Chapter 3

Design of RNA-Based Translational Repressors

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Abstract

The toehold switch is an RNA-based riboregulator that activates translation in response to a cognate trigger RNA and provides high ON/OFF ratios, excellent orthogonality, and logic capabilities. Riboregulators that provide the inverse function – turning off translation in response to a trigger RNA – are also versatile tools for sensing and efficiently implementing logic gates such as NAND or NOR. Toehold and three-way junction (3WJ) repressors are two de novo designed translational repressors devised to provide NOT functions with an easily programmable and intuitive structural design. Toehold and 3WJ repressors repress translation upon binding to cognate trigger RNAs by forming strong hairpin and three-way junction structures, respectively. These two translational repressors can be incorporated into multi-input NAND and NOR gates. This chapter provides methods for designing these translational repressors and protocols for in vivo characterization in *E. coli*.

Key words RNA-based regulator, Toehold switch, Toehold repressor, 3WJ repressor, Ribocomputing

1 Introduction

Synthetic biology is a nascent field aimed at designing biological systems with novel functionality at the genetic level [1, 2]. A wide range of synthetic circuits have been designed including bistable switches, oscillators, logic gates, and memory devices [3–5]. Historically, many protein regulators, including transcription factors or enzymes, have been used to construct genetic circuits, but the narrow dynamic range and unwanted cross talk displayed by many components has hindered the task of implementing more complex circuits [7]. In this respect, nucleic acid-based regulatory circuits provide an alternative to constructing genetic circuits with design flexibility and high programmability due to predictable Watson-Crick base pairing rules. In particular, RNA-based regulators have the advantage of requiring much smaller encoding space compared to protein regulators, which helps to overcome the limited ability to carry out genetic programs in a single cell [8–10].

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A representative example of an RNA-based regulator is the toehold switch, which controls gene expression at the translational level [6]. The toehold switch inhibits translation initiation by hiding the ribosome binding site (RBS) and start codon (AUG) inside the RNA hairpin stem-loop structure, precluding access of ribosomes (*see* Fig. 1a). In the presence of the cognate trigger RNA, the toehold switch can unwind the stem-loop structure as the trigger RNA binds to the toehold region and branch migration occurs. Through this, the RBS and AUG that were hidden in the stem loop can be exposed, allowing access to ribosomes and translation initiation. The toehold switch can be implemented in NOT logic gates through direct hybridization of a third species, the deactivating RNA, to a trigger RNA to silence the trigger and prevent trigger-switch binding. Alternatively, if the trigger-switch complex has formed, extended single-stranded domains of the trigger RNA also provide a binding region for the deactivating RNA, leading to the separation of the trigger RNA from the switch RNA.

We reported that the programmable toehold switch design can be implemented as a multi-input AND and OR logic processing ribocomputing devices with good performance [11]. However, NOT logic gates composed of only two strands, a trigger and a switch RNA, enable versatile logic gates like NAND or NOR to be implemented more intuitively and efficiently. Thus, we developed two de novo designed translational repressor models: the toehold repressor and the three-way junction (3WJ) repressor [12]. The toehold repressor has a strong hairpin structure and a downstream exposed RBS and start codon that enables translation to be initiated (*see* Fig. 1b). In the presence of the trigger RNA, the hairpin structure is released, and a secondary hairpin is formed downstream, isolating the RBS and start codon and turning off translation. On the other hand, the 3WJ repressor has an unstable hairpin structure, which makes it easy to unwind the stem-loop and exposes RBS and AUG to enable downstream gene translation (*see* Fig. 1c). In the presence of the 3WJ trigger RNA, the trigger RNA binds to a pair of single-stranded domains on either side of the hairpin, forming a three-way junction structure to inhibit translation.

These two translational repressors can be used for implementing NAND and NOR logic gates through RNA-RNA interactions alone. To implement the NAND gate with toehold repressors, two different triggers were designed to hybridize to each other through complementary bridging domains to unwind the repressor hairpin structure (*see* Fig. 2a, b). In the case of 3WJ repressor, two orthogonal repressor RNAs are connected in a single RNA transcript such that translation can be inhibited only when both input triggers are present (*see* Fig. 2c, d). The NOR gate was implemented by including multiple sequestered trigger RNAs upstream of the

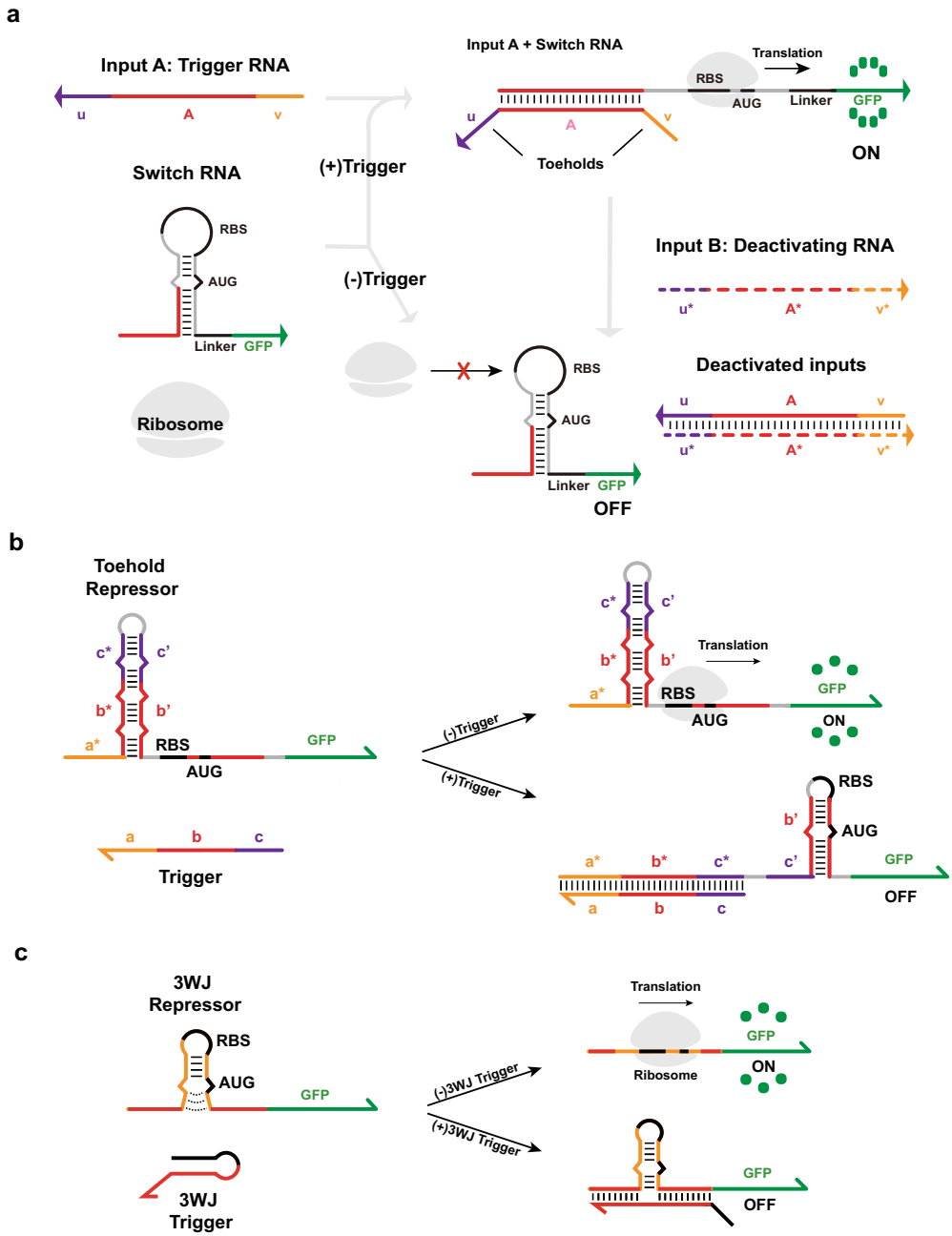

Fig. 1 De novo designed synthetic RNA translational regulators. (**a**) Schematic of the toehold switch mechanism. The ribosomal binding site (RBS) and start codon (AUG) of the switch RNA are exposed only when the cognate trigger RNA (input A) disrupts the secondary structure. A AND (NOT B) logic can be implemented by a deactivating RNA (input B), which induces strand displacement to abolish trigger RNA (input A) activity. (**b**) Schematic of the toehold repressor mechanism. In the presence of the trigger RNA, the hairpin structure is released, and a secondary hairpin is formed, isolating the RBS and start codon. (**c**) Schematic of the 3WJ repressor mechanism. In the presence of the 3WJ trigger RNA, the trigger RNA binds to the domains around the bottom of repressor hairpin, forming a three-way junction structure that inhibits translation

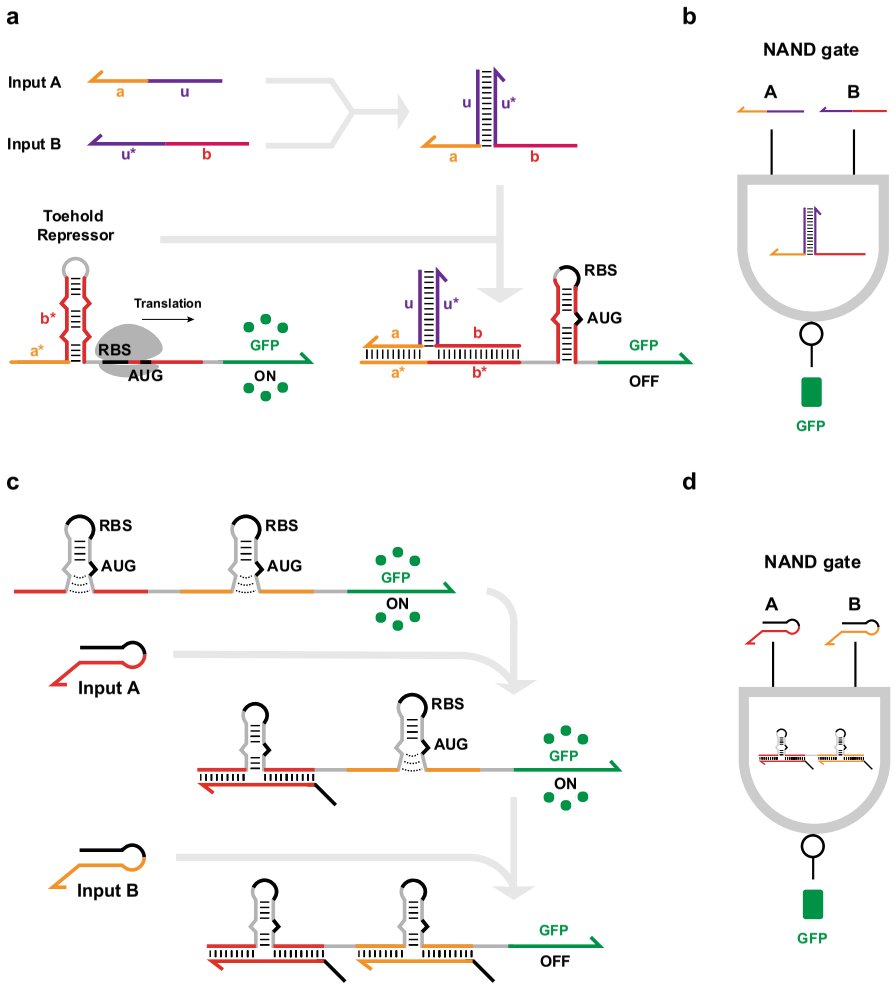

Fig. 2 Two-input NAND logic gates using toehold and 3WJ repressor. (**a**, **b**) Design of a toehold repressor NAND gate where two input RNAs hybridize to each other to unwind the repressor hairpin. The downstream gene is repressed only if both input A and input B are present. (**c**, **d**) Design of a 3WJ repressor NAND gate. In the gate RNA, two orthogonal repressor RNAs are inserted in-frame and upstream of the reporter gene so that translation can be inhibited only when both input triggers are present

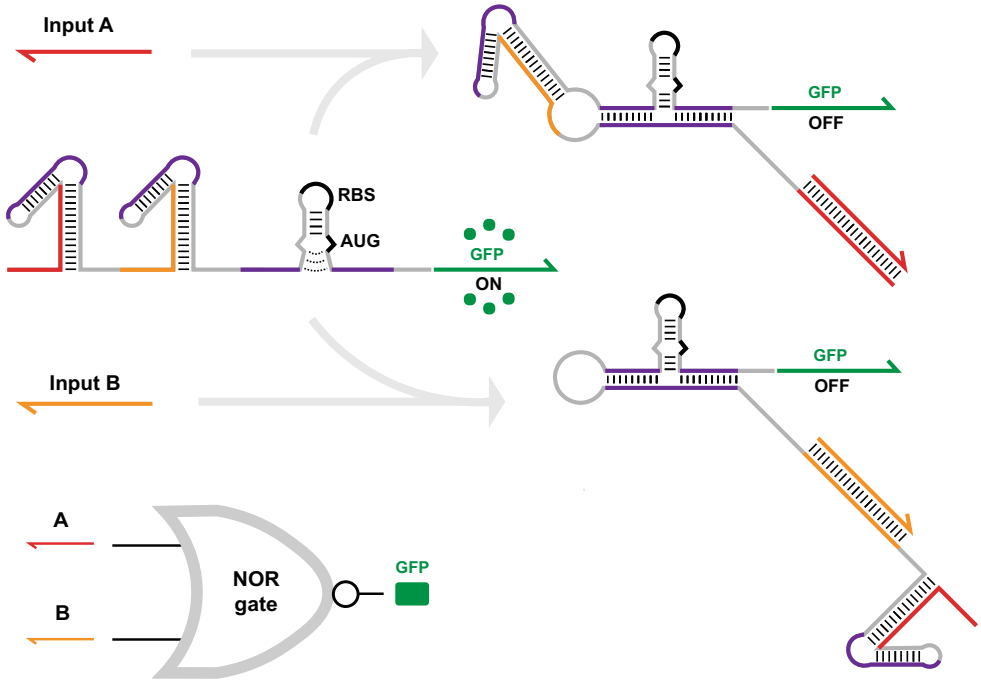

Fig. 3 NOR logic gate implemented by 3WJ repressor. The gate RNA contains a repressor module that regulates the output gene and two sequestered trigger RNAs upstream of the repressor. Binding of either input RNA causes the corresponding hairpin to unwind, inducing trigger binding to the 3WJ repressor module and inhibiting translation

3WJ repressor to induce intramolecular interactions only in the presence of the input RNAs (*see* Fig. 3). These NAND and NOR gates could be extended to multi-input ribocomputing devices that recognize up to four independent input RNAs.

This chapter briefly introduces how to design toehold and 3WJ repressors, how to clone them, and how to identify their repressor activity in vivo by measuring GFP fluorescence.

2 Materials

Prepare all solutions using distilled water (DW) and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise.

2.1 Toehold Repressor and Three-Way Junction (3WJ) Repressor

1. An example of toehold repressor is as follows:

   Switch sequence:

   5′- GGGAAAGUGAAAGUAAGAAUAAAGUGGAAAGAGA CGAGAAUAAGAAGAGACAUCAUAUCUUCUUAUU CUCGUCUCUUUCUACUUUAUAAAC **AGAGGA-GA**AUAAAG**AUG**AAAGAGACGAACCUGGCGGCAG CGCAAAAGAUGCGUAAA-3′ (*see* **Note 1**)

Trigger sequence:

5′-GGGCGAUAGCUAUACCUGGAUAGCUAUCGCAUU CUUCUUAUUCUCGUCUCUUUCCACUUUAUUC UUACUUUCACUUUACU-3′

Decoy sequence:

5′-GGGUCUCACGCCCUCAGCUGGGCGUGAGAUGAG CCUCGUCUCCAGAUGACGAGGCAACGUAGGAUC UGACUGAUCCUACUAU-3′ (*see* **Note 2**)

2. An example of 3WJ repressor

Switch sequence:

5′- GGGAUGAAUGAUAUACACUUGUUAUAGUUAUG AAC**AGAGGAGA**CAUAAC**AUG**AACAAGCACGAAU UGACUACACUAAACCUGGCGGCAGCGCAAAAGA UGCGUAAA-3′

Trigger sequence:

5′- GGGACGAAUUGAUUUGUCAAUUCGUGCGUGUA UAUCAUUCAUCAU-3′

Decoy sequence:

5′-GGGUCUCACGCCCUCAGCUGGGCGUGAGAUGAG CCUCGUCUCCAGAUGACGAGGCAACGUAGGAUC UGACUGAUCCUACUAU-3′

2.2 Suitable Vectors

1. Switch RNA expressed from a relatively low copy vector is suitable for proper switch RNA-trigger RNA interaction (*see* **Note 3**).
2. Trigger RNA is expressed from a relatively high copy vector (*see* **Note 3**).

2.3 Cloning

1. 14 mL round bottom tube.
2. Plasmid miniprep kit.
3. 2× Pfu PCR master mix, store at −20 °C.
4. Thermal cycler.
5. 1% agarose gel: agarose, 1× TAE buffer.
6. 1 kb DNA ladder.
7. SYBR™ Safe DNA Gel Stain.
8. Gel imaging system.
9. 20,000 units/mL DpnI, store at 20 °C.
10. 1× rCutsmart™ buffer: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100 μg/mL recombinant albumin, pH 7.9, store at −20 °C.
11. Gibson assembly master mix, store at −20 °C.
12. 10 units/μL T4 polynucleotide kinase (T4 PNK), store at −20 °C.

13. Type IIS restriction enzyme (BsaI-HFv2, BsmBI-v2), store at −20 °C.
14. 350 units/μL T4 DNA ligase, store at −20 °C.
15. 10× T4 DNA ligase buffer: 660 mM Tris-HCl, pH 7.6, 66 mM $MgCl_2$, 100 mM DTT, 1 mM ATP, store at −20 °C.

2.4 Chemically Competent E. coli

1. Inoue transformation buffer: 10 mM pipes, pH 6.7, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl.
2. Dimethyl sulfoxide (DMSO).
3. Liquid nitrogen.

2.5 Strains and Growth Condition

1. BL21 DE3 (F− ompT hsdSB (rB−mB−) gal dcm (DE3)).
2. BL21 Star DE3 (F− ompT hsdSB (rB−mB−) gal dcm rne131 (DE3)) (*see* **Note 4**).
3. BL21-AI™ (F-ompT hsdSB (rB- mB-) gal dcm araB::T7RNAP-tetA) (*see* **Note 5**).
4. DH5α (endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17 (rK−mK+) λ−).
5. Lysogeny Broth (LB) medium: 5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl.
6. 30 mg/mL kanamycin in DW, filter sterilize and store 1 mL aliquots at −20 °C.
7. 50 mg/mL ampicillin in DW, filter sterilize and store 1 mL aliquots at −20 °C.
8. 25 mg/mL spectinomycin in DW, filter sterilize and store 1 mL aliquots at −20 °C.
9. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) in DW, filter sterilize and store 1 mL aliquots at −20 °C.
10. 20% w/v arabinose in DW, filter sterilize and store 1 mL aliquots at −20 °C.
11. 96-well deep plate.
12. Breathable sealing film.

2.6 Plate Reader

1. 96-well black/clear plate.
2. Microtiter plate reader with monochromator or lasers and filters for simultaneous measurement of green, red, yellow, and cyan fluorescent proteins.

2.7 Flow Cytometry

1. Flow cytometer with appropriate lasers and filters for simultaneous measurement of green, red, yellow, and cyan fluorescent proteins.
2. 1× phosphate buffered saline (PBS).
3. Paraformaldehyde powder.

3 Methods

3.1 Design of RNA Translational Repressors

To generate de novo RNA translational repressors, use the NUPACK sequence design package (http://nupack.org/design/new) [13].

1. Enter the design code, select an appropriate RNA energy parameter, and write structural information and sequence information to NUPACK (*see* **Note 6**, Figs. 4 and 5).
2. Click the update button.
3. Check that the structure generated within the preview window is the correct one.
4. Click the design button.
5. Choose candidates by considering normalized ensemble defects and ensuring that they lack in-frame stop codons downstream of the translation start site.
6. Use NUPACK's analysis function to examine whether candidates form the intended structures (*see* **Note 7**).

3.2 Cloning of RNA Translational Repressor Expression Vectors

1. Choose an optimal cloning method based on the following recommendations. If your sequence has a strong structure (like a toehold repressor), or repeating sequences, choose CRATES [14] as described in **steps 2–6**. If you change a small part (few bases deletion, insertion, substitution) of existing sample, choose site-directed plasmid mutagenesis protocol [15] as described in **steps 7–10**. Otherwise, choose Gibson assembly [16] as described in **steps 11–15**.
2. For easy colony selection, prepare a backbone plasmid containing a constitutively expressed reporter gene cassette with each end having type IIS restriction sites. Determine the concentration of plasmid by measuring the absorption at 260 nm according to Beer-Lambert's law using sequence-specific extinction coefficients.
3. Design DNA blocks using compatible 4 nt junctions. For detailed instructions, see [14].
4. Phosphorylate the single stranded DNA oligo: Combine 2 μL of 20 nM single-stranded DNA oligo with 5 μL of 10× T4 DNA ligase buffer, 1 μL of T4 PNK, and 42 μL of DW to a total volume of 50 μL. Incubate for 30 min at 37 °C and heat inactivate by incubating at 65 °C for 20 min.
5. Anneal the single-stranded DNA oligo to double-stranded DNA block with 4 nt junctions: add 25 μL of phosphorylated (+) strand oligo to 25 μL of phosphorylated (−) strand oligo. Anneal in a PCR machine by heating up to 95 °C and cooling down to 15 °C at rate of about −1 °C/min.

```
# Sample NUPACK code for a toehold repressor

# specify RNA energy parameters, values = rna1995 or rna1999
# number of designs, values = 1 to 10
material = rna1999
temperature = 37.0
trials = 10

# The GGG sequence is only required when using T7 promoter. If there is
# a required sequence for other promoters, design with that sequence.
domain preG = GGG
domain a = N15
domain b = N18
domain c = N12

# cp_star domain specifies the top right portion of the switch stem.
# It is not fully complementary to domain c.
# bp_star domain specifies the bottom right portion of the switch
# stem. It is not fully complementary to domain b.
domain cp_star = N12
domain bp_star = N18

# The first 6 nt in this bpp domain specifies the 6-nt spacer between
# RBS and AUG that is required for efficient translation. This domain is
# not fully complementary to bp_star due to the start codon bulge
domain bpp = N6 AUG N9
domain loop1 = N9

# AGAGGAGA sequence is the RBS sequence to allow efficient translation.
domain loop2 = N4 AGAGGAGA

# Common linker sequence from Green et al.[1]
domain linker = AACCUGGCGGCAGCGCAAAAG

# target switch structure
structure Tr = U3 U45
structure Sw = U3 U15 D7 (U1 D7 (U1 D7 (U1 D6 (U9) U1) U1) U1) U12 U18
        U21
structure Sw_Tr = U3 D45 (U9 U12 D9 (U3 D6 U12 U3) U21 + U3)

Tr.seq = preG c* b* a*
Sw.seq = preG a b c loop1 cp_star bp_star loop2 bpp linker
Sw_Tr.seq = preG a b c loop1 cp_star bp_star loop2 bpp linker preG c* b*
        a*

# prevent sequence patterns
prevent = AAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR, SSSSSS, WWWWWW,
        YYYYYY
```

Fig. 4 Example of NUPACK code for toehold repressor

```
# Sample NUPACK Code for a 3WJ repressor

# specify RNA energy parameters, values = rna1995 or rna1999
# number of designs, values = 1 to 10
material = rna1999
temperature = 37.0
trials = 10

# target structures
structure Sw = U3 U15 U5 U3 D6 U11 U3 U5 U12 U7 U21
structure Tr = U3 D8 U6 U4 U13
structure Sw_Tr = U3 D15 (D5 (U3 D6 U11 U3) D12 (U7 U21 + U3 U12))

# sequence domains
domain preG = GGG
domain targ_stem = N8
domain targ_loop = N6
domain toehold = N15
domain stem1 = UUGUU
domain stem2 = AUA
domain stem3 = GUUAUG

# AGAGGAGA sequence is the RBS sequence to allow efficient translation.
# bases added after binding site to ensure the 3WJ repressor and gene
# are in the same reading frame
domain loop = AACAGAGGAGA
domain stem2_star = AUG
domain post_hairpin1 = N2
domain post_hairpin2 = N10
domain post_hairpin3 = N7

# Common linker sequence from Green et al.[6]
domain linker = AACCUGGCGGCAGCGCAAAAG

Sw.seq = preG toehold stem1 stem2 stem3 loop stem3* stem2_star stem1*
        post_hairpin1 post_hairpin2 post_hairpin3 linker
Tr.seq = preG targ_stem targ_loop targ_stem* post_hairpin1* toehold*
Sw_Tr.seq = preG toehold stem1 stem2 stem3 loop stem3* stem2_star stem1*
        post_hairpin1 post_hairpin2 post_hairpin3 linker preG targ_stem
        targ_loop targ_stem* post_hairpin1* toehold*

# prevent sequence patterns
prevent = AAAA, CCCC, GGGG, UUUU, KKKKKKKK, MMMMMM, RRRRRR, SSSSSS,
        WWWWWW, YYYYYY
```

Fig. 5 Example of NUPACK code for 3WJ repressor

6. Digest backbone plasmid and assemble with double-stranded DNA blocks in one-pot reaction: mix 20 fmol backbone plasmid, 400 fmol each double stranded DNA blocks, 2 μL of T4 DNA ligase buffer, 2000 units of T4 DNA ligase, 10 units of type IIS restriction enzyme, and DW to a total of 20 μL. Incubate for 2 min at 42 °C and 5 min at 16 °C for 25 cycles. Incubate for 10 min at 60 °C and heat inactivate by incubating at 80 °C for 10 min.
7. Design forward and reverse primers (*see* **Note 8**).
8. Polymerase chain reaction: Combine 10 μL of Pfu master mix with 2 μL of forward primer, 2 μL of reverse primer, 1 μL of template plasmid, and 5 μL of DW to a total of 20 μL. Denature template plasmid for 5 min at 95 °C. Incubate for 1 min at 95 °C, for 1 min at T_m of nonoverlapping region – 5 °C, and for 1 min/kb at 72 °C for 12–15 cycles. Incubate for 1 min at T_m of overlapping region – 5 °C and for 2 min/kb at 72 °C.
9. Check the size of the PCR product by gel electrophoresis (*see* **Note 9**).
10. Digest template plasmid: Add 1 μL of DpnI and 2 μL of CutSmart Buffer to 17 μL of PCR product. Incubate 45–60 min at 37 °C.
11. Design forward and reverse primers to make insert DNA and backbone DNA (*see* **Note 10**).
12. Polymerase chain reaction: Combine 10 μL of Pfu master mix with 2 μL of forward primer, 2 μL of reverse primer, 1 μL of template plasmid, and 5 μL of DW to a total of 20 μL. Denature template plasmid for 2 min at 95 °C. Incubate for 20 sec at 95 °C, for 40 sec at T_m – 5 °C, and for 1 min/kb + 1 min at 72 °C for 30 cycles. Incubate for 2 min/kb – 1 min at 72 °C.
13. Check the size of the PCR product by gel electrophoresis (*see* **Note 11**).
14. Digest template plasmid: Add 1 μL of DpnI and 2 μL of CutSmart Buffer to 17 μL of PCR product. Incubate 45–60 min at 37 °C.
15. Gibson assembly: Combine 5 μL of Gibson assembly master mix with 1 μL of insert PCR product, 1 μL of backbone PCR product, and 3 μL of DW to a total of 10 μL. Incubate 45–60 min at 50 °C.
16. Transform 50 μL of chemically competent *E. coli* DH5a cells with the 5 μL of CRATE product, site-directed plasmid mutagenesis product, or Gibson assembly product using heat shock.
17. Incubate transformed bacteria with LB medium without antibiotics for 1–2 h at 37 °C in a shaker.
18. Plate on selective LB agar plates.

3.3 Making of Chemically Competent E. coli for Cloning and Experimental Transformant

1. Plate *E. coli* (DH5a, BL21 DE3) on LB agar plates followed by incubation at 37 °C overnight (*see* **Note 12**).
2. Select a single colony from the plate to inoculate 7 mL of LB medium. Incubate overnight at 37 °C with shaking.
3. Inoculate 5 mL of overnight cell culture to 500 mL of fresh LB in a 2 L narrow mouth Erlenmeyer flask. Incubate at 37 °C with shaking until OD600 of 0.35 (*see* **Note 13**).
4. Aliquot the cells ino 50 mL conical tubes (50 mL per tube) and chill on ice for 10 min (*see* **Note 14**).
5. Centrifuge the cells at 2012 × *g* for 10 min at 4 °C.
6. Resuspend each pellet in 6 mL of ice-chilled Inoue transformation buffer and chill on ice for 10 min.
7. Centrifuge the cells at 2012 × *g* for 10 min at 4 °C.
8. Resuspend all pellets in 20 mL of ice-chilled Inoue transformation buffer with 7% v/v DMSO and chill on ice for 10 min.
9. Aliquot the cells into 200 μL PCR tubes (50 μL per tube) and flash freeze with liquid nitrogen. Store at −70 °C freezer.

3.4 Experiments with RNA Translational Repressors

1. Thaw 50 μL of competent *E. coli* on ice, add 150 ng of each plasmid for expressing the switch RNA and trigger RNA to the cells, and incubate for 10 min on ice. To apply a heat shock, incubate the reaction tube for 30 s at 42 °C using a water bath. Immediately after the heat shock, chill the cells on ice for 2 min. Recover the cells with 1 mL of LB media without antibiotics for 1 h (in case of single transformant) to 2 h (in case of double transformant) in shaker. Plate on selective LB agar plates.
2. Select three single colony from the plate to inoculate 1 mL of LB media with antibiotics in 96-well deep plate with shaking at 800 rpm and 37 °C (*see* **Note 15**).
3. Dilute overnight cultures by 100-fold into fresh LB medium with antibiotics and return to the shaker (800 rpm, 37 °C). After 80 min, cells are induced using IPTG at a final concentration of 1 mM from a 100 mM stock solution in DW (in case of BL21 DE3 and BL21 Star DE3 strain) or L-arabinose at a final concentration of 0.2% (w/v) from a 20% stock solution in DW (in case of BL21 AI strain) for 3 h and 30 min or 6 h (in case of two-input logic gate test).
4. After IPTG or L-arabinose induction, transfer 200 μL of induced cultures to a 96-well black/clear plate. Measure the fluorescence level and OD600 in the plate reader. Calculate normalized fluorescence level as follows:

   In the case of GFP,

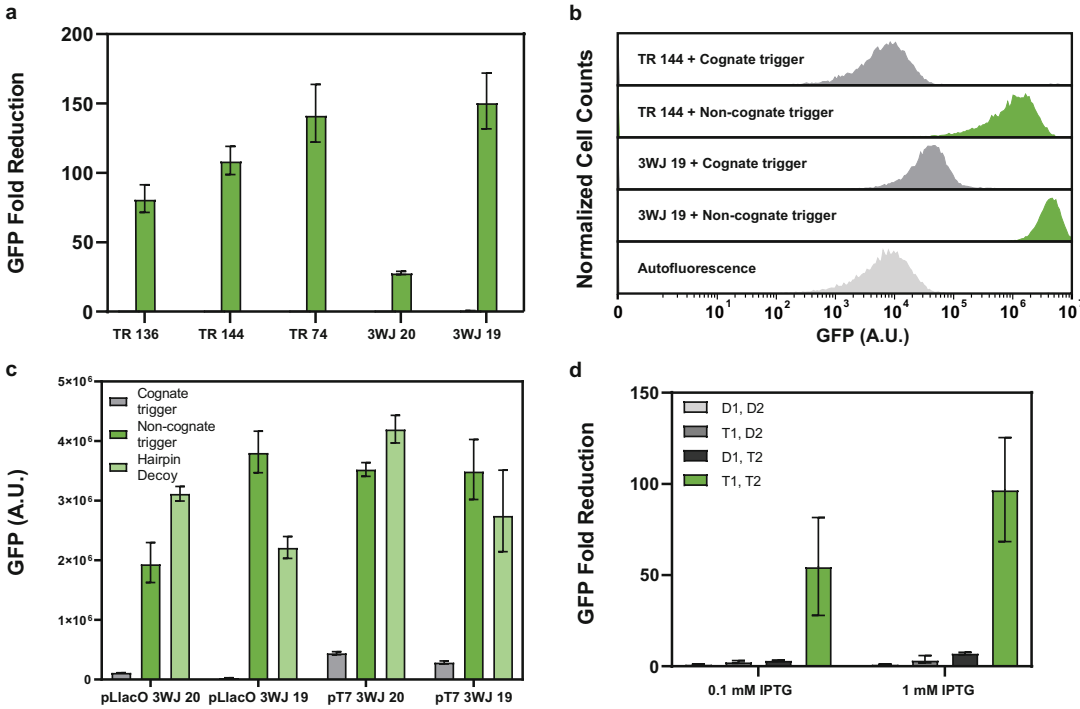

Fig. 6 GFP fluorescence measurements of toehold and 3WJ repressor. (**a**) GFP fold reduction of toehold repressors (TR 136, TR144, TR 74) and 3WJ repressors (3WJ 20, 3WJ 19) [12]. *E. coli* cells were induced with 0.1 mM IPTG. (**b**) Flow cytometry GFP fluorescence histograms for TR 144 and 3WJ 19 compared to *E. coli* autofluorescence. Autofluorescence level was measured in cells transformed with a control plasmid that does not contain the GFP sequence. (**c**) GFP fluorescence result of 3WJ repressors with the promoters pLlacO and pT7 (*see* **Note 17**). *E. coli* cells were induced with 0.1 mM IPTG. (**d**) GFP fold reduction of 3WJ NAND gate with two different IPTG concentrations: 0.1 mM and 1 mM (*see* **Note 18**). Error bars represent standard deviations of three biological replicates

$$\text{Normalized GFP} = \frac{GFP\ of\ test\ sample - GFP\ of\ LB\ with\ antibiotics}{OD600\ of\ test\ sample - OD600\ of\ LB\ with\ antibiotics}.$$

5. Flow cytometry can be used for more precise analysis of cell fluorescence (*see* **Note 16**). Figure 6 shows the results of the flow cytometry assay.

4 Notes

1. The first GGG sequence is a conserved sequence for proper transcription via the T7 promoter. The bold sequences are ribosome binding site and initiation codon.
2. The decoy is an RNA that minimally binds to the switch RNA. The experimental result using the decoy represents the output

of the noninteracting state (in the case of repressors, this is the ON state). The most suitable decoy sequence may be different for each switch, but in general, hairpin RNA can be used which forms a strong secondary structure by itself.

3. In these experimental conditions, the copy number differences in the plasmids expressing switch and trigger RNAs led to an approximately 6- to 8-fold excess of trigger compared to switch molecules as determined by fluorescence measurements of the GFP reporter expressed separately from each plasmid [6]. pCOLADuet (kanamycin resistance, ColA origin) for switches and pET15b (ampicillin resistance, ColE1 origin) and pCDFDuet (spectinomycin resistance, CDF origin) for triggers are used.
4. The BL21 Star DE3 strain with an IPTG inducible genomic T7 RNA polymerase and decreased RNase activity is used for repressor characterization.
5. The BL21-AI™ strain with an arabinose inducible genomic T7 RNA polymerase is used in experiments that require tight expression control.
6. Online NUPACK sequence design package does not recognize the wobble base pair, so be careful. Alternatively, you can use the python NUPACK library or Linux software, which offers many of the latest functions. The example of NUPACK scripts for the repressors in Figs. 4 and 5 can also be found at https://github.com/AlexGreenLab/DesignofRNA-basedTranslationalRepressors.
7. If you want more accurate results, include the first ~100 nt of the reporter gene after the switch RNA, and include the T7 terminator sequence after the trigger RNA. Short hairpin regions with stems 6–10 bp long can also be added to the 5′ end of the transcripts to increase their stability.
8. Each primer contains a nonoverlapping region at the 5′ end and overlapping region at the 3′ end. The melting temperature of nonoverlapping region is 5–10 °C higher than the melting temperature of overlapping region (we use parameter of SantaLucia et al. [17]). This difference minimizes forward and reverse primer dimerization and enables the primers to use the PCR products as the template. The mutation site (insertion, deletion, substitution) can be placed in any region.
9. If you get smeared or unwanted bands, increase annealing temperature, or use more dilute template DNA.
10. The length of Gibson region, which overlaps the insert and backbone DNA, is 15–30 bp. We usually use a T7 promoter and its upstream sequence [for the forward primer] and the linker sequence or T7 terminator sequence [for the reverse primer] to design primers.

11. If you get smeared or unwanted bands, increase the annealing temperature, or use more dilute template DNA. However, if the size of the unwanted backbone band is under 1500 bp, it can be ignored, and you can proceed to the next step. DNA bands under 1500 bp cannot form an intact plasmid that contains the antibiotic resistance gene and the origin of replication, so this product is simply removed at the antibiotic selection step.
12. If you need to make a triple transformant for NAND or NOR gate experiments, it helps to prepare a chemically competent *E. coli* that contains a gate RNA. Plate a single transformant on a selective agar plate, and all the next steps are carried out in selective LB medium.
13. If you need a small number of competent cells, inoculate 300 μL of overnight cell culture to 30 mL of fresh LB in 50 mL conical tube. And incubate at 37 °C with shaking with a slightly opened lid until reaching an OD600 of 0.35. You might get approximately 40 vials of competent cells (50 μL per vial).
14. Performing aliquot, chilling, and resuspension steps in a cold room is recommended for better competency.
15. If your gene is cytotoxic, adding 0.2% glucose is recommended to prevent recombination.
16. After **step** 3.4, 3, dilute cells with 1× PBS (adjusting OD600 to 0.1) to make a final volume of 100 μL. Detect cells with a flow cytometer using a forward scatter (FSC) trigger, recording at least 15,000 cell events for each measurement. Analyze acquired data using CytExpert software as follows: cell populations were gated according to their FSC and side scatter (SSC) distributions using auto polygon tool (gated cells should be at least 80% of the total populations). Calculate geometric mean of GFP fluorescence histograms to extract the average fluorescence from three biological replicates. If the flow cytometer is not available due to instrument availability issues, IPTG-induced cells can be fixed with paraformaldehyde and measured later. Collect IPTG-induced cells by centrifugation, resuspend the pellet in 1% paraformaldehyde solution, and wait for 30 min. When stored in a 4 °C refrigerator, fixed cells are guaranteed to be stable for up to 7 days.
17. The 3WJ repressor system can work well enough with promoters other than the T7 promoter (*see* Fig. 6c). Also, pLlacO 3WJ 19 and pT7 3WJ 19 with non-cognate trigger and hairpin decoy data show that the hairpin decoy does not necessarily have a minimum interaction with switches.

18. When three or more input RNAs are required, such as a NAND gate, it can be better to perform induction with a higher concentration of IPTG (*see* Fig. 6d). This is presumably due to the requirement of higher concentrations of RNA components for multicomponent complexes.

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