# Prokaryotic Toehold Switch Sensors

## 1. Overview

A toehold switch is an engineered mRNA that carries its own off-switch. It is translated only when a chosen RNA (the trigger) is present in the cell. On its own, the switch folds into a hairpin that buries the two things a ribosome needs to start translation: the ribosome-binding site (RBS), held inside the hairpin loop, and the start codon (AUG), held in a bulge of the stem. The 5′ end is left single-stranded; this is the trigger-binding site (TBS). The trigger binds there first and then unzips the hairpin, base pair by base pair. RBS and AUG become accessible, and the gene downstream is translated (Figure 1). That gene can be anything we want to place under this control; in our experiments it is GFP, used as a reporter.

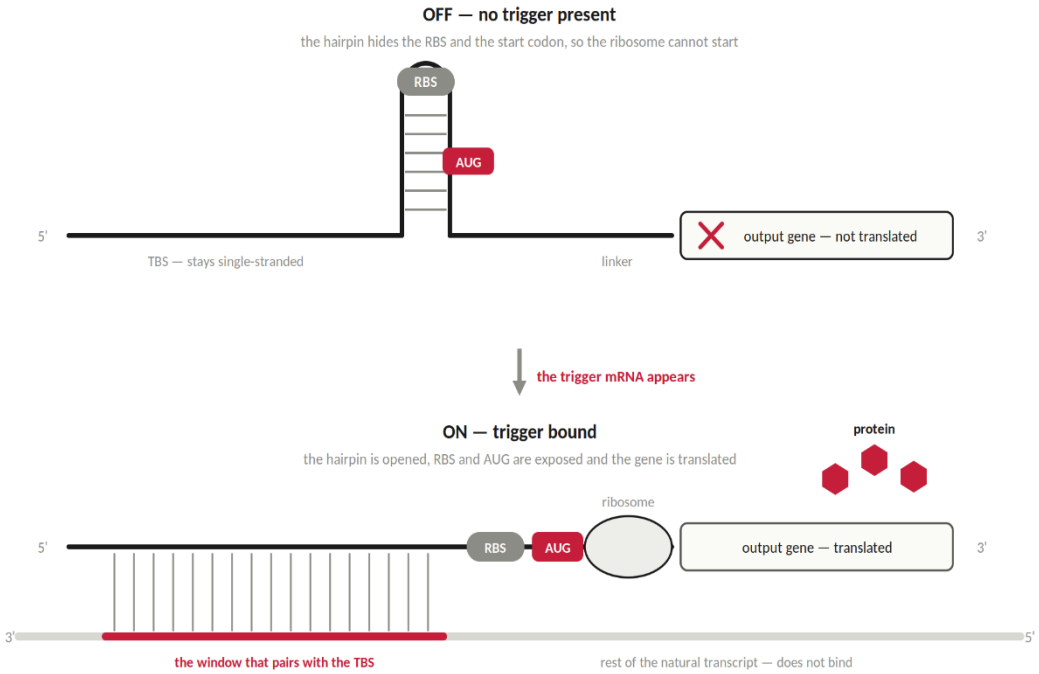


**Figure 1.** How the switch works. Without the trigger the hairpin hides the RBS and the AUG, so no protein is made. The trigger lands on the free TBS, opens the hairpin, and the ribosome can start.

The trigger here is a natural transcript, the mCherry mRNA, and we cannot edit it. Every design choice therefore sits on the switch: which window of the target it reads, how long the TBS and the stem are, and what sequence follows the start codon.

NucSyn [2] proposes candidate switches for each trigger window. Every candidate is folded once with ViennaRNA at 37 °C, in two states: OFF is the switch alone, ON is the switch together with its trigger. Five checks can remove a candidate outright; all other checks only attach a warning. Whatever survives is ranked by a single score, and a small set is ordered for the lab (Figure 2).

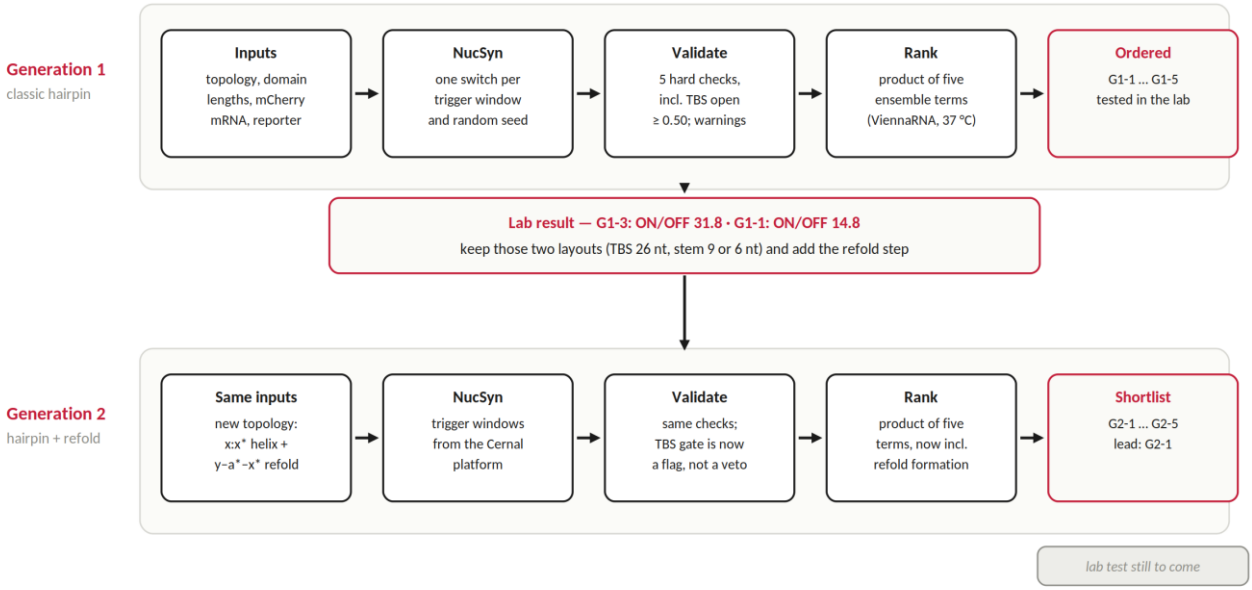


**Figure 2.** The process described in this report, end to end. Generation 1 was designed, ordered and measured; its result set the starting point for generation 2, which has been designed but not yet tested.

Generation 1 (the classic hairpin) produced five ordered designs, G1-1 to G1-5. All five passed the TBS gate, and two worked best in the lab: G1-3 (ON/OFF 31.8) and G1-1 (ON/OFF 14.8), against 35.4 for the best mCherry switch of Green et al. Generation 2 keeps the TBS and stem lengths of those two and adds a refolding step taken from Green et al. (2014), which lowers the energy cost of opening the hairpin. Five candidates were selected, G2-1 to G2-5, with G2-1 as the lead on computational grounds (score 0.5816, TBS open 0.615, refold formation 0.979).

Generation 2 has no experimental results yet. Until a measured ON/OFF ratio exists, every number in this report ranks designs against one another.

# 2. Introduction

## 2.1 Why a toehold switch, and why at translation

A circuit designed to distinguish between cell states must detect specific RNA markers. Regulating gene expression at the translation level provides a minimally invasive approach, requiring no additional promoter, transcription factor, or intermediate protein. The only requirement is that translation stay blocked while the marker is absent and starts once it appears.

The marker (the trigger) is a natural transcript and cannot be edited, so the switch must be built around whatever sequence the trigger happens to have. Throughout this report the two states are defined on two molecules: OFF is the switch folded on its own, and ON is the switch folded together with its trigger.

## 2.2 How the switch is built

In the OFF state the hairpin keeps the start of translation out of reach. The RBS sits in the hairpin loop, and the AUG sits in the stem facing a 3-nt bulge on the opposite arm, forming a 3×3 internal loop. The 5′ end stays unpaired (the TBS).

Because the TBS is already unpaired, the trigger can form its first base pairs without having to open a helix first, this is what makes the design work. Once anchored, the trigger continues into the stem and opens its base pairs one at a time until the hairpin is gone. In the ON state the RBS and the AUG are exposed, the ribosome loads, and the reporter is translated (Green et al., 2014).

The design therefore reduces to a few numbers: where the trigger window starts on the target transcript, how long the TBS is, and how long the main stem is. Figure 3 shows the switch as a chain of domains, read from the 5′ end. A domain is simply a named stretch of sequence with one job: the TBS, a stem arm, the loop carrying the RBS, or the linker.

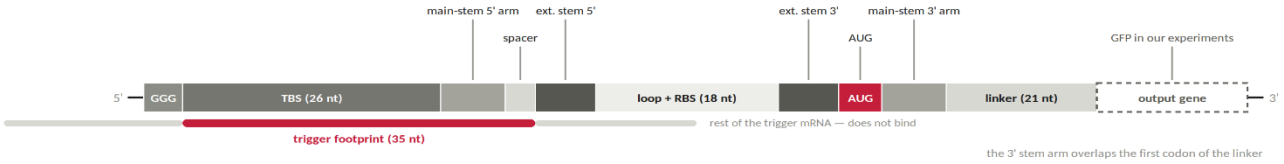


**Figure 3.** The generation 1 switch as a chain of domains, drawn 5′ to 3′. The red bar marks the stretch the trigger pairs with.

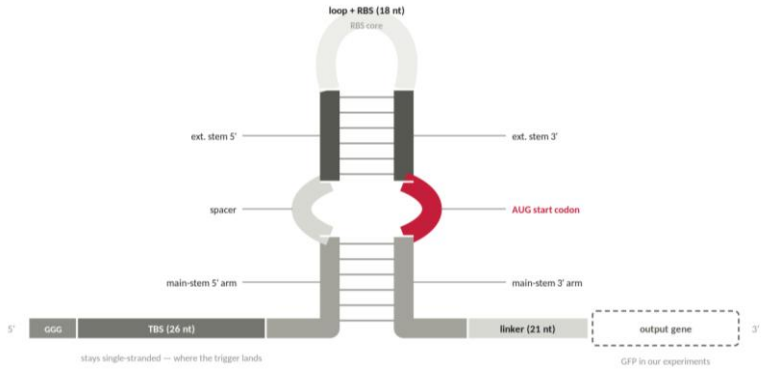


**Figure 4.** The same switch folded into its closed (OFF) state, with every designed domain named. The base-pair rungs mark the two helices; the TBS stays single-stranded.

### 2.3 What the model assumes

The scoring code rests on the four assumptions below, and the rest of this report should be read against them.

- **Ensemble, not a single structure.** The minimum-free-energy (MFE) structure is the single most stable fold; the ensemble is every fold the molecule can adopt, each weighted by its Boltzmann probability. We read pairing from base-pair probabilities, because for an RNA of this size the MFE fold is rarely the dominant one.
- **“Open” means unpaired, not reachable.** A nucleotide counts as open when it is unpaired with high probability. An unpaired RBS or AUG can still be physically unreachable if it sits in a small internal loop, so the ON values are upper bounds on ribosome access.
- **Equilibrium only.** No kinetics, no ribosome, no competing cellular RNA. Capture of the trigger by the TBS is a kinetic process, and the model sees it only indirectly, through TBS accessibility.
- **One binding register.** The trigger is assumed to pair in a single antiparallel register with the TBS and the 5′ arm of the main stem. The GGG leader is excluded from all TBS and binding metrics.

## 3. Pipeline stages and algorithm

The same five-step pipeline is used in both generations (Figure 5): it produces candidates, removes the ones that cannot work, ranks the rest, and ends with a short list to order. Algorithm 1 gives it in pseudocode, as run for generation 1; generation 2 changes two lines (Section 5.3). Symbols are defined in Appendix A.

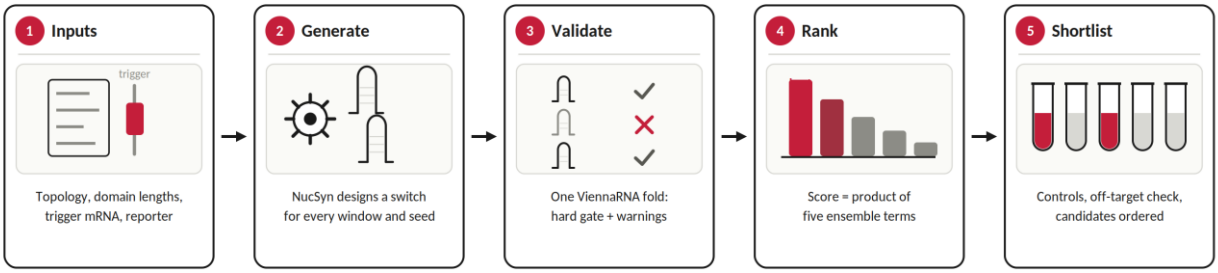


**Figure 5.** The design pipeline, step by step. Sections 3.1 to 3.4 follow these five steps in order.

### Algorithm 1. Design, validate and rank toehold switches

**Input:** M, template, N_w, N_s, T
**Output:** ranked, the validated designs ordered by score

| | |
|---|---|
| **M** | the target mRNA (mCherry); every trigger is a stretch of it |
| **template** | the part of the switch that never changes: domain lengths, GGG, loop with the RBS, linker, reporter |
| **N_w = 10** | number of trigger windows, i.e. places on M where a trigger can bind |
| **N_s = 20** | number of designs per window, each built from a different random start |
| **seed s** | the random start of NucSyn's search; the same seed gives the same design again |
| **T = 37 °C** | folding temperature |

| | | |
|---|---|---|
| 1 | windows ← SELECT_WINDOWS(M, N_w) | NucSyn scans the target and keeps the most accessible sites |
| 2 | designsØ ← | |
| 3 | **for each** w **in** windows **do** | each window is TBS_size + L_stem nt of the target |
| 4 | **for** s = 1 **to** N_s **do** | the design is repeated from 20 random starts |
| 5 | d ← NUCSYN(w, template, seed = s) | builds a switch whose predicted fold matches the template |
| 6 | designs ← designs ∪ {d} | N_w × N_s = 200 designs in total |
| 7 | **sort** designs by sim_score, ascending | lower sim_score = closer to the intended fold |
| 8 | rankedØ ← | |
| 9 | **for each** d **in** designs **do** | one fold per design; three ensembles come out of it |
| 10 | OFF ← RNAfold(d) | switch alone: is the hairpin shut and the TBS free? |
| 11 | ON ← RNAcofold(d, trig) | switch + trigger: does the hairpin open? |
| 12 | TRG ← RNAfold(trig) | trigger alone: needed for ΔG binding |
| 13 | **if** d fails any hard check **then** discard d | stop codon, missing RBS, frameshift, spacer AUG, closed TBS |
| 14 | record warnings(d) | weak switching, blocked AUG … - the design is kept |
| 15 | S(d) ← T_off · Δ_sw · D(ΔG_m) · B · (1 − P_def) | Equation (1) - a product, so every step must work |
| 16 | ranked ← ranked ∪ {(d, S(d))} | |
| 17 | **sort** ranked by S, descending | best-scoring design first |
| 18 | **return** ranked | the shortlist is then chosen by hand from the top |

Generation 2 changes two lines: the windows come from the CERNAL trigger-selection algorithm (line 1), and the score becomes Equation (2) (line 15).

## 3.1 Steps 1-2: inputs and generation with NucSyn

NucSyn [2] is run by hand, one set of inputs at a time, through a runner script in the NucSyn package. We specify: a prokaryotic switch for E. coli at 37 °C; the topology and the length of every domain; the trigger as the full mCherry mRNA, from which NucSyn cuts candidate trigger windows of length TBS_size + main stem (10 windows in our runs, a number set in the runner script); the reporter gene, preceded by a fixed 21-nt linker (AACCUGGCGGCAGCGCAAAAG); a 3-nt GGG leader as prefix; the 18-nt loop sequence carrying the RBS; and an RBS-to-AUG offset of 6 nt, following Green.

For each window NucSyn repeats the design with 20 random seeds and returns every design sorted by sim_score, its own measure of how close the predicted fold is to the intended one in dot-bracket notation (lower

is closer). The windows themselves are chosen by NucSyn, using an RNA-accessibility criterion on the target transcript. NucSyn returns the resulting designs, and each one is validated and scored separately.

### 3.2 Step 3: hard checks and warnings

Each design is folded once with ViennaRNA at 37 °C - RNAfold for the switch alone and RNAcofold for the switch with its trigger, taking both the MFE structure and the partition function - and that fold is then checked (Figure 6). Five checks have veto power and remove the design from the ranked pool:

- an in-frame stop codon in the reporter;
- the RBS motif missing from the loop;
- a reading frame that is not a multiple of three from the AUG;
- an AUG or a stop codon in the spacer between the RBS and the AUG;
- TBS accessibility in OFF below 0.50.

A design that fails any of the five is discarded. Only the last of these is thermodynamic: a TBS that is mostly paired cannot capture the trigger, so the switch can never open. Every other check only attaches a warning and the design stays in: a switching delta below 0.4 (the main-stem 3′ arm after the AUG should open when the trigger binds), an internal in-frame AUG, RBS open probability below 0.70 in either state, a helix of at least 4 bp over the AUG in ON, a loop self-fold of at least 4 bp, trigger binding below 0.70, and a ΔG margin above −5 kcal/mol.

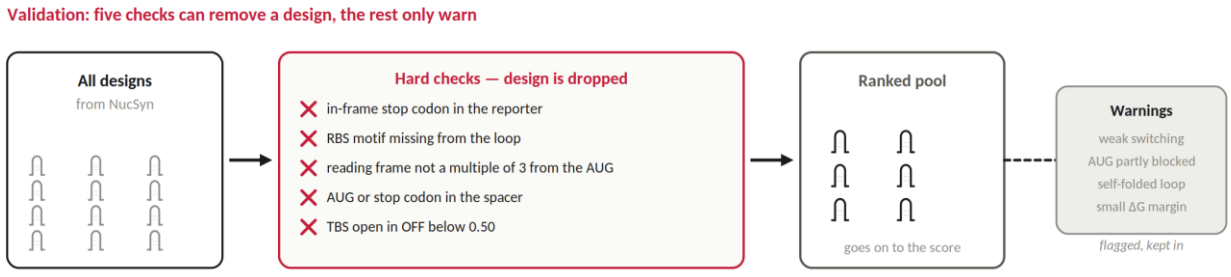


**Figure 6.** Validation. Five failures are fatal; everything else is reported as a warning and the design continues to the ranking.

### 3.3 Step 4: scoring and ranking

Each candidate is folded once, at 37 °C. That single fold gives three ensembles: the switch alone (OFF), the switch together with its trigger (ON), and the trigger alone. From the partition function we read how likely each nucleotide is to be unpaired (TBS, RBS, AUG, main-stem 3′ arm) and how much of the trigger footprint is actually paired with the trigger. From the MFE energies we read ΔG alone, ΔG complex, the ΔG margin (ΔG complex − ΔG alone) and ΔG binding (ΔG margin − ΔG of the trigger alone).

Designs that pass the checks are then ranked by a single number $S_1$, Equation (1):

$$(1)\; S_1 = T_off \cdot \Delta_sw \cdot D(\Delta G_m) \cdot B \cdot (1 - P_def)$$

**where:**

**T_off** - OFF tightness: the mean of TBS open, stem paired and spacer paired, in the OFF state (0-1).

**Δ_sw** - switching delta: the unpaired probability of the main-stem 3′ arm after the AUG, in ON minus OFF (0-1).

**D(ΔG$_m$)** - driving force: D(ΔG$_m$) = 1 / (1 + e^((ΔG$_m$ + 2.5) / 1.5)), with ΔG$_m$ = ΔG_complex − ΔG_alone in kcal/mol (0-1).

**B** - binding: the fraction of the trigger footprint paired with the trigger (0-1).

**P_def** - defect penalty: P_def = L_block / 8, where L_block is the longest helix blocking the AUG or inside the loop, in base pairs; capped at 1.

The terms of Equation (1) are multiplied rather than added, because a switch must work at every step: being strong in one term cannot make up for failing another. Candidates are then sorted by $S_1$, descending. Figure 7 shows where on the molecule each term is measured.

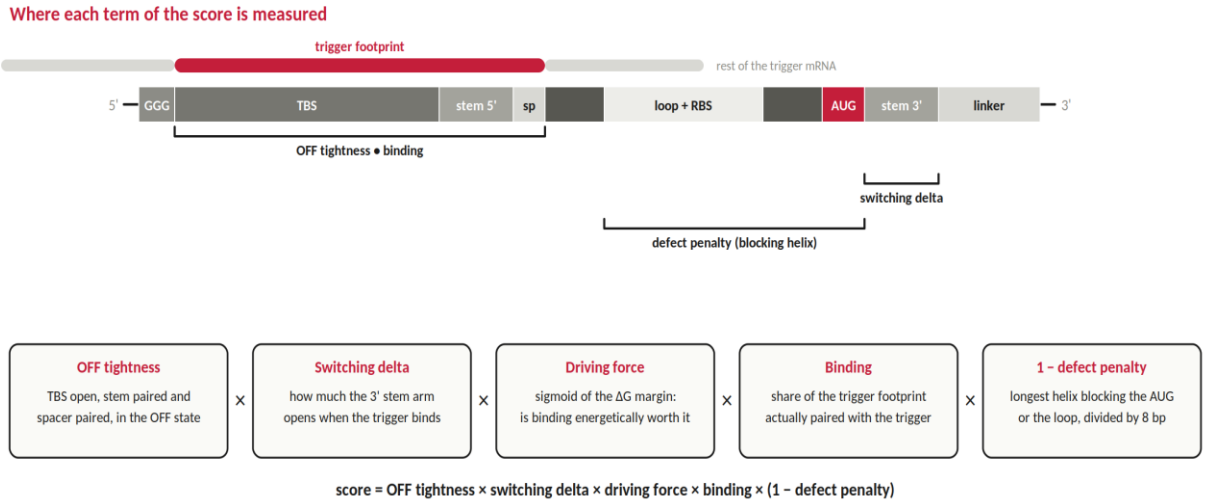


**Figure 7.** The five terms of the generation 1 score and the part of the molecule each one looks at.

### 3.4 Step 5: shortlist, controls and specificity

We chose five candidates from the ranked tables so that the set covers a small grid: TBS 26 versus 30 nt at a 6-nt stem, and stems of 6, 9 and 12 nt at a 26-nt TBS (G1-1 to G1-4, all from trigger family A). G1-5 uses a separate, non-overlapping window (family B), so that the series does not rest on a single target site. A 40-nt TBS was generated and then dropped: in its MFE fold, part of the TBS paired with bases deep inside the linker for every trigger tested.

Specificity was checked against the engineered mCherry mRNA, a recoded version of the target that encodes the same protein. Its duplex energy with our switches was 43-52% of the intended binding, with no dangerous match.

## 4. Generation 1: results

Five designs were ordered (Table 1; sequences in Appendix B). All five passed the TBS gate, at 50.0-59.0% open. In the lab, G1-3 and G1-1 performed best, with ON/OFF ratios of 31.8 and 14.8 (Figure 8); for comparison, the best mCherry switch of Green et al. reaches 35.4. G1-1 sits exactly on the gate (0.50 for the 29-nt window including the GGG prefix, 0.498 for the TBS alone). G1-2 did not switch at all (0.87), despite having the most open TBS (59%) and a 100%-GC external stem- which is what pushed generation 2 towards a lower-GC stem.

| Cand. | TBS / stem | Trigger | Sim. score | ΔG margin (kcal/mol) | TBS open | RBS open OFF / ON | AUG open ON | Trigger bound | Lab ON/OFF |
|---|---|---|---|---|---|---|---|---|---|
| **G1-1** | 26 / 6 nt | A | 6.91 | −48.5 | 50.0% | 87.8% / 94.6% | 98.5% | 99.2% | 14.80 |
| **G1-2** | 30 / 6 nt | A | 7.62 | −54.6 | 59.0% | 96.5% / 97.1% | 99.0% | 98.0% | 0.87 |
| **G1-3** | 26 / 9 nt | A | 6.98 | −55.1 | 50.1% | 96.7% / 97.1% | 95.4% | 99.8% | 31.77 |
| **G1-4** | 26 / 12 nt | A | 7.46 | −60.4 | 52.1% | 94.7% / 96.6% | 96.0% | 98.0% | 5.68 |
| **G1-5** | 26 / 9 nt | B | 8.33 | −58.8 | 51.9% | 96.5% / 95.6% | 90.6% | 99.7% | 1.87 |

**Table 1.** Generation 1 candidates, ordered in the first round. Shaded rows performed best in the lab. TBS open is averaged over the TBS (26 nt, or 30 nt for G1-2) without the GGG leader; RBS open is an unpaired probability. Sequences are in Appendix B.

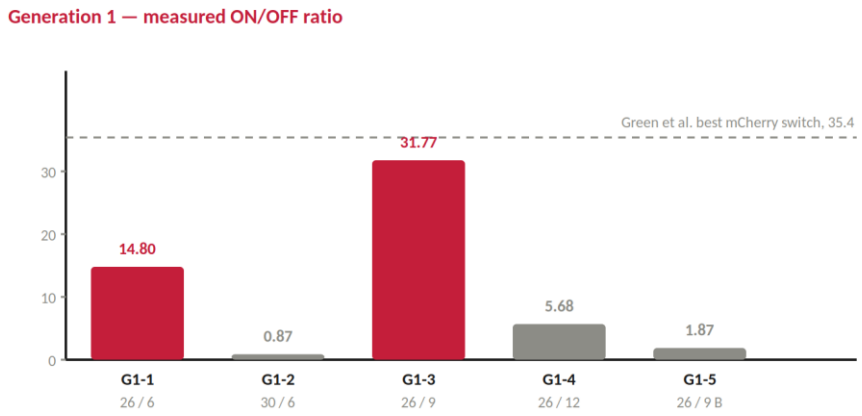


**Figure 8.** Measured ON/OFF ratios for the five ordered designs. TBS and main-stem length in nt below each bar; Family B for G1-5; red= the two best performance.

- **Binding is not the limiting step.** At equilibrium, at least 98% of the trigger footprint is bound in every candidate, with ΔG margins from −48 to −60 kcal/mol.
- **The external stem is very stable and stays shut.** Folded alone with its loop it has ΔG −6.9 kcal/mol in G1-1 and −8.7 kcal/mol in G1-3 (92% and 83% GC), and the model predicts it stays closed in both states. Whether it also hinders ribosome access is a hypothesis we have not tested.
- **TBS accessibility is our best guess for the weak point.** The candidates sit only just above the gate (50-59%), and the value depends on the trigger: different triggers give different results. NucSyn already picked what it scored as the best triggers, so a closer look at the trigger itself may help. In G1-1 and G1-3, for example, the TBS contains a repeat (GAUGUU) that can fold back on itself.

# 5. Generation 2: the next step

## 5.1 Why a second generation

Generation 1 gave us the first measurements for this design approach; generation 2 is the attempt to do better. It targets one specific thing: how easily the hairpin opens once the trigger has already bound.

The idea comes from Green et al. (2014). The best of their 168 first-generation switches worked better than its hairpin thermodynamics predicted. The reason was that after the trigger opened part of the stem, the newly exposed bases paired with the downstream linker, and that extra fold pulled one more base pair open (Supplement S14.1). In the mRNA-sensor design of the same study, this effect was built in deliberately (Supplement S14.2). Generation 2 adds this refolding mechanism to our switches.

## 5.2 What changes in the molecule

Generation 2 keeps the pipeline of Section 3 and changes the switch itself. Table 2 lists every difference; the one that needs explaining in words is the refold. Three new domains are added downstream of the hairpin: y, a* and x*, and NucSyn designs their sequences. Figure 9 shows where they sit.

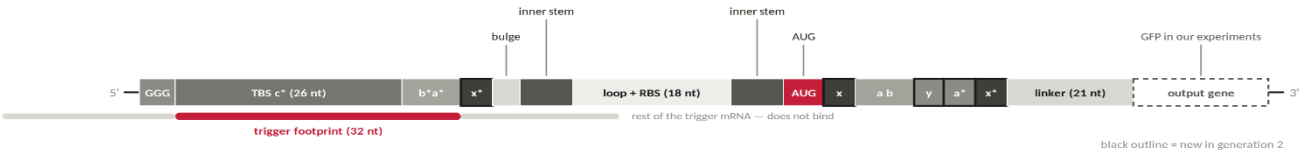


**Figure 9.** A generation 2 switch, drawn 5′ to 3′. Domains with a black outline are new; the red bar marks the trigger footprint.

In the OFF state two things hold the hairpin shut below the AUG loop: the main stem (b*a* paired with a b) and a short x : x* helix of 3 bp. The trigger lands on the TBS and unzips the main stem, which frees a and b. The freed

a now has a second partner waiting downstream (a*), and x can let go of the upstream x* and pair with the downstream one instead. Together, a and a* close a new small hairpin whose loop is made of b and y. Because x has left the upstream x*, those 3 base pairs open as well.

In energy terms: the trigger pays to break the main-stem pairs, and forming the new hairpin gives some of that energy back, so the activated complex sits lower in energy and 3 more base pairs open than the trigger could manage on its own. Figure 10 follows this in three steps.




**Figure 10.** The refold, step by step. Curves below the chain mark which domains are paired with; the trigger is shown in red above it.

This is also why x is kept low in GC. It pairs with x* both before and after the refold, so it must be able to change partners cheaply. Weak, AU-rich pairs allow that; a GC-rich x forms stronger pairs and invites alternative structures instead. The physical reading here is an interpretation, not a measurement.

| Feature | Generation 1 | Generation 2 |
|---|---|---|
| **Topology** | Classic hairpin plus an external stem around the RBS loop; AUG in a 3×3 internal loop | Same hairpin core, plus an x : x* helix and downstream y - a* - x* refold domains |
| **TBS** | 26 or 30 nt | Fixed at 26 nt |
| **Main stem** | 6, 9 or 12 nt | 6 or 9 nt (a + b) |
| **Stem under the RBS loop** | External stem, 6 bp (67-100% GC) | Inner stem, 6 bp (50% GC) |
| **Loop with RBS** | 18 nt, AGACAAGAACAGAGGAGA (Green et al., 2014, Table S4) | Unchanged |
| **Trigger windows** | Taken by NucSyn from the mCherry mRNA (families A and B) | Taken from the trigger-choosing algorithm of the full Cernal platform: mCherry 355-389, 246-277 and 251-282 |
| **Free sequence choice** | None beyond what NucSyn designs | 3-nt x filler (GAA, UCU, GUU, CUU, GAG) |
| **TBS gate (open ≥ 0.50)** | Hard fail: the design is dropped | Flag: the design stays in the table |
| **Ranking score** | Product of five terms | Product of five terms, including refold formation, with weights on GC |

**Table 2.** What differs between the two generations. Everything else (loop, linker, reporter and folding engine) is shared.

## 5.3 What changes in the pipeline

**Engine.** In generation 2 the code is merged into the CERNAL engine, so trigger windows are now chosen by its trigger-selection algorithm instead of by NucSyn.

**Gate.** The 0.50 threshold on TBS accessibility is now applied as a flag instead of a veto, so that flagged designs stay visible in the ranked table.

**Score.** The generation 2 score is again a plain product $S_2$, Equation (2):

$$(2)\ S_2 = T_off \cdot O_bind \cdot R_up \cdot F_ref \cdot (1 - d)$$

**where:**

**T_off** - as in Equation (1), but over the TBS alone (0-1).

**O_bind** - binding occupancy: the fraction of the ON ensemble with the trigger bound (0-1).

**R_up** - upstream release: the mean unpaired probability of b*, a* and x* in ON (0-1).

**F_ref** - refold formation: the fraction of the ON ensemble that adopts the intended refolded structure (0-1).

**d** - defects: unformed designed ON-state base pairs divided by the number of designed ON-state base pairs (0-1).

The five terms now follow the refold. TBS open (OFF) replaces OFF tightness. Binding occupancy (the fraction of the ensemble with the trigger bound) replaces driving force and binding. Upstream release (the opening of b*, a* and x*) replaces switching delta. The defect penalty becomes defects, the share of designed ON-state pairs that fail to form. Refold formation is new: the fraction of the ON ensemble that adopts the intended refolded structure. Generation 1 had no refold, so it could not measure this. GC content is weighted inside NucSyn's design objective, and the ranking then prefers the less GC-rich designs.

**Shortlist.** The final candidates use three trigger sites on the mCherry mRNA, selected by the CERNAL trigger-selection algorithm. The five candidates themselves were chosen by hand from the top of the ranked table, as in Section 3.4. The last two of which overlap: 355-389 (9-nt stem, x = GAA and UCU), 246-277 (6-nt stem, x = GUU and CUU) and 251-282 (6-nt stem, x = GAG). None of them carries a start or stop codon in the stem.

### 5.4 The final candidates

| Cand. | Design (mCherry window · stem · x) | Score | TBS open | Switch GC | Upstream release | Refold | Gate | ΔG margin |
|---|---|---|---|---|---|---|---|---|
| **G2-1** | 355-389 · 9 nt · GAA (lead) | 0.5816 | 0.615 | 51.0% | 0.996 | 0.979 | PASS | −53.1 |
| **G2-2** | 355-389 · 9 nt · UCU | 0.5691 | 0.616 | 51.0% | 0.997 | 0.954 | PASS | −55.3 |
| **G2-3** | 246-277 · 6 nt · GUU | 0.3781 | 0.399 | 49.3% | 0.988 | 0.991 | FLAG | −49.4 |
| **G2-4** | 246-277 · 6 nt · CUU | 0.3747 | 0.399 | 49.3% | 0.985 | 0.986 | FLAG | −48.2 |
| **G2-5** | 251-282 · 6 nt · GAG | 0.3027 | 0.411 | 50.0% | 0.900 | 0.868 | FLAG | −48.1 |

**Table 3.** Generation 2 final candidates. Gate = TBS open ≥ 0.50. Switch GC is the GC content of the whole switch; the external stem is 50% GC in all five. ΔG margin in kcal/mol.

G2-1 is the best candidate on computational grounds. It has the highest score (0.5816), a TBS that passes the gate (0.615), very high upstream release (0.996) and refold formation (0.979), and a strong ΔG margin (−53.1 kcal/mol). Its 26-nt TBS with a 9-nt stem is also the layout of the best generation 1 switch, G1-3 (ON/OFF 31.8). Its one weakness is the accessibility of its trigger window inside the mCherry mRNA: 0.345, against a gene average of 0.399.

G2-2 is the backup. It uses the same trigger and the same stem with x = UCU, as a hedge against unexpected behavior of GAA. Its TBS is just as open (0.616), but its refold formation is lower (0.954).

## 6. Conclusions and next steps

The pipeline works end to end for prokaryotic toehold switches: NucSyn proposes designs, a validation gate removes those that cannot work, and an ensemble score ranks the rest. In generation 1 binding is strong, and we take TBS accessibility to be the limiting factor. In generation 2 the refolding topology, with GC weights that bring the stem under the RBS loop down to 50% GC, gives five candidates, with two (G2-1 and G2-2, on the mCherry 355-389 window) that clear the TBS gate (0.615 and 0.616) with predicted refold formation of 0.979 and 0.954.

Next steps, in order of value:

- Test generation 2 in the lab and measure the ON/OFF ratio.

- Compare the 355-389 candidates (G2-1, G2-2) with the 246-277 candidates (G2-3, G2-4), to test whether trigger-window accessibility predicts the measurement.
- Calibrate the score against the measured fold change, then re-weigh or extend its terms.
- If the results are weak, sweep the stem under the RBS loop further towards an AU-rich, Green-like sequence (GUUAUGCAUAAC), and scan more trigger windows.

## References


[1] Green AA, Silver PA, Collins JJ, Yin P. Toehold switches: de-novo-designed regulators of gene expression. Cell 159(4):925-939 (2014). doi:10.1016/j.cell.2014.10.002, including the Supplemental Information (S14).

[2] NucSyn Platform. GitHub repository, citation file: https://github.com/YehudaLandau/NucSyn-Platform/blob/main/CITATION.md

[3] Lorenz R, Bernhart SH, Höner zu Siederdissen C, et al. ViennaRNA Package 2.0. Algorithms for Molecular Biology 6:26 (2011). doi:10.1186/1748-7188-6-26.


## Appendix A. Notation

| Symbol | Meenning | Units |
|---|---|---|
| **TBS_size** | Length of the trigger-binding site. Generation 1: 26 or 30 nt. Generation 2: 26 nt. | nt |
| **a, b, x, y** | Domains of the main stem: a = 6 or 3 nt and b = 3 nt (9- or 6-nt stem); x and y are 3 nt. | nt |
| **a*, b*, x*** | Reverse complements of a, b and x; b*a* is the main-stem 5′ arm. | nt |
| **ΔG alone, ΔG complex** | MFE of the switch alone (OFF) and of the switch cofolded with its trigger (ON). | kcal/mol |
| **ΔG margin** | ΔG complex − ΔG alone: net drive for the trigger to bind; more negative is better. | kcal/mol |
| **ΔG binding** | ΔG margin − ΔG of the trigger alone. | kcal/mol |
| **TBS open (OFF)** | Mean unpaired probability over the TBS in the switch alone; gate at 0.50. | 0-1 |
| **Binding occupancy** | Fraction of the trigger footprint (ensemble) paired with the trigger. | 0-1 |
| **Switching delta** | Open probability of the main-stem 3′ arm after the AUG, in ON minus OFF. | 0-1 |
| **Upstream release** | Opening of b*, a* and x* once the trigger is bound (ON). | 0-1 |
| **Refold formation** | Fraction of the ON ensemble that adopts the intended refolded structure. | 0-1 |
| **Defects** | Designed ON-state base pairs that fail to form, out of the designed pairs. | count |
| **sim_score** | NucSyn similarity score; lower is closer to an ideal switch. | - |
| **Gate / flag** | TBS open (OFF) below 0.50: hard fail in generation 1, flag in generation 2. | - |

## Appendix B. Sequences (RNA, 5′ → 3′)

Generation 1 sequences end at the linker. Generation 2 sequences continue through the first 33 nt of the GFP coding sequence.

### G1-1 (26 nt TBS, 6 nt stem, family A)

**Trigger** CGUACAACGUCAACAUCAAGUUGGACAUCACCUCC

**Switch** GGGGGAGGUGAUGUUUGAUUUGAUGUUGAUGUUGUAUGCCGCGGAGACAAGAACAGAGGAGACCGUGGAUGACAAACCUGGCGGCAGCGCAAAAG

### G1-2 (30 / 6, family A)

**Trigger** CGUACAACGUCAACAUCAAGUUGGACAUCACCUCCCACA

**Switch** GGGUGUGGGAGGUGGUGUUUAGUUUGAUGUUGAUGUUGUAUGCCGCCCAGACAAGAACAGAGGAGAGGGCGGAUGACAAACCUGGCGGCAGCGCAAAAG

### G1-3 (26 / 9, family A)

**Trigger** GCGCGUACAACGUCAACAUCAAGUUGGACAUCACCUCC

**Switch** GGGGGGGGUGAUGUUUAAUUUGAUGUUGAUGUUGUAUGUGCUCCCGCAGACAAGAACAGAGGAGAGCGGGAAUGUAUACAAACCUGGCGGCAGCGCAAAAG

### G1-4 (26 / 12, family A)

**Trigger** UCCCACAACGAGGACUACACCAUCGUGGAACAGUACGAACG

**Switch**

GGGUGUUCGUAUUGUUUCAUGAUGGUGUAGUCCUCGUUGUGGGAGCGACCAGACAAGAACAGAGGAGAGGUCGUAUGUAUAACGAGAACCUGGCGGCAGCGCAAAAG

### G1-5 (26 / 9, family B)

**Trigger** CCCCUCCGACGGCCCCGUAAUGCAGAAGAAGACUAUGG

**Switch** GGGCUAUAGUCUUUUUCUGUAUUAUGGGGUCGUCGGAGGGGUCCUGCAGACAAGAACAGAGGAGAGCAGGAAUGUUUCGAAACCUGGCGGCAGCGCAAAAG

### G2-1 (mCherry 355-389, 9 nt stem, x = GAA; lead)

**Trigger** GCAGGACGGCGAGUUCAUCUACAAGGUGAAGCUGC

**Switch**

GGGGCAGCUUCACCUUGUAGAUGAACUCGCCGUCCUGCUUCAAGGUUUCCAGACAAGAACAGAGGAGAGGAAACAUGGAAGCAGGACGGAUAUCCUGCUUCAACCUGGC
GGCAGCGCAAAAGAUGCGUAAAGGAGAAGAACUUUUCACUGGAGUU

### G2-2 (mCherry 355-389, 9 nt stem, x = UCU)

**Trigger** GCAGGACGGCGAGUUCAUCUACAAGGUGAAGCUGC

**Switch**

GGGGCAGCUUCACCUUGUAGAUGAACUCGCCGUCCUGCAGAAAGGAUUCCAGACAAGAACAGAGGAGAGGAAUCAUGUCUGCAGGACGGAUAUCCUGCAGAAACCUGGC
GGCAGCGCAAAAGAUGCGUAAAGGAGAAGAACUUUUCACUGGAGUU

### G2-3 (mCherry 246-277, 6 nt stem, x = GUU)

**Trigger** CCGACAUCCCCGACUACUUGAAGCUGUCCUUC

**Switch**

GGGGAAGGACAGCUUCAAGUAGUCGGGGAUGUCGGAACAAACUGUACAGACAAGAACAGAGGAGAGUACAGAUGGUUCCGACAAAACGGAACAACCUGGCGGCAGCGCA
AAAGAUGCGUAAAGGAGAAGAACUUUUCACUGGAGUU

### G2-4 (mCherry 246-277, 6 nt stem, x = CUU)

**Trigger** CCGACAUCCCCGACUACUUGAAGCUGUCCUUC

**Switch**

GGGGAAGGACAGCUUCAAGUAGUCGGGGAUGUCGGAAGAAAGAAUCCAGACAAGAACAGAGGAGAGGAUUCAUGCUUCCGACAAAACGGAAGAACCUGGCGGCAGCGCA
AAAGAUGCGUAAAGGAGAAGAACUUUUCACUGGAGUU

### G2-5 (mCherry 251-282, 6 nt stem, x = GAG)

**Trigger** AUCCCCGACUACUUGAAGCUGUCCUUCCCCGA

**Switch**

GGGUCGGGGAAGGACAGCUUCAAGUAGUCGGGGAUCUCAAAGUAUCCAGACAAGAACAGAGGAGAGGAUACAUGGAGAUCCCCAAAGAUCUCAACCUGGCGGCAGCGCA
AAAGAUGCGUAAAGGAGAAGAACUUUUCACUGGAGUU