Supplementary Information for

Construction of an *in vitro* bistable circuit from synthetic transcriptional switches

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Supplementary text

The choice of domain lengths on the synthetic transcriptional switches are based on earlier experiments which used different DNA sequences and transcription reagents. Here, we describe the earlier switch designs and the changes we made for improved switch functionality.

1. **Toehold**: We tested different toehold lengths for effective implementation of the inhibition mechanism. A toehold length of 6, 8, or 10 bases showed fast kinetics in initiation of branch migration, however a toehold length of 10 decreased the transcription rate from an ON state switch template. Thus, we kept the toehold length at 8 bases for increased programmability compared to 6 bases and for increased transcription efficiency compared to 10 bases.

2. **Branch migration region**: Initially, we used 17 base branch migration regions upstream of the T7 RNAP promoter sequence. However, the ON state switch template transcription rate dropped dramatically after a few hours. We interpreted this as the binding between the template and activator being relatively weak and sensitive to the change of buffer condition as the transcription reaction progressed. When we increased the branch migration region to 27 bases, the transcription rates of ON state templates became more stable for up to 12 hours.

3. **Position of Nick**: We tested different nick positions from -8 to -14 in the T7 RNAP promoter sequence. The ON state transcription rates were similar for various nick positions, but the OFF state transcription rates were higher as the nick position moved from -8 to -14. To test crosstalk, we used a DNA activator with a different branch migration sequence together with the OFF state template, and tested whether the leaky transcription from the OFF state template increased. The nick position -10 showed increased leaky transcription from an OFF state template when the unrelated DNA activator is used in excess, while the nick position -12 had no detectable change in the leaky transcription. To prevent crosstalk, we kept the nick position at -12 of the promoter region.

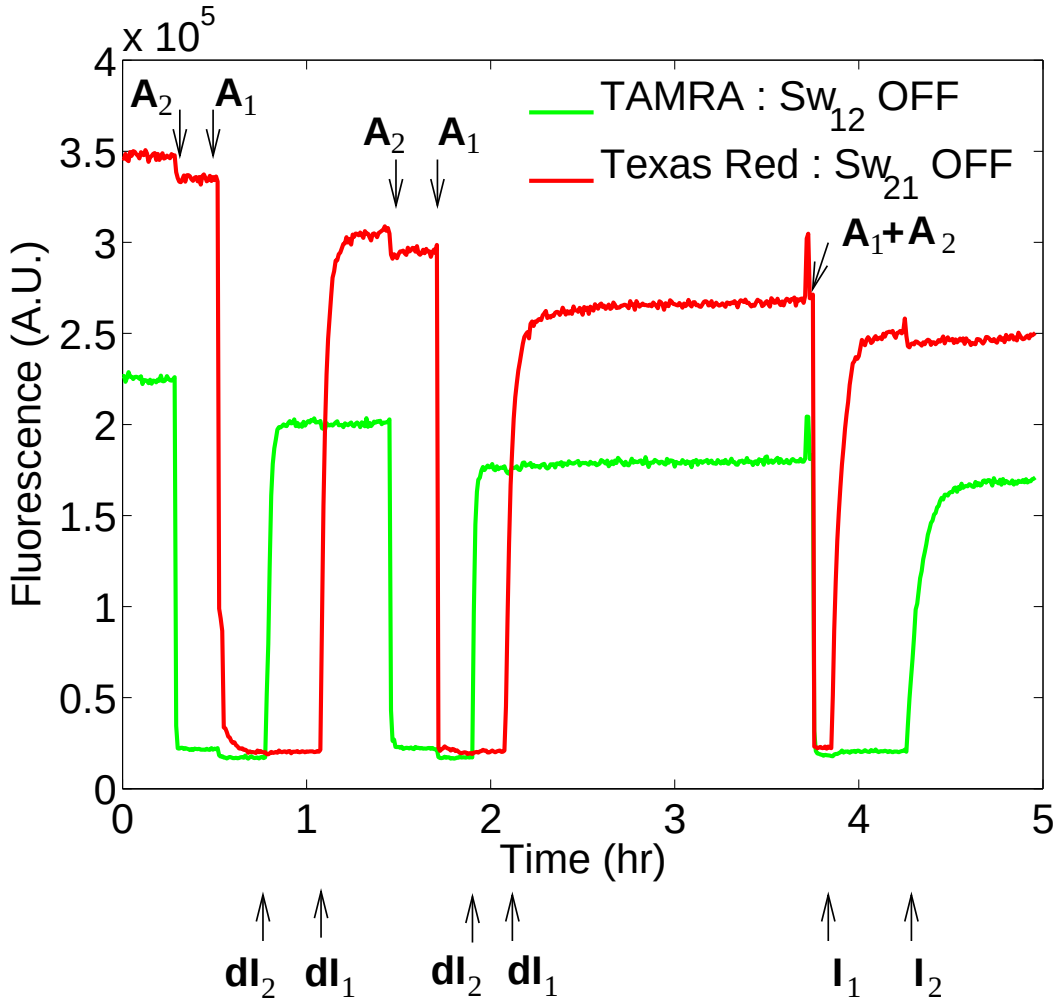

Figure S1: Specificity of template-activator interactions. Initially, the 70 μL reaction mixture contains 50 nM Texas Red-labeled switch template T_{21}, 50 nM TAMRA-labeled switch template T_{12}, and 10% (v/v) transcription buffer. Quencher-labeled activators and DNA/RNA inhibitors are added in the following order: (1) 0.3 μL of 50 μM activator A_2 at 15 minutes, (2) 0.3 μL of 50 μM activator A_1 at 30 minutes, (3) 0.5 μL of 50 μM DNA inhibitor dI_2 at 45 minutes, (4) 0.5 μL of 50 μM DNA inhibitor dI_1 at 65 minutes, (5) 0.5 μL of activator A_2 at 85 minutes, (6) 0.6 μL of activator A_1 at 100 minutes, (7) 0.5 μL of DNA inhibitor dI_2 at 115 minutes, (8) 0.5 μL of DNA inhibitor dI_1 at 125 minutes, (9) 0.5 μL of activator A_1 and 0.5 μL of activator A_2 at 225 minutes, (10) 0.4 μL of 80 μM RNA inhibitor I_1 at 230 minutes, (11) 0.4 μL of 70 μM RNA inhibitor I_2 at 255 minutes. The TAMRA signal changes only upon the introduction of A_2, dI_2, and I_2, while the Texas Red signal changes only upon the introduction of A_1, dI_1, and I_1, demonstrating the specificity of interactions. The fluorescence crosstalk between two channels is about 2% of the total fluorescence signals. The maximum fluorescence level dropped about 30% by the end of repeated hybridization reactions. One-third of the signal drop can be explained by dilution. Other sources of signal loss include absorption and loss of fluorophore-labeled DNA on pipet tips during mixing (data not shown).

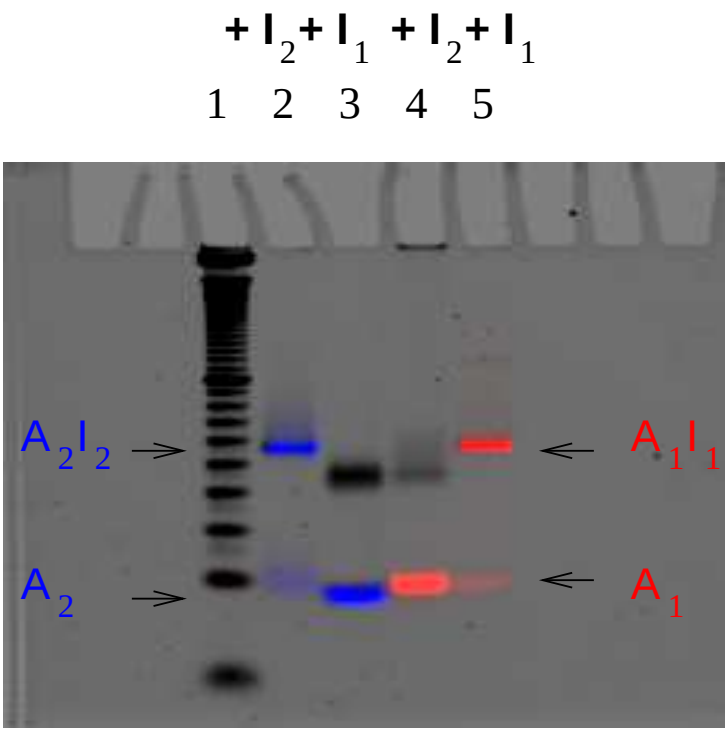

Figure S2: Specificity of activator-inhibitor interactions analyzed in a 10% non-denaturing gel. The gel is scanned for FAM fluorescence (blue, excitation: 488 nm, emission: 500-560 nm) and Cy5 fluorescence (red, excitation: 645 nm, emission: 670-720 nm). After staining with SYBRgold, the gel is scanned for SYBRgold signal (black). These three images were digitally aligned and superimposed. Lane 1 contains 10-base ladder. Lane 2 contains 1 μM FAM-labeled activator A_2 and 0.6 μM RNA inhibitor I_2. Lane 3 contains 1 μM FAM-labeled activator A_2 and 2 μM RNA inhibitor I_1. Lane 4 contains 1 μM Cy5-labeled activator A_1 and 2 μM RNA inhibitor I_2. Lane 5 contains 1 μM Cy5-labeled activator A_1 and 0.6 μM RNA inhibitor I_1. Activator A_2 and A_2I_2 complex (blue) can be identified in lane 2, but activator A_2 (blue) and RNA inhibitor I_1 (black) migrate separately and no A_2I_1 complex is identified in lane 3. Activator A_1 and A_1I_1 complex (red) can be identified in lane 5, but activator A_1 (red) and RNA inhibitor I_2 (black) migrate separately and no A_1I_2 complex is identified in lane 4.

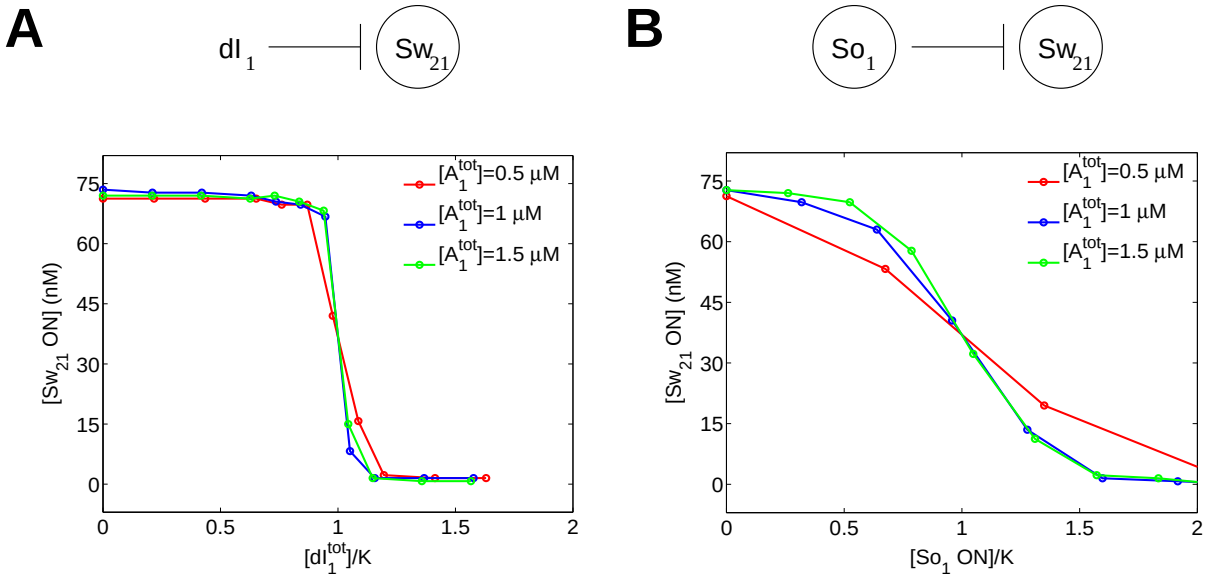

Figure S3: Rescaled transfer curves of switch Sw_{21} and a feedforward circuit. The data sets are the same as those used in Figures 2B and 2E. The inputs are normalized with respect to K's (the amount of inputs required for half repression). The transfer curves with higher total activator concentrations show sharper transitions. Experimental data are plotted as circles with lines drawn to guide the eye.

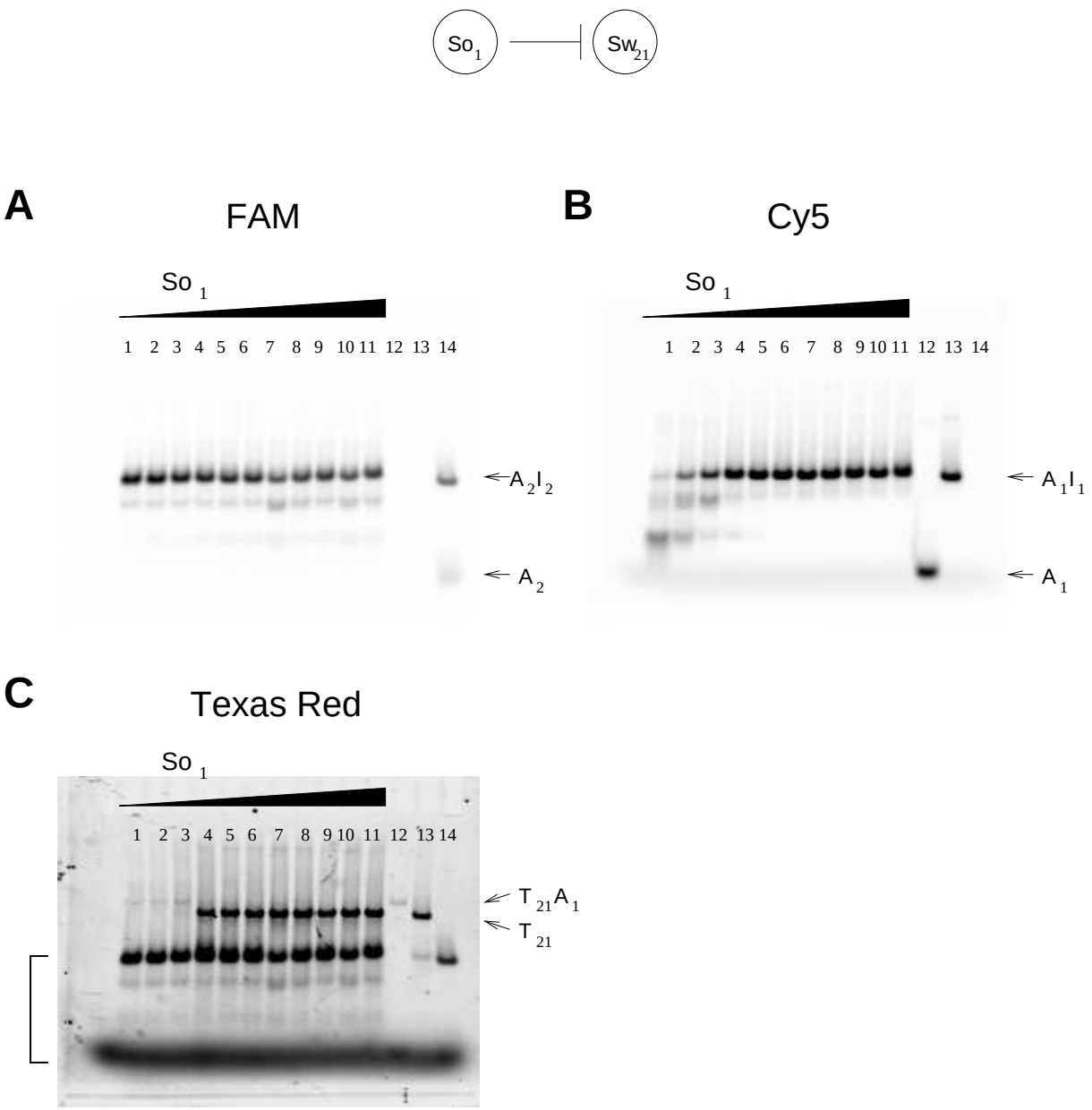

Figure S4: Feedforward circuit analyzed in a 10% non-denaturing gel. The gel is scanned for FAM fluorescence (excitation: 488 nm, emission: 500-560 nm), Cy5 fluorescence (excitation: 645 nm, emission: 670-720 nm) and Texas Red fluorescence (excitation: 532 nm, emission: 580-630 nm). These three images are digitally aligned and superimposed in Figure 3D. **(A)** FAM-labeled activator A_2 and A_2I_2 complex can be identified. The A_2 band in lane 14 is faint because almost stochiometric amount of I_2 is used. The unidentified bands may be A_2 bound to a partially degraded I_2. **(B)** Cy5-labeled activator A_1 and A_1I_1 complex can be identified. The unidentified bands may be A_1 bound to a partially degraded I_1. **(C)** Texas Red-labeled switch template T_{21} and $T_{21}A_1$ can be identified. The ON switch template $T_{21}A_1$ has low fluorescence (it is barely discernable in lanes 1, 2, 3, and 12), presumably due to fluorescence resonance energy transfer from Texas Red on T_{21} to Cy5 on A_1. Because the Texas Red fluorescence is relatively weak, emission from FAM-labeled species and XCFF loading dye show up as well (bracket).

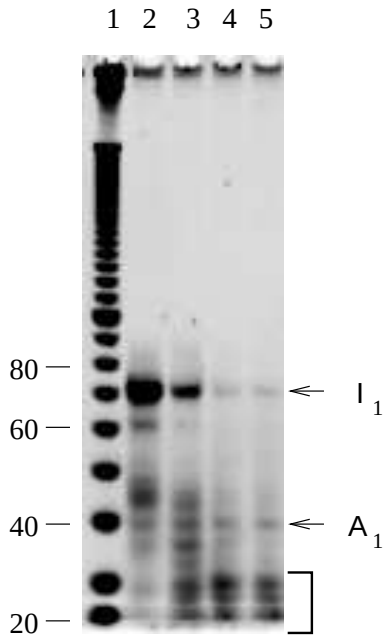

Figure S5: Ribonuclease H activity. The reaction mixture contains 4 μM RNA inhibitor I_1, 100 nM activator A_1, 100 nM RNAP, 20 nM RNase H, 10% (v/v) transcription buffer, and 40% (v/v) NTP. The reaction mixture is incubated at 37°C and samples are taken at different times to be analyzed in a 8% denaturing gel. Lane 1 contains 10-base ladder, lane 2 to 5 contain samples taken at 0, 20, 40, and 60 minutes, respectively. About 3 μM of inhibitor I_1 is processed during the first 20 minutes. Note that smaller RNA species are not degraded after 40 minutes (bracket). These are presumably the 5' overhang and 3' hairpin structures in inhibitor I_1, which are not complementary to activator A_1.

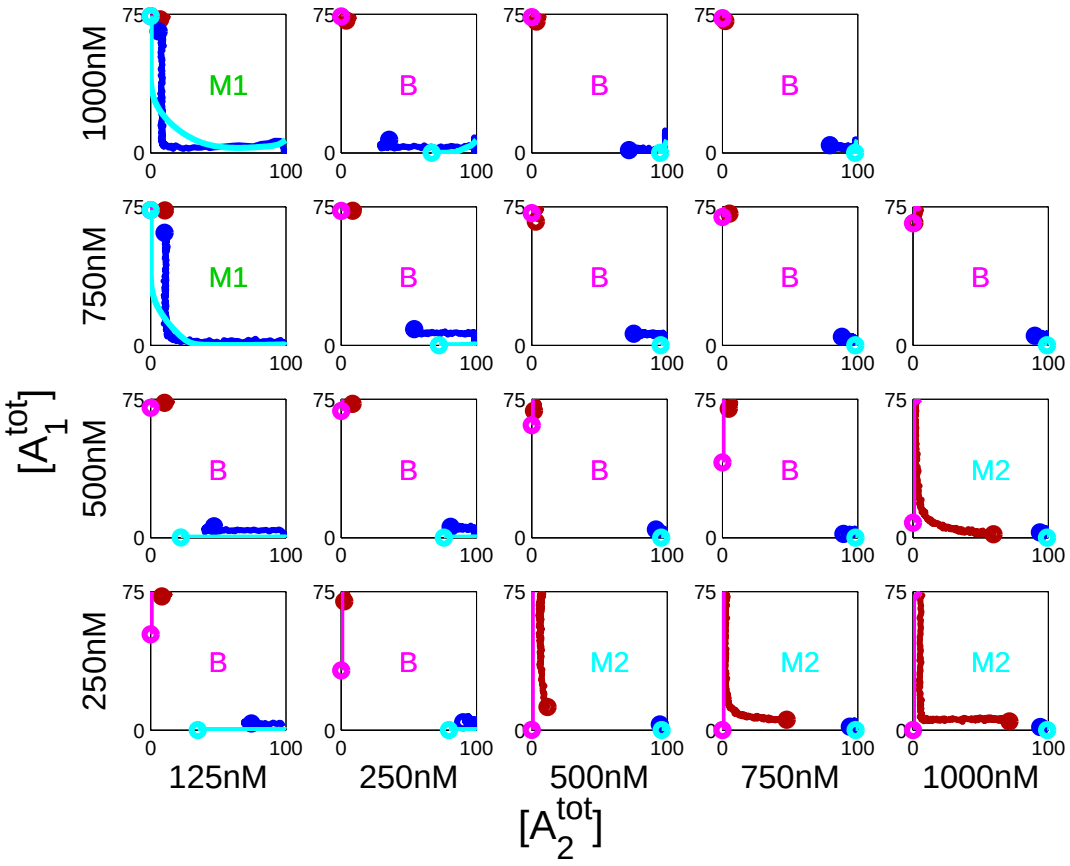

Figure S6: Kinetic trajectories of the bistable circuit used for the bifurcation diagram (Figure 5B). Switch activity phase planes with the switch Sw_{12} activity as x axes and the switch Sw_{21} activity as y axes are embedded in the bifurcation diagram for the activator concentrations. Each phase plane contains trajectories starting from the opposite corners: (switch Sw_{21} ON, switch Sw_{12} OFF) or (switch Sw_{21} OFF, switch Sw_{12} ON). Both experimental trajectories (red and blue) and simulation trajectories (magenta and cyan) are shown with final points as circles. Bistability is achieved when the two trajectories do not converge. The stability assessment for experimental results are shown as letters: only (switch Sw_{21} ON, switch Sw_{12} OFF) is stable (M1), only (switch Sw_{21} OFF, switch Sw_{12} ON) is stable (M2), or both initial conditions are stable (B).

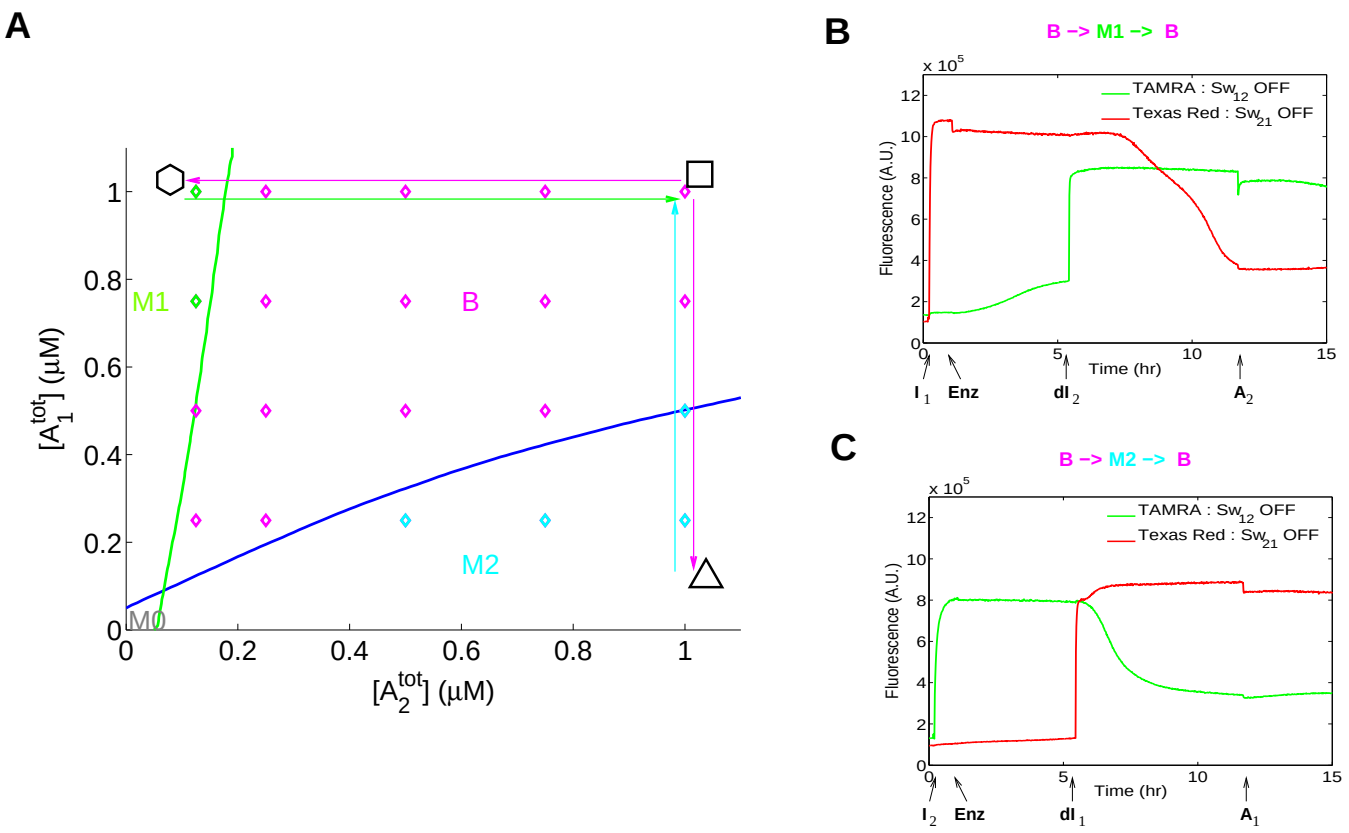

Figure S7: Hysteresis of the bistable circuit. **(A)** Experimental design. The bistable circuit is perturbed in two different ways to test hysteresis. The activator concentrations were shifted from the bistable regime (square) to one of the monostable regimes (hexagon and triangle) and back to the bistable regime. The circuit maintained the state acquired in the monostable parameter regime when returned to the bistable parameter regime. **(B and C)** Fluorescence time-courses. Initially, the 70 μL reaction mixture contains 75 nM Texas Red-labeled switch template T_{21}, 100 nM TAMRA-labeled switch template T_{12}, 1 μM quencher-labeled activator A_1 and A_2 with transcription buffer and NTP. The reaction condition is the same as that of Figure 5C except for the amount of initial RNA inhibitors. Initial fluorescence time-courses of (B) and (C) closely match those of Figure 5A right when converted to the switch activities. **(B)** Other reagents are added in the following order: (1) 3.5 μL of 20 μM inhibitor I_1 at 10 minutes, (2) 2.1 μL RNAP and 0.16 μL RNase H (an equivalent amount of this RNase H batch as 0.24 μL used in other reactions of bifurcation diagram results (Figure S6)) at 60 minutes, (3) 1.25 μL of 50 μM DNA inhibitor dI_2 at 320 minutes, (4) 1.25 μL of 50 μM quencher-labeled activator A_2 at 700 minutes. The TAMRA signal stays low (switch Sw_{12} ON) and the Texas Red signal stays high (switch Sw_{21} OFF) initially, but the signal state switches after the addition of DNA inhibitor dI_2 because the circuit has moved to a monostable parameter regime (Hexagon in (A): switch Sw_{12} OFF, switch Sw_{21} ON). The fluorescence signals stay at the same level even after the addition of activator A_2, which brings the circuit back to the bistable regime. **(C)** Other reagents were added in the following order: (1) 3.5 μL of 20 μM inhibitor I_2 at 10 minutes, (2) 2.1 μL RNAP and 0.16 μL RNase H at 60 minutes, (3) 1.25 μL of 50 μM DNA inhibitor dI_1 at 320 minutes, (4) 1.25 μL of 50 μM quencher-labeled activator A_1 at 700 minutes. The TAMRA signal stays high (switch Sw_{12} OFF) and the Texas Red signal stays low (switch Sw_{21} ON) initially, but the signal state switches after the addition of DNA inhibitor dI_1 because the circuit has moved to a monostable parameter regime (Triangle in (A): switch Sw_{12} ON, switch Sw_{21} OFF). The fluorescence signals stay at the same level even after the addition of activator A_1, which brings the circuit back to the bistable regime.

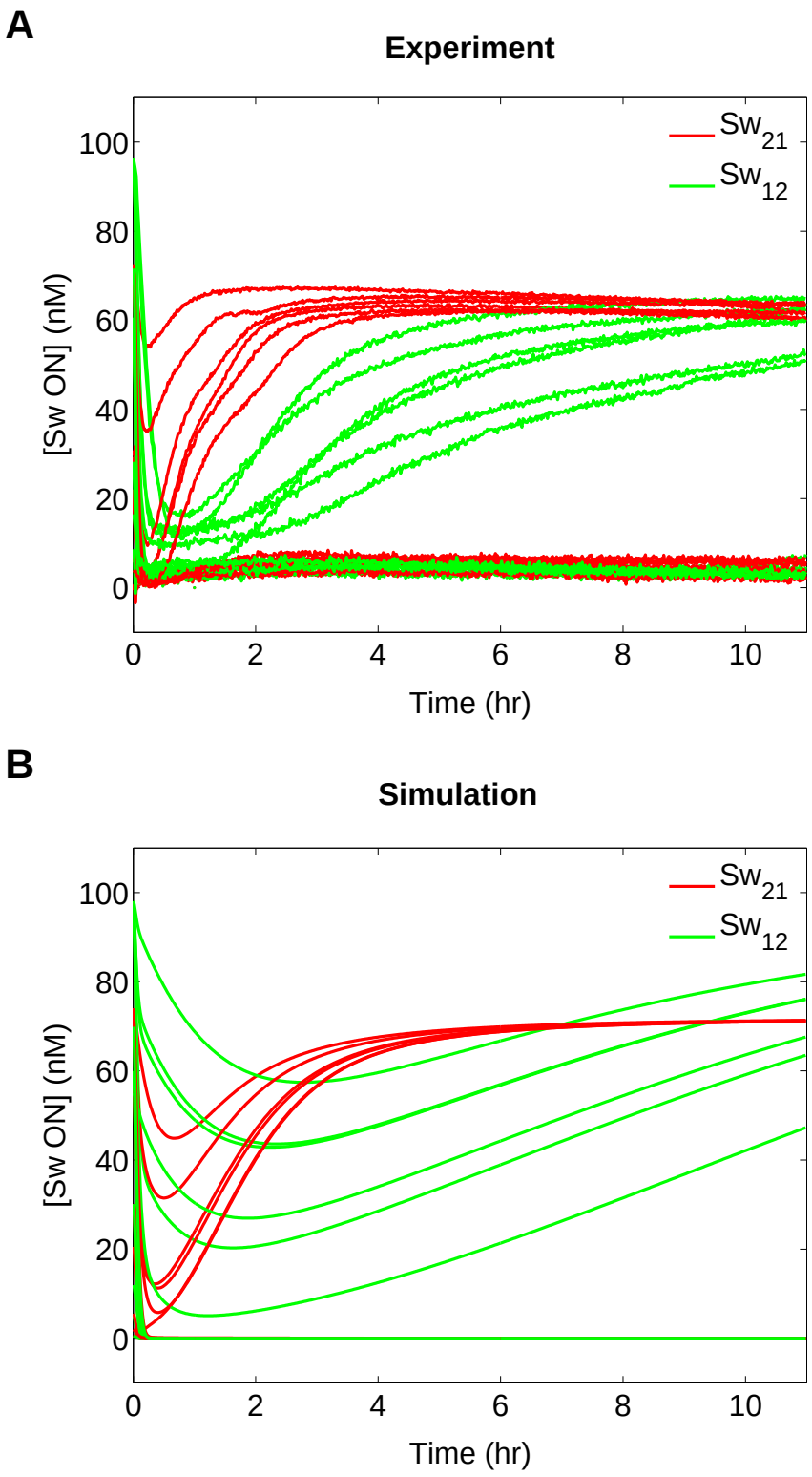

Figure S8: Kinetic trajectories of the bistable circuit used for the phaseplane. **(A)** Experimental switch activity time-courses of the bistable circuit (Figure 5C). **(B)** Switch activity time-courses of the bistable circuit generated from the model (Figure 5D).