first characterized the translation and expression reactions of the PURE system in the
112 absence of riboregulation. To do so, we prepared by PCR a linear DNA fragment coding
113 for a green fluorescent protein (GFP) with no upstream regulatory region, called cr⁻DNA.
114 It is composed of a T7 RNAP promoter, a ribosome binding site, the GFP-coding sequence,
115 and a T7 terminator. In addition, we prepared by in vitro transcription the corresponding
116 messenger RNA, cr⁻RNA, from cr⁻DNA. We successively used cr⁻RNA and cr⁻DNA as
117 the coding nucleic acid input of the TX-TL system. We varied the concentration of the
118 input and we measured the fluorescence emitted by the GFP produced over time (Figure 2).
119 Starting from cr⁻RNA, the translation module of the TX-TL system actively produced GFP
120 during 2 hours. The translation kinetics displayed three different phases: during about 5 min
121 no signal was discernable from the background level, then followed a phase of quasi-linear
122 increase during 100 min, that slowed down until a plateau was reached (Figure 2A). In the
123 range 0 – 80 nM of cr⁻RNA, both the final intensity and the maximum rate of fluorescence
124 growth, v_{tl}^{max} , increased linearly with the initial quantity of coding RNA (Figure 2B). For
125 higher concentrations there was a saturation: putting more RNA template did not increase
126 significantly the final yield or the maximal production rate. When using cr⁻DNA as the
127 initial input, the dynamics of the fluorescence intensity showed both common and contrasting
128 features with the previous case (Figure 2C). Three phases were still observed: delay, growth

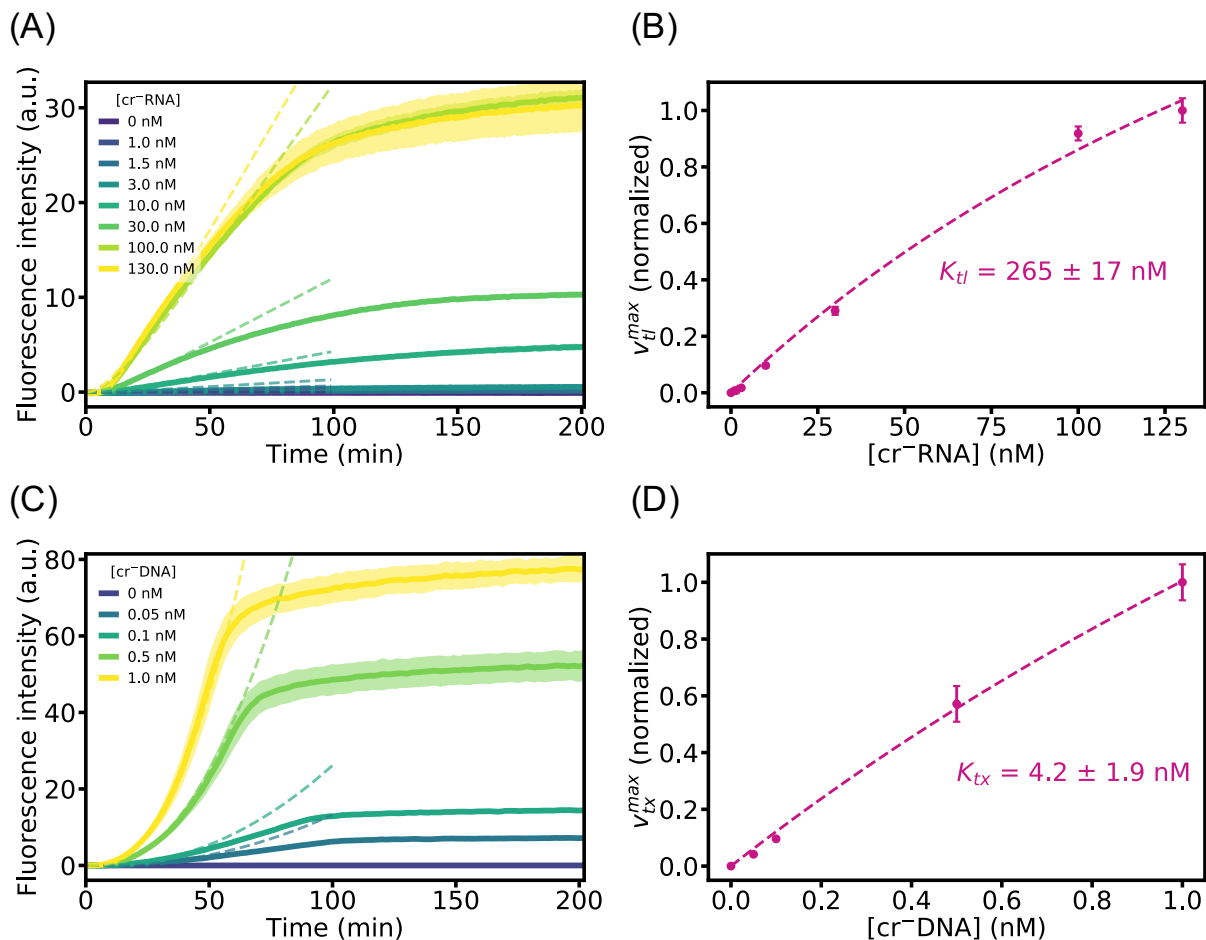


Figure 2: Characterization of the TX-TL system in the absence of riboregulation. Translation dynamics (A) and maximum fluorescence production rate (B) for increasing concentrations of an unregulated mRNA fragment coding for GFP. Expression (transcription and translation) dynamics (C) and maximum fluorescence production rate (D) for increasing concentrations of an unregulated linear DNA fragment coding for GFP. Solid lines (A,C) and disks (B,D) represent data, dotted lines are fits to the model. All experiments were performed in triplicate. Shading around the lines and error bars correspond to one standard deviation.

129 and a plateau. However, the delay observed before an increase of fluorescence was now of 15
 130 min. Finally, the quantity of DNA required to saturate the maximum rate of fluorescence
 131 growth, v_{tx}^{max} , was almost two orders of magnitude lower than the quantity of RNA that
 132 saturated translation (Figure 2D).

133 We propose a simple analytical kinetic model that fits our data. To take into account the
 134 saturation of the production rates we assigned Michaelis-Menten kinetics to the transcription
 135 and the translation reactions. As a plausible source of the initial delay in the translation
 136 reaction, we included a first-order step of maturation of the non-fluorescent GFP protein,
 137 noted P, into the functional fluorescent protein P^* . This is in accordance to published
 138 maturation times.³⁹ We neglected DNA and RNA degradation and we did not take into
 139 consideration the depletion of resources because we analyzed our data between 0 and 50 min.
 140 For these reasons, our model did not reach a plateau in P^* concentration (Figure 2A,C).
 141 These approximations are valid as long as the RNA molecules do not deteriorate and the
 142 enzymatic resources, more specifically the ribosomes, are not depleted. We thus write the
 143 following mechanism



144 where D_{act} and R_{act} are, respectively, cr⁻DNA and cr⁻RNA and r_{tx} , r_{tl} and r_m are, re-
 145 spectively, the transcription, translation and maturation rates. With the aforementioned
 146 hypotheses, this mechanism is associated with the rate equations

$$\frac{dR_{act}}{dt} = r_{tx} = \frac{k_{tx} \cdot D_{act}}{K_{tx} + D_{act}} \quad (4)$$

$$\frac{dP}{dt} = r_{tl} - r_m = \frac{k_{tl} \cdot R_{act}}{K_{tl} + R_{act}} - k_m \cdot P \quad (5)$$

$$\frac{dP^*}{dt} = r_m = k_m \cdot P \quad (6)$$

147 where k_x and K_x are, respectively, the rate and the Michaelis-Menten constants of reaction x
 148 and species concentrations are noted in italics. Equations (4-6) have exact solutions both for
 149 initial conditions corresponding to the translation ($D_{act}(0) = 0$, $R_{act}(0) \neq 0$) and expression
 150 experiments ($D_{act}(0) \neq 0$, $R_{act}(0) = 0$) (SI Section 3). For translation we obtain (SI Section
 151 3.1)

$$P^*(t) = \frac{R_{act}(0)}{K_{tl} + R_{act}(0)} \frac{k_{tl}}{k_m} (e^{-k_m t} + k_m t - 1) \quad (7)$$

152 The term $(e^{-k_m t} - 1)$, due to protein maturation, makes the kink of the curves in Figure 2A
 153 at $t = 10$ min, while the linear term in time dominates for $t = 20 - 50$ min. Note that when
 154 the ribosome is not saturated, $R_{act}(0) \ll K_{tl}$, and for $t \gg k_m^{-1}$ we can write

$$P^*(t) \approx \frac{k_{tl}}{K_{tl}} \cdot R_{act}(0) \quad (8)$$

155 explicitly showing that translation acts as a linear amplifier of the initial concentration of
 156 active RNA.

157 For expression, the exact solution is given in SI Section 3.3. Here we provide an approx-
 158 imated solution when $R_{act}(t) \ll K_{tl}$ (SI Section 3.2),

$$P^*(t) \approx \frac{D_{act}(0)}{K_{tx} + D_{act}(0)} \frac{k_{tx} k_{tl}}{2K_{tl}} \left(t^2 - \frac{2}{k_m} t + \frac{2}{k_m^2} (1 - e^{-k_m t}) \right) \quad (9)$$

159 Again, if $D_{act}(0) \ll K_{tx}$ and $t \gg k_m^{-1}$, expression quadratically amplifies $D_{act}(0)$ into a
 160 fluorescence signal.

161 Considering that the fluorescence intensity is proportional to P^* we fitted (7) and (9)
 162 to the data in Figure 2. We obtained $K_{tx} = 4.2 \pm 1.9$ nM, $K_{tl} = 265 \pm 17$ nM and $k_m =$
 163 0.10 ± 0.01 min⁻¹, in fair agreement with previous measurements reporting $K_{tx} = 4 - 9$ nM
 164 for T7 RNAP,^{33,34,40} $K_{tl} = 66$ nM³³ and $k_m = 0.2$ min⁻¹.^{33,41} Note that although cell-extract
 165 TX-TL uses *E. coli* instead of T7 RNAP, the reported⁴¹ value of K_{tx} is similar, 1-10 nM.

166 In summary, the saturation of transcription by DNA occurs at a concentration two-orders
 167 of magnitude lower than the saturation of translation by RNA. Below saturation, the TX-
 168 TL system acts as a linear amplifier of the concentration of active RNA, R_{act} , and as a
 169 quadratic amplifier of D_{act} with a readout of intensity fluorescence. As a result we can use
 170 GFP fluorescence as a measure of the concentration of R_{act} .

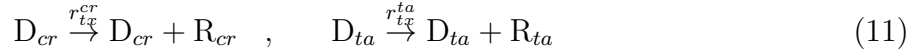
171 Analytical model of translational riboregulation and quantitative 172 definition of the dynamic range of a riboregulator

173 When riboregulators are used in vivo the DNA sequences D_{cr} and D_{ta} , respectively coding
 174 for the cis-repressed and trans-activator RNA R_{cr} and R_{ta} , can either be inserted in the
 175 chromosome, in the same plasmid or in two different plasmids. The performance of a ri-
 176 boregulator in vivo is assayed by fusing D_{cr} with a GFP and measuring the dynamic range,
 177 defined as

$$\rho_{ON/OFF} = \frac{\text{GFP fluorescence in the presence of } D_{ta}}{\text{GFP fluorescence in the absence of } D_{ta}} \quad (10)$$

178 In vivo it is common to use a two-plasmid strategy,^{20,42} trying to improve $\rho_{ON/OFF}$ by
 179 inserting D_{ta} in a high-copy plasmid. The simplicity of in vitro TX-TL allows to provide a
 180 quantitative definition of $\rho_{ON/OFF}$ and to test the effect of D_{ta} concentration on $\rho_{ON/OFF}$. We
 181 start by writing the simplest model of riboregulation dynamics from DNA that is consistent
 182 with the results of the previous section. Within the TX-TL system the two DNA molecules,
 183 D_{cr} and D_{ta} , are transcribed into the corresponding RNA strands, which associate into a

184 coding RNA, R_{act} . The production of P mainly comes from the translation of R_{act} but also
 185 may come from R_{cr} , when cis-repression is not very effective. We thus write the following
 186 mechanism,



187 We model reactions (11-14) with the following set of ODEs, that takes into account the
 188 competition for transcriptional resources,

$$\frac{dR_{cr}}{dt} = r_{tx}^{cr} = \frac{k_{tx}^{cr} \cdot D_{cr}}{K_{tx} + D_{cr} + D_{ta}} \quad (15)$$

$$\frac{dR_{ta}}{dt} = r_{tx}^{ta} = \frac{k_{tx}^{ta} \cdot D_{ta}}{K_{tx} + D_{cr} + D_{ta}} \quad (16)$$

$$R_{act} = \frac{R_{cr}R_{ta}}{K_d} \quad (17)$$

$$\frac{dP}{dt} = r_{tl}^{act} + r_{tl}^{cr} \approx \frac{k_{tl}^{act}}{K_{tl}} R_{act} + \frac{k_{tl}^{cr}}{K_{tl}} R_{cr} \quad (18)$$

$$\frac{dP^*}{dt} = r_m = k_m \cdot P \quad (19)$$

189 where we have assumed that D_{cr} and D_{ta} may have different transcription rate constants k_{tx}^i .
 190 We have also assumed, as previously, that transcription follows Michaelis-Menten dynamics,
 191 that translation can be considered a non-saturated Michaelis-Menten ($R_{cr}, R_{act} \ll k_{tl}^{act}$) and
 192 further that the hybridization reaction (12) is fast compared with the others and thus can
 193 be considered at equilibrium. We have seen in the previous section that maturation reaction
 194 (14) introduces an additional term ($e^{-k_m t} - 1$) that vanishes when $t \gg k_m^{-1} = 10$ min. To

195 facilitate subsequent calculations we will suppose $t \gg k_m^{-1}$ and thus $P = P^*$.

196 We define $\alpha = \frac{D_{ta}}{D_{cr}}$ and integrate (15-18) to obtain (SI Section 4)

$$P^*(\alpha) = \frac{k_{tl}^{act} \alpha}{3K_d k_{tl}^{act}} \frac{k_{tx}^{ta} k_{tx}^{cr}}{\left(1 + \alpha + \frac{K_{tx}}{D_{cr}}\right)^2} t^3 + \frac{k_{tl}^{cr}}{2k_{tl}^{act}} \frac{k_{tx}^{cr}}{1 + \alpha + \frac{K_{tx}}{D_{cr}}} t^2 \quad (20)$$

197 where we have indicated explicitly that P^* is a function of α . We can naturally define the
198 dynamic range of the riboregulator as

$$\rho_{ON/OFF}^{th} = \frac{P^*(\alpha)}{P^*(\alpha=0)} = \frac{1 + \frac{K_{tx}}{D_{cr}}}{\left(1 + \alpha + \frac{K_{tx}}{D_{cr}}\right)} \left[1 + \frac{2}{3} \frac{k_{tx}^{ta} k_{tl}^{act}}{K_d k_{tl}^{cr}} \frac{\alpha}{\left(1 + \alpha + \frac{K_{tx}}{D_{cr}}\right)} t \right] \quad (21)$$

199 where the superscript *th* indicates that this is an theoretical quantity defined in the frame-
200 work of model (15-18). This equation reveals three important points. Firstly, $\rho_{ON/OFF}^{th}$
201 depends linearly on time and thus it is difficult to compare $\rho_{ON/OFF}$ between two experi-
202 ments, in vivo or in vitro, if they have not been calculated at the same time. This linear
203 dependence comes from the fact that protein production from the riboregulator is cubic in
204 time, while the leak production from D_{cr} alone is quadratic (20). In vivo one may expect
205 that $\rho_{ON/OFF}^{th}$ reaches a plateau due to degradation. However a linear increase of $\rho_{ON/OFF}$
206 was observed over 4 h for riboregulator 7 in reference 20. Secondly, $\rho_{ON/OFF}^{th}$ is proportional
207 to the aggregate factor $\beta = \frac{k_{tx}^{ta} k_{tl}^{act}}{K_d k_{tl}^{cr}}$. Thus, $\rho_{ON/OFF}^{th}$ is proportional to the transcription rate
208 constant k_{tx}^{ta} , and thus will differ between different RNAPs and promoters, it is also propor-
209 tional to the ratio between the translation rate constant of the active and the inactive state,
210 which is intuitive, and it is inversely proportional to the equilibrium constant of dissociation
211 between R_{cr} and R_{ta} . Finally, $\rho_{ON/OFF}^{th}$ is strongly and non-trivially dependent on the con-
212 centration of D_{cr} and D_{ta} (through $\alpha = D_{ta}/D_{cr}$). This is, to the best of our knowledge, the
213 first rigorous definition of the dynamic range of a translational riboregulator. The maximum
214 of $\rho_{ON/OFF}^{th}$ is obtained for (SI Section 4.2)

$$D_{ta} = D_{cr} + K_{tx} \quad (22)$$

215 **The dynamic range of a riboregulator strongly depends on the con-**
 216 **centration of D_{ta}**

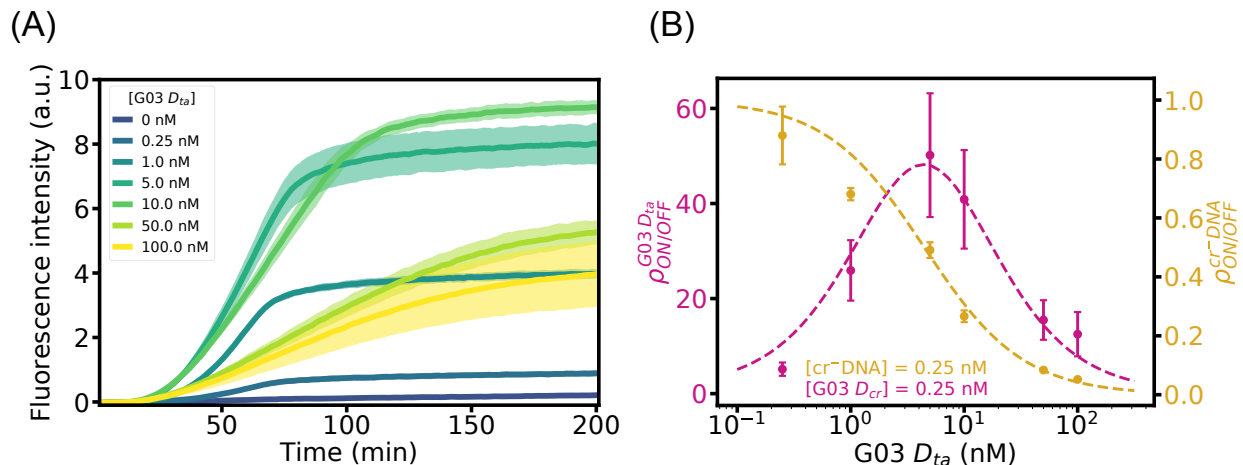


Figure 3: The dynamic range of a riboregulator strongly depends on the concentration of D_{ta} . (A) Fluorescence intensity vs. time for the in vitro expression of 0.25 nM of D_{cr} DNA, coding for GFP, with increasing concentrations of D_{ta} DNA, for riboregulator G03. (B) Dynamic range $\rho_{ON/OFF}$ at time 75 min for a D_{cr} with, G03 (pink disks, left axis), or without, cr^- (yellow disks, right axis), cis regulatory region as a function of the concentration of D_{ta} from riboregulator G03. The dashed lines correspond to a fit to equation (21) with a single free parameter $\beta = (k_{tx}^{ta} k_{tl}^{act}) / (K_d k_{tl}^{cr})$ (pink) and to SI equation (30) without fit (yellow), both using $K_{tx} = 4.2$ nM as measured in Figure 2D. All experiments were performed in triplicate. Shading around the lines and error bars correspond to one standard deviation.

217 To test the model's prediction that $\rho_{ON/OFF}$ strongly depends on α and thus on D_{ta} ,
 218 we titrated riboregulator G03 (Table S1) by keeping $D_{cr} = 0.25$ nM constant, varying D_{ta}
 219 in the range 0 – 100 nM and recording GFP fluorescence over time (Figure 3). Increasing
 220 D_{ta} in the range 0 – 5 nM resulted in an increased fluorescence signal. However, for $D_{ta} >$
 221 5 nM the fluorescence signal dramatically decreased until reaching 10% of the maximum
 222 production rate at $D_{ta} = 100$ nM. Equation (21) fits the data with a single free parameter
 223 $\beta = 286$ (Figure 3B, pink line), indicating that the bell-like shape of $\rho_{ON/OFF}$ arises from the
 224 competition of D_{ta} and D_{cr} for transcriptional resources. To further test this interpretation
 225 we titrated cr^- DNA, which lacks the cis-regulatory region, with the D_{ta} of riboregulator
 226 G03. The data were quantitatively predicted by equation (21) taking the limit $K_d \rightarrow \infty$
 227 (SI equation (30)) without fitting parameters. Importantly, the addition of non-transcribing

228 DNA had little effect on the expression dynamics (SI Figure S3).

229 The observation that an increase in non-coding DNA concentration reduces protein ex-
230 pression in TX-TL systems has already been reported^{25,30,43,44} and it has been modeled⁴⁵
231 in the absence of riboregulation, although a quantitative comparison between the model
232 and the data has not been reported. Our model (15-18) explicitly takes into account the
233 competition between the two DNA substrates D_{ta} and D_{cr} and the predicted dependence
234 of $\rho_{ON/OFF}^{th}$ on D_{ta} is in agreement with the data. Hu et al. recently proposed a kinetic
235 model for transcriptional riboregulators³¹ and compared their model with in vitro TX-TL
236 experiments. Our models are of the same type in the sense that they describe the kinetics
237 with a set of ODEs at the level of concentrations. In contrast, the model of Hu et al has
238 significantly more parameters than ours, 13 instead of 5, and takes into account the degra-
239 dation of both RNA and protein. As a result, the authors cannot provide analytical results
240 that clearly pinpoint the important parameters to design functional riboregulators, such as
241 equation (21).

242 Saturation of transcriptional resources is particularly important in the context of riboreg-
243 ulators, where the non-coding DNA produces a regulatory RNA that has an important effect
244 in the GRN. To the best of our knowledge, the bell-like curve in Figure 3B has not been
245 reported before. The similar value of K_{tx} for T7 and *E. coli* RNAP, together with previous
246 observations of transcriptional saturation in *E. coli*-based TX-TL systems, suggests that
247 this behavior is not due to a particular property of the T7 RNAP. Our model and in vitro
248 results thus predict that inserting D_{ta} in a high-copy plasmid will decrease the performance
249 of the riboregulator activator and suggests a trade-off between resource competition and the
250 over-expression of antisense RNAs. This prediction shall be tested in a future work.

251 **Comparison of the dynamic range of riboregulators in vivo and in**
 252 **vitro**

253 To evaluate how dynamic ranges determined in vitro compared with in vivo measurements,
 254 we investigated five riboregulators of two different types, two loop-mediated²¹ and three
 255 toehold-mediated²⁰ (Table S1). In the former, the RBS is buried inside the hairpin and the
 256 R_{ta} binds first to the loop on the hairpin. In the later, the start codon is protected by the
 257 hairpin and the R_{ta} binds to a toehold sequence on the 5' side of the hairpin (Figure 1B).
 258 We performed in vitro GFP expression experiments at 1 nM D_{cr} in the presence and in
 259 the absence of 5 nM of the corresponding D_{ta} . We chose D_{cr} and D_{ta} that verified (22)
 260 to determine the maximum dynamic range. In vivo, the dynamic range is generally defined
 261 without subtracting the autofluorescence of the cells.^{20,21} In the previous section, to compare
 262 with $\rho_{ON/OFF}^{th}$, we computed the experimental $\rho_{ON/OFF}$ by dividing fluorescence signals that
 263 had been subtracted from the autofluorescence of the PURE system. In this section, to
 264 compare with in vivo measurements, we computed $\rho'_{ON/OFF}$, where the prime indicates that
 265 autofluorescence was not subtracted.

Table 1: Comparison of the performance of five riboregulators in vivo and in vitro. Dynamic range calculated without subtracting the autofluorescence, $\rho'_{ON/OFF}$, in vivo and in TX-TL, ON and OFF raw fluorescence signals, I_{ON} and I_{OFF} , in TX-TL, and ratio of $\rho'_{ON/OFF}$ in vivo relative to in vitro. In vivo data were extracted from ref. 20 for GXX and from ref. 21 for RAJXX. TX-TL data were measured at $D_{cr} = 1$ and $D_{ta} = 5$ nM at $t = 75$ min. The typical value of autofluorescence was 0.07 ± 0.01 a.u.. Error bars correspond to one standard deviation of a triplicate experiment.

| Device | in vivo $\rho'_{ON/OFF}$ | TX-TL $\rho'_{ON/OFF}$ | TX-TL I_{ON} (a.u.) | TX-TL I_{OFF} (a.u.) | ratio $\rho'_{ON/OFF}$ in vivo/ TX-TL |
|--------|-----------------------------|---------------------------|--------------------------|---------------------------|--|
| G01 | 290 ± 20 | 37 ± 10 | 116 ± 25 | 3.1 ± 0.5 | 8 ± 2 |
| G03 | 260 ± 30 | 26 ± 6 | 81 ± 10 | 3.1 ± 0.6 | 10 ± 3 |
| G80L18 | 500 ± 150 | 23 ± 3 | 93 ± 8 | 4.1 ± 0.4 | 22 ± 8 |
| RAJ11 | 11 ± 2 | 1.9 ± 0.3 | 2.1 ± 0.3 | 1.1 ± 0.1 | 6 ± 1 |
| RAJ12 | 8 ± 1 | 1.2 ± 0.1 | 1.2 ± 0.2 | 1.1 ± 0.1 | 7 ± 1 |

266 The agreement between the values obtained in vivo and in vitro is remarkable. Of course,
 267 the absolute values of $\rho'_{ON/OFF}$ in vivo and in vitro are different, which is expected because

268 $\rho_{ON/OFF}^{th}$ is proportional to time and in vivo and in vitro data were obtained at different
 269 times (Table 1). In contrast, the relative order of $\rho'_{ON/OFF}$ is similar in vivo and in vitro.
 270 Moreover the ratio between the two is constant for all riboregulators except for G80L18
 271 that is twice more active in vivo, indicating that TX-TL experiments predict well $\rho'_{ON/OFF}$
 272 in vivo. The measured value of $\rho'_{ON/OFF}$ for RAJ12 is close to unity. However, increasing
 273 the DNA concentrations to $D_{cr} = D_{ta} = 50$ nM, which increases protein production (20)
 274 while respecting (22), demonstrated that RAJ12 was indeed functional and we obtained
 275 $\rho'_{ON/OFF} = 7$ at these concentrations. Comparing the values of I_{OFF} shows that RAJXX
 276 leaked significantly less than GXX while G80L18 leaked slightly more than G01 and G03.
 277 Finally, our experiments show that the remarkable $\rho'_{ON/OFF}$ values of GXX devices come
 278 from their high I_{ON} , and thus a very active ON state. We thus conclude that in vitro TX-
 279 TL provides values of $\rho'_{ON/OFF}$ that correlate well with in vivo measurements, in agreement
 280 with previous reports comparing protein expression in vivo and in vitro.^{30,45,46}

281 **Translation from RNA characterizes the reaction between the cis-** 282 **repressed and the trans-activator RNA**

283 The regulatory step of translational riboregulators takes place when the two RNA fragments,
 284 R_{cr} and R_{ta} , hybridize and thereby change the accessibility of the ribosome to a site needed
 285 for initiating translation (RBS or AUG). The core of the riboregulation process can thus
 286 be described with reactions (12) and (13), where the first one involves the hybridization
 287 of R_{cr} with R_{ta} to form an active RNA complex, R_{act} , that can be translated, and the
 288 second being the translation of R_{act} into protein P. We have seen that the thermodynamics
 289 of the first reaction play an important role in $\rho_{ON/OFF}^{th}$ through K_d (21). However K_d is
 290 not straightforward to determine. One possibility is to use an electrophoretic mobility shift
 291 assay in a polyacrylamide gel. Another way uses the property of a reverse transcriptase to
 292 terminate on stable RNA duplexes.¹³ In both cases these assays characterize the species R_{act}
 293 for being a duplex RNA but they are not sensitive to its translational activity. Here, instead,

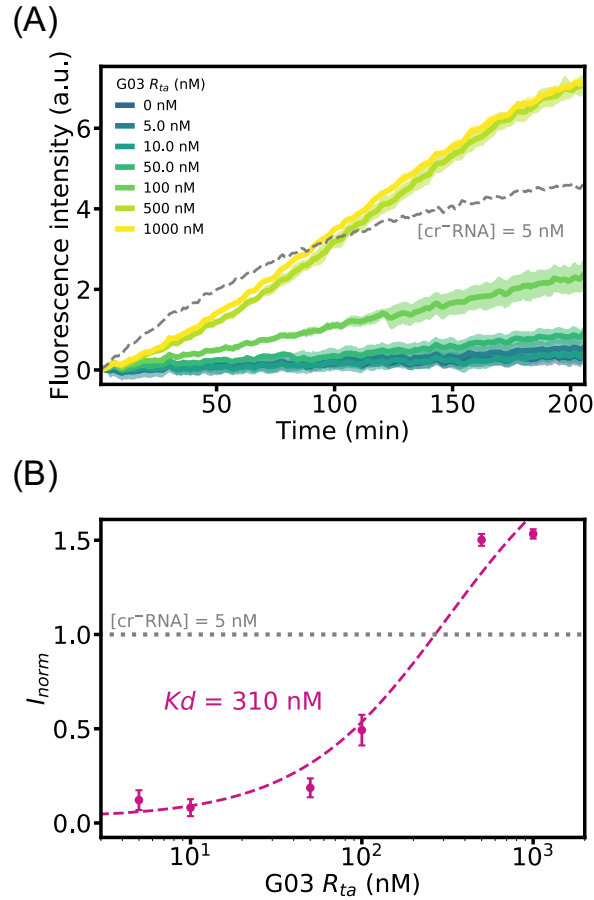


Figure 4: Titration of a riboregulator at the RNA level measures the dissociation constant of the riboregulator complex. GFP fluorescence produced over time (A) and normalized maximum fluorescence (B) for different trans-activator concentrations, R_{ta} for riboregulator G03. As a control, panel A shows the fluorescence intensity produced by the translation of 5 nM of an unregulated cr⁻RNA (grey dashes). In (B) disks correspond to experimental data and the dashed line is a fit of (23) to the data. All experiments were performed in triplicate. Shading around the lines and error bars correspond to one standard deviation.

294 we probed the equilibrium concentration of R_{act} that is active for translation. Our method
 295 is thus more meaningful to evaluate the design performances of a riboregulator.

296 To characterize reaction (12) we in vitro transcribed the five riboregulators described
 297 previously (Figure S1). We studied their translation dynamics by titrating 5 nM R_{cr} with
 298 increasing concentrations of its corresponding R_{ta} in the range 0 – 1000 nM (Figure 4 and
 299 SI Figure S4). Because translation linearly amplifies R_{act} (Figure 2B and (8)), measuring
 300 the GFP intensity at a given time is directly proportional to the concentration of R_{act} that
 301 is translationally active. We thus plotted the normalized GFP fluorescence at 200 min as
 302 a function of the log of R_{ta} concentration. For a bimolecular equilibrium such as (12) one
 303 expects these plots to be described by

$$I_{norm} \sim \bar{R}_{act} = \frac{1}{2} R_{cr}^0 \left(\frac{K_d + R_{cr}^0 + R_{ta}^0}{R_{cr}^0} - \sqrt{\left(\frac{K_d + R_{cr}^0 + R_{ta}^0}{R_{cr}^0} \right)^2 - 4 \frac{R_{ta}^0}{R_{cr}^0}} \right) \quad (23)$$

304 where \bar{R}_{act} is the equilibrium concentration of R_{act} and superscript 0 indicates initial con-
 305 centrations (SI Section 6). Our experimental data followed this trend (Figures 4 and S4).
 306 We thus fitted (23) to the data and found dissociation equilibrium constants in the range
 307 10 – 2000 nM (Table 2), in agreement with K_d values of the order of 100 nM that have
 308 already been reported for loop-mediated activators.¹³ Values of K_d obtained from different
 309 batches of PURE were within 50 % (Figure S5).

310 In the case of G01, however, after a normal sigmoidal increase of I_{norm} vs. R_{ta} , I_{norm}
 311 decreased for $R_{ta} > 200$ nM (Figure S4). To evaluate why in this particular case high R_{ta}
 312 inhibited translation, we performed a control experiment where a well-behaved regulator,
 313 G80L18, activated with 50 nM of its corresponding R_{ta} , was titrated with increasing concen-
 314 trations of R_{ta} -G01 (Figure 5). We observed again that very high concentrations of R_{ta} -G01
 315 significantly reduced the final GFP concentration. In contrast, similarly high concentrations
 316 of R_{ta} -RAJ11 did not have a significant effect in translation. We thus concluded that R_{ta} -G01
 317 poisoned the translation machinery, which could occur by nonspecific binding to other RNA

318 components, including tRNAs, ribosomes or mRNA, with about 1 μ M affinity. However, a
 319 sequence alignment between riboregulator’s sequence and tRNA and rRNA from *E. coli* did
 320 not show significant differences among riboregulators (Figure S6).

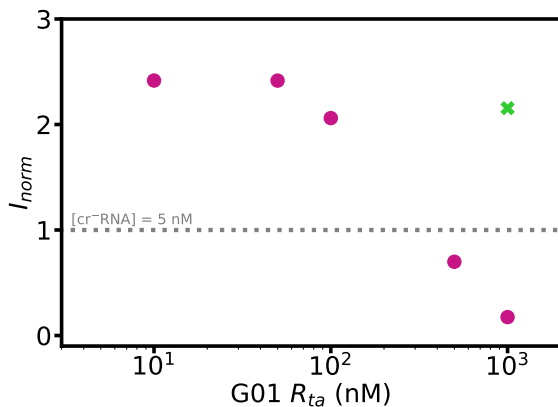


Figure 5: Titration of activated riboregulator G80L18 ($R_{cr} = 5$ nM $R_{ta} = 50$ nM) with increasing concentrations of R_{ta} from riboregulators G01 (disks) and RAJ11 (green cross).

321 To assess the performance of our method for measuring K_d , we independently measured
 322 it with a standard mobility-shift assay performed with capillary gel electrophoresis. We used
 323 the same purified R_{cr} and R_{ta} that we mixed together at 37°C in a buffer with identical salt
 324 composition than the TX-TL system during 10 min before performing the electrophoresis
 325 assay. R_{cr} concentration was 8.3 nM and the R_{ta} concentration was ranging from 0 to 200
 326 nM. Figures 6 and S7 show the electropherograms for riboregulator G03, where a peak in
 327 intensity at a given time point corresponds to an RNA structure. We detected three main
 328 peaks corresponding to R_{ta} at 28 s (Figure S7) and R_{cr} and R_{act} complex between 37 and
 329 40 s (Figure 6A). Interestingly, species R_{cr} and R_{act} yielded well-resolved peaks for toehold-
 330 mediated but not for loop-mediated riboregulators (Figure S8), which suggests a structural
 331 difference between the two. As a result this method only provided K_d for some but not all
 332 of the tested riboregulators, in contrast with the TX-TL method. The values obtained were
 333 of the same order of magnitude of those obtained by TX-TL. However, mobility-shift assay
 334 yielded K_d in a narrower range of 100 – 250 nM, while TX-TL was able to better discriminate
 335 K_d for the same species and provided values in the range 15 – 2200 nM (Table 2).

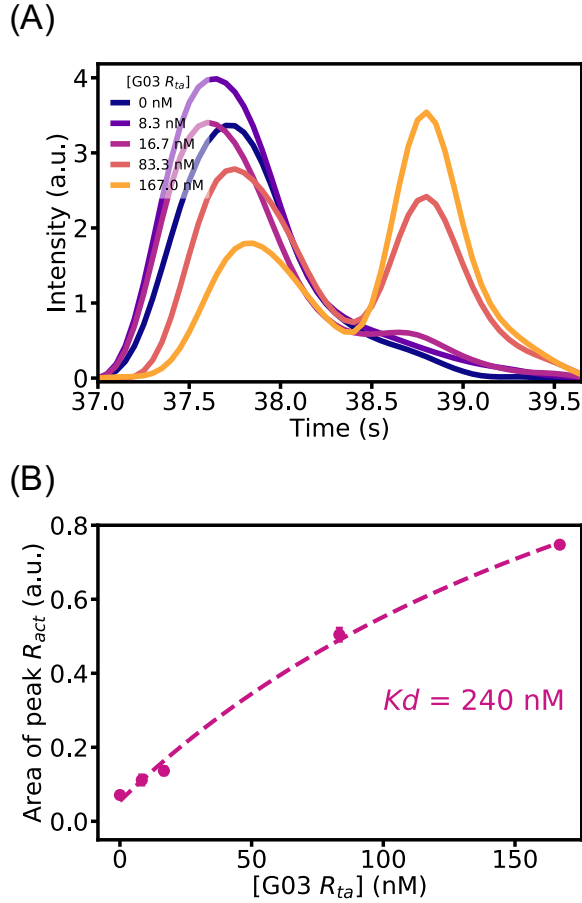


Figure 6: Titration of translational riboregulator G03 by mobility-shift capillary electrophoresis. (A) Corrected electropherograms vs. elution time and (B) peak area for different concentrations of R_{ta}. Experiments were performed in triplicate. Error bars correspond to one standard deviation. Dashed line is a fit of (23) to the data.

Table 2: Dissociation constants K_d at 37°C for the studied riboregulator devices. K_d was measured using the cell-free translation method (txtl) and the mobility-shift method (ms). N.M. indicates that the electropherogram showed ill-defined peaks from which K_d could not be extracted. Error bars correspond to one standard deviation of the fit. Values for G03 and RAJ11 were fitted to data in triplicate.

| Device | K_d^{txtl} (nM) | K_d^{ms} (nM) |
|--------|-------------------|-----------------|
| G01 | 46 ± 56 | 180 ± 20 |
| G03 | 310 ± 154 | 240 ± 110 |
| G80L18 | 31 ± 19 | 110 ± 90 |
| RAJ11 | 15 ± 14 | N.M. |
| RAJ12 | 2220 ± 950 | N.M. |

336 Conclusion

337 We have demonstrated that in vitro transcription-translation (TX-TL) systems are an at-
338 tractive platform to quantitatively characterize translational riboregulators. To do so we
339 have taken advantage of the ribosome as a molecular machine that not only recognizes RNA
340 complexes that are translationally active but also measures their concentration. The sim-
341 plicity of the TX-TL system allowed us to propose an analytical expression for the dynamic
342 range of a riboregulator. In quantitative agreement with this model we have shown that
343 increasing the DNA concentration of the trans-activating species first promotes and later in-
344 hibits expression. This result suggests that inserting trans-activating elements in high-copy
345 plasmids in vivo could limit the efficiency of translational activators, a prediction that shall
346 be tested in future work. Furthermore, relative dynamic ranges measured in vitro were in
347 agreement with those reported in vivo for four out of five measured riboregulators. Finally,
348 by titrating the cis-repressed gene with the trans-activating species at the RNA level we
349 could determine dissociation constants, K_d , for the RNA hybridization reaction in a very
350 simple manner. In particular, we could obtain K_d 's for riboregulators that could not be
351 resolved by mobility-shift assays. Our method thus provides a simple and rapid way for the
352 quantitative characterization of riboregulators.

353 Combined with other biomolecular techniques such as molecular beacons³⁴ and automated-
354 based designs,⁴⁷ cell-free transcription-translation systems are becoming essential for a wide
355 brand of applications. They allow to verify theoretical predictions on both RNA structures
356 and behaviour of large scale regulatory networks. Their versatility is a real asset for conceiving
357 new synthetic biological features⁴⁸ and creating innovative biomolecular tools.⁴⁹ The use of
358 an in vitro step in the design and elaboration of complex synthetic regulatory networks will
359 maximise the chance of expected in-vivo performances.

360 **Methods**

361 **DNA and RNA preparations**

362 DNA templates were prepared by PCR amplification of plasmids encoding for the RNA
363 translational regulators, followed by affinity column purification using Monarch PCR Pu-
364 rification Kit (New England BioLabs) or PureLink PCR Purification Kit (Thermo Fisher
365 Scientific). Primers used for PCR amplification contained a T7 promoter or a T7 terminator
366 (Biomers). RNA templates were prepared by in vitro transcription followed by purification
367 using MEGAclear Transcription Clean-Up Kit (Ambion). The DNA and RNA integrity was
368 determined by a 1.5% agarose gel (Figure S1) and the concentrations were determined by
369 absorbance at 260 nm using a NanoDrop 2000 UV-Vis spectrophotometer. The sequences
370 of the riboregulator domains (Table S1), of the PCR primers (Table S2) and of the plasmids
371 are compiled in the SI.

372 **Preparation of the PURE TX-TL system**

373 The PURE TX-TL system was prepared according to reference 50 to reach the following
374 composition: 1 units/ μ L of RNase inhibitor Murine (New England Biolabs), 50 mM HEPES-
375 KOH pH 7.6, 13 mM magnesium acetate, 100 mM potassium glutamate, 2 mM spermidine,
376 1 mM dithiothreitol (DTT), 2 mM of each ATP and GTP, 1 mM of each CTP and UTP,
377 20 mM creatine phosphate, 0.3 mM 20 amino acids, 56 A260/ml tRNA mix (Roche), 10
378 μ g/mL 10-formyl-5, 6, 7, 8-tetrahydrofolic acid, 0.1 mM each of amino acids, and factor
379 mix. The factor mix contained 1.2 μ M ribosome, 10 μ g/ml IF1, 40 μ g/ml IF2, 10 μ g/ml
380 IF3, 50 μ g/ml EF-G, 100 μ g/ml EF-Tu, 50 μ g/ml EF-Ts, 10 μ g/ml RF1, 10 μ g/ml RF2,
381 10 μ g/ml RF3, 10 μ g/ml RRF, 600-6000 U/ml of each ARS and MTF 4.0 μ g/ml creatine
382 kinase (Roche), 3.0 μ g/ml myokinase (Sigma), 1.1 μ g/ml nucleoside-diphosphate kinase, 1.0
383 U/ml pyrophosphatase (Sigma), and 10 μ g/ml of T7 RNAP.

384 **Fluorescence measurements in real-time PCR machine**

385 Rotor-GeneQ real-time PCR (Qiagen) was used to record fluorescence from GFP expression
386 (excitation 470 ± 10 nm, emission 510 ± 5 nm) in an 8 or 15 μL volume. The temperature
387 was set to 37°C and fluorescence recorded every minute for at least 3 h. In some experiments
388 (Figure 5) we used PURExpress in vitro protein synthesis kit (NEB).

389 **Data processing**

390 Data were processed using in-house Python routines. For each condition of template—DNA
391 or RNA— concentration, fluorescence intensity plots were shifted to the origin by removing
392 the mean value of the three first minutes and by subtracting the fluorescence due to the
393 PURE TX-TL system without any template. I_{norm} was computed by dividing this corrected
394 fluorescence by the final intensity of the cr⁻RNA control. Corrected data were filtered using
395 a Savitzky–Golay filter (window length: 21, polynomial order: 3) to remove residual noise
396 before being derived to compute v^{max} .

397 **Electrophoretic mobility shift assays**

398 Electrophoretic mobility shift assays were performed with a 2100 Bioanalyzer System (Ag-
399 ilent Technologies) and an RNA Nano chip Kit. Samples were prepared by mixing RNA
400 strands in 50 mM Hepes-KOH pH 7.6, 13 mM magnesium acetate, 100 mM potassium glu-
401 tamate, 2 mM spermidine, 1 mM DTT and nuclease free water. They were incubated at 37°C
402 for 10 min before being loaded into the electrophoresis chip. Electropherograms were manu-
403 ally aligned along the time axis. Affine curves corresponding to the backgrounds of zones of
404 interest were subtracted. Areas under peaks were determined by numerical integration and
405 were normalized using an RNA marker provided in Agilent’s kit.

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411 **Supporting Information Available**

412 Sequences for the RNA regulators, the PCR primers and the plasmids used, additional
413 figures S1–S8, tables S1–S2, the detailed solution of translation and expression kinetics
414 in the absence and in the presence of regulation, derivation of the equation used for Kd
415 determination, supplementary data on RNA titration and details on the sequence alignment.

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1 Graphical TOC entry

