

## Supplementary Material for

### Characterizing and Prototyping Genetic Networks with Cell-Free Transcription-Translation Reactions

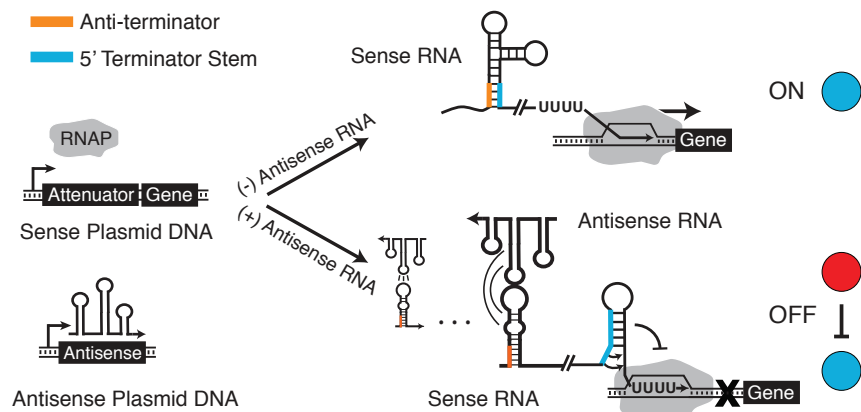
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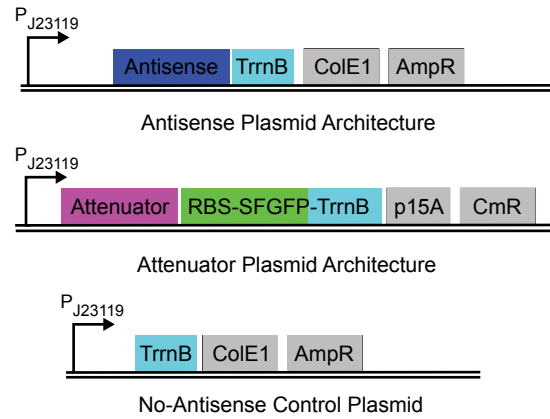
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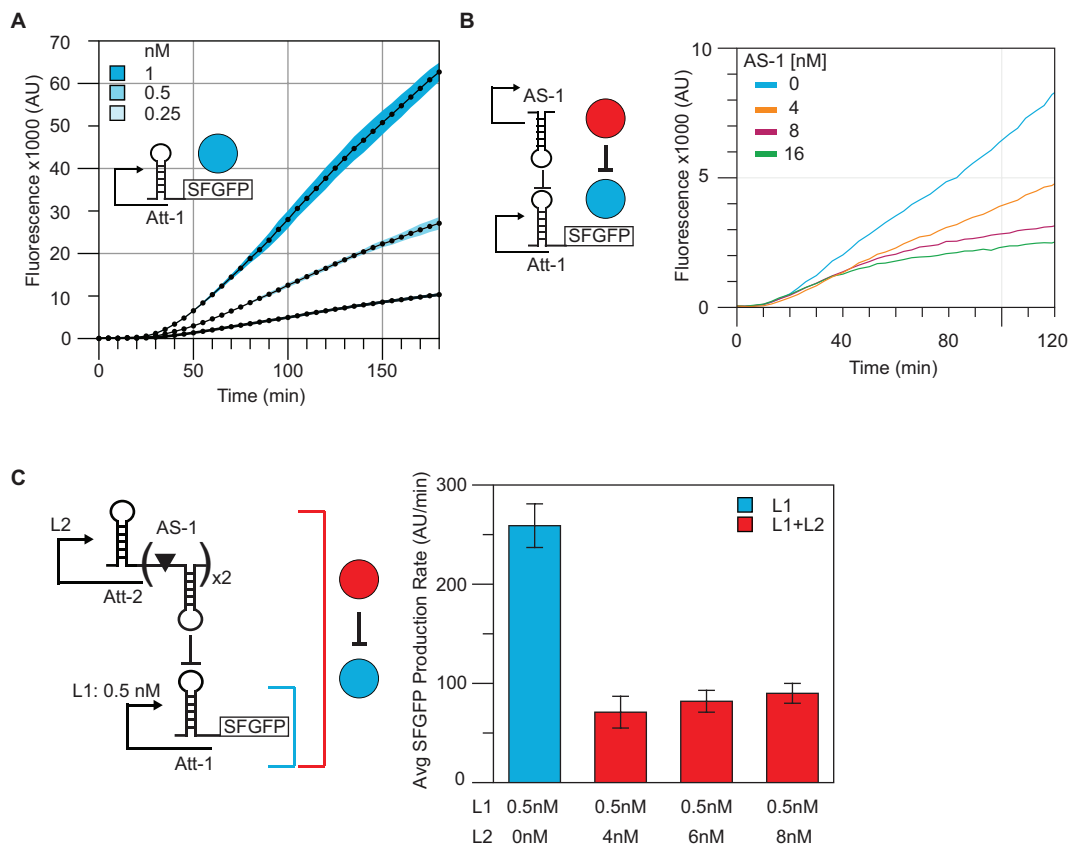
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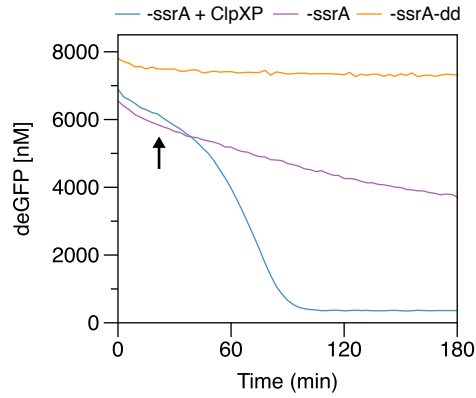
**Figure S1.** The transcriptional attenuation mechanism from the *Staphylococcus aureus* plasmid pT181 [1,2]. The attenuator lies in the 5' untranslated region of the transcript and can fold into a structure that will allow transcription to continue if antisense RNA is not present (ON). Antisense RNA binding to the attenuator causes the formation of a transcription terminator hairpin, stopping transcription before the gene of interest (OFF, indicated by x symbol). Colored circles represent an abstract view of the switch. The antisense RNA (red circle) represses (blunt end line) the attenuator-reporter gene target (blue circle).



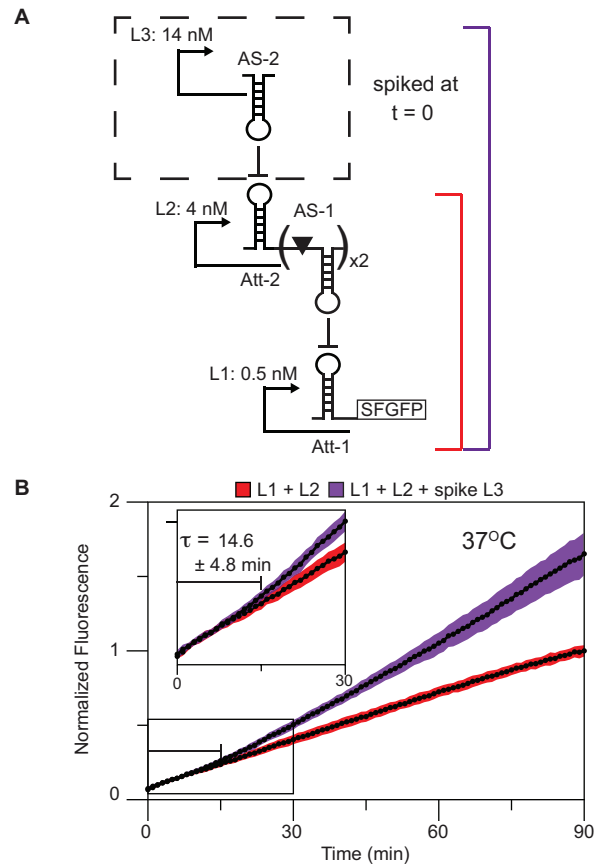
**Figure S2.** Plasmid architecture for attenuator and antisense plasmids. Antisense plasmids have the ColE1 origin and ampicillin resistance (AmpR). Attenuator plasmids have the p15A origin and chloramphenicol resistance (CmR). The J23119 *E. coli* consensus promoter ([http://partsregistry.org/Part:BBa\\_J23119](http://partsregistry.org/Part:BBa_J23119)), modified to include a Spel site right before the start of transcription, was used for all plasmids. TrnB is a transcriptional terminator. RBS = ribosome binding site; SFGFP = super folder green fluorescent protein coding sequence.



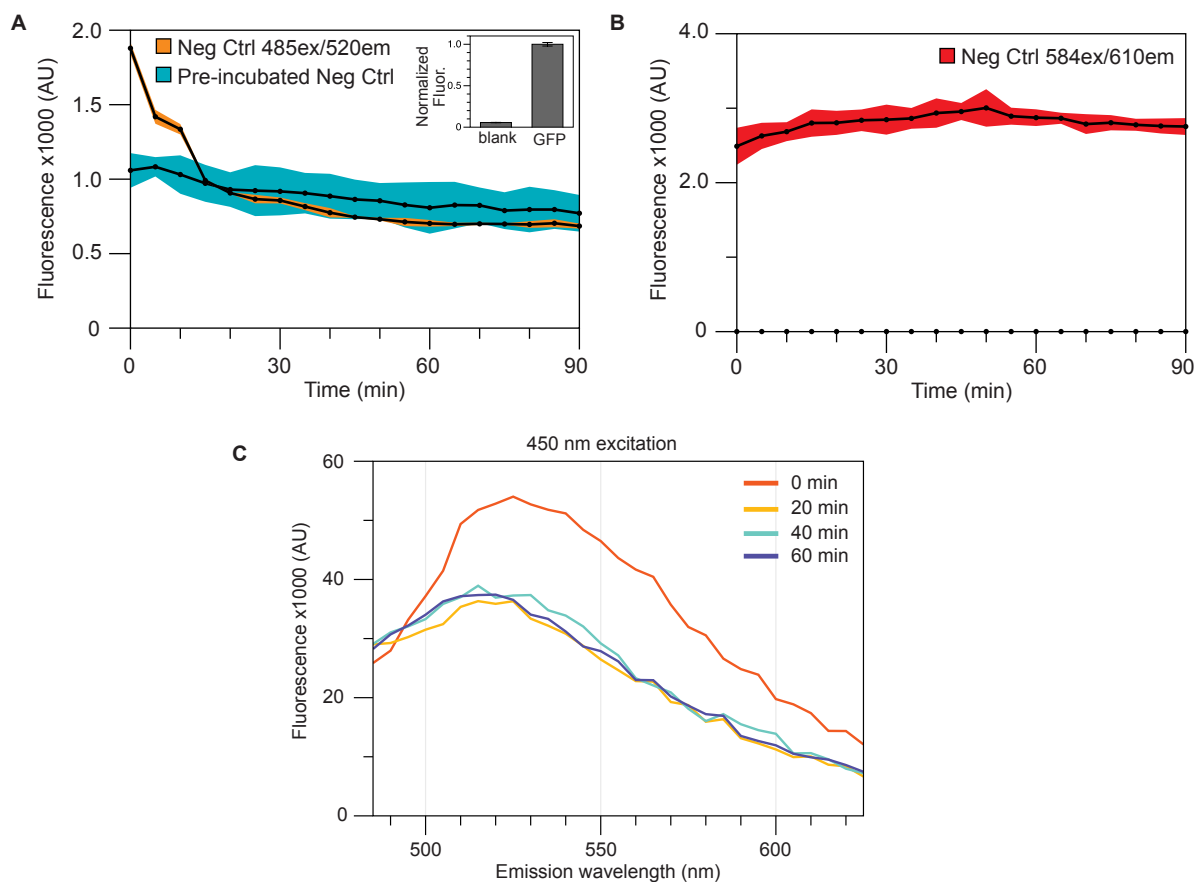
**Figure S3.** Titration data for the RNA network example in Figure 2. (A) Average fluorescence time courses of TX-TL reactions containing the pT181 attenuator plasmid with varying (0.25 – 1nM) concentrations. Shaded regions represent standard deviations of four independent reactions calculated at each time point. (B) Schematic of repression of the pT181 attenuator (Att-1) by its antisense RNA (AS-1). Fluorescence time courses for TX-TL reactions with 0.5 nM attenuator plasmid and varying antisense RNA concentrations (0 – 16nM). Traces represent a single TX-TL reaction collected on a different plate reader, thus a different fluorescence output than A, C, and Figure 2. (C) Schematic of the first two levels of the cascade in Figure 2. L1 is the same pT181 attenuator (Att-1) reporter plasmid used in Figure 2 (A) – (D). In the plasmid for L2, the pT181-mut attenuator (Att-2) controls transcription of two copies of the pT181 antisense (AS-1), each separated by a ribozyme (triangle). Average SFGFP production rates for TX-TL reactions with 0.5 nM L1 and varying concentrations of L2 (4 – 8nM). Error bars represent standard deviations from four independent reactions. Figure adapted from Takahashi et al., ACS Synth. Biol., 4 (2015) 503-515 [3].



**Figure S4.** Protein degradation by ClpXP. ClpXP selectively degrades –ssrA tagged proteins in TX-TL reactions. 4.5 $\mu$ M of either –ssrA or defectively –ssrA (-ssrA-dd) tagged, purified deGFP is added to a TX-TL reaction either supplemented with nothing or with 1nM of a strong-expressing plasmid expressing ClpX and ClpP. The black arrow indicates the point at which expressed ClpX and ClpP start to become active.



**Figure S5.** Determining cascade response time. (A) Schematic of a spike experiment. L3 (or the no-antisense control plasmid) was spiked into an ongoing L1+L2 TX-TL reaction at time,  $t = 0$  (represented by dashed box). Concentrations of DNA used are indicated beside the levels. (B) Normalized fluorescence curves combining three separate experiments performed at 37 °C with a total of 11 replicates over multiple days. An L1 (0.5 nM) + L2 (4 nM) reaction was setup for 20 min at which point L3 (14 nM, purple curve) or no-antisense control DNA (14 nM, red curve) was spiked into the reaction and time reset to 0. Inset shows the response time of the circuit to the addition of L3; defined as the time at which the L3 spike curve is statistically different from the L1+L2 curve ( $\tau = 14.6 \pm 4.8$  min). Figure adapted from Takahashi et al., ACS Synth. Biol., 4 (2015) 503-515 [3].



**Figure S6.** Fluorescence of TX-TL reaction negative control. (A) Fluorescence trajectories of the negative control with (blue) and without (orange) pre-incubation measured at 485 nm excitation and 520 nm emission. A pre-incubation of the buffer and extract for 20 min at 37°C eliminates the decrease in fluorescence seen over the first 20–40 min of the reaction. Inset shows the fluorescence of the negative control relative to SFGFP fluorescence from the Att-1 construct in Figure 2A after a 2 hour reaction. Error bars represent standard deviations from three independent reactions. (B) Fluorescence trajectory of the negative control without pre-incubation measured at 584 nm excitation and 610 nm emission. Shaded regions represent standard deviations from three independent reactions. (C) TX-TL buffer and extract were mixed, added to a 384-well plate, and incubated at 37°C. Emission spectra were collected (450 nm excitation) at 0, 20, 40, and 60 min after mixing.

## References

- [1] R.P. Novick, S. Iordanescu, S.J. Projan, J. Kornblum, I. Edelman, pT181 plasmid replication is regulated by a countertranscript-driven transcriptional attenuator, *Cell*. 59 (1989) 395–404.
- [2] S. Brantl, E.G. Wagner, Antisense RNA-mediated transcriptional attenuation: an in vitro study of plasmid pT181, *Mol Microbiol.* 35 (2000) 1469–1482.
- [3] M.K. Takahashi, J. Chappell, C.A. Hayes, Z.Z. Sun, J. Kim, V. Singhal, et al., Rapidly Characterizing the Fast Dynamics of RNA Genetic Circuitry with Cell-Free Transcription-Translation (TX-TL) Systems, *ACS Synth. Biol.* 4 (2015) 503-515. doi:10.1021/sb400206c.