Chapter 19

Screening of Genetic Switches Based on the Twister Ribozyme Motif

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Abstract

The recent description of a new class of small endonucleolytic ribozymes termed twister opened new avenues into the development of artificial riboswitches, providing new tools for the development of artificial genetic circuits in bacteria. Here we present a method to develop new ligand-dependent riboswitches, employing the newly described catalytic motif as an expression platform in conjugation with naturally occurring or in vitro-selected aptameric domains. The twister motif is an outstandingly flexible tool for the development of highly active ribozyme-based riboswitches able to control gene expression in a ligand-dependent manner in *Escherichia coli*.

Key words Ribozyme, Riboswitch, Biosensor, Aptamer, Aptazyme

1 Introduction

The family of small endonucleolytic ribozymes is composed of RNA motifs that have a size in the range of 50–150 nucleotides and which are able of sequence-specific cleavage [1]. Until recently, only five classes of small endonucleolytic ribozymes were described: the hammerhead [2], the hairpin [3], the hepatitis delta virus (HDV) [4], the Varkud satellite (VS) [5], and the glmS ribozymes [6]. Lately, a new widespread class of small endonucleolytic motifs has been identified by Breaker and coworkers using a bioinformatics pipeline [7]. Breaker and coworkers coined the name "twister" because the secondary structure resembles the ancient hieroglyph "twisted flax." Twister ribozymes occur in both *Bacteria* (mostly in the class *Clostridia*) and *Eukarya* (fungi, plants, cnidarians, flatworms, insects, and fish). They are also found in a variety of environmental sequences (i.e., not lab-cultivated organisms). Although the physiological functions remain unknown, it was observed that the genetic contexts of twister ribozymes are often similar to those of the hammerhead ribozymes found in the same organism [7].

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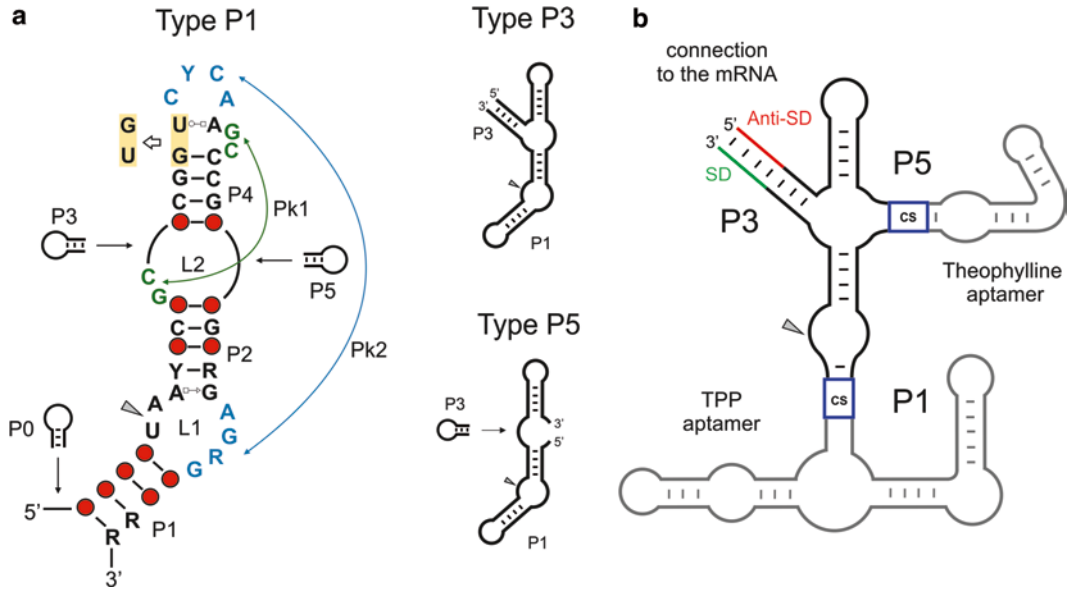

Fig. 1 The twister ribozyme as expression platform of artificial riboswitches. (**a**) Conserved consensus secondary structure of twister. The three twister types are represented with the possible structural variations. Conserved (75–97 %) nucleotides are shown. The positions in which the nucleotide identity is less conserved are represented by *red circles*. Pseudoknot 1 (Pk1) and pseudoknot 2 (Pk2) are represented in *green* and *blue*, respectively. The *arrowheads* identify the cleavage sites. The inactivating mutation is highlighted in *yellow*. (**b**) Example of an artificial two-input twister-based riboswitches. A type P3 twister was used as expression platform. The Shine-Dalgarno (SD) sequence of the reporter gene is inserted into stem P3 of the twister motif. Two different sensor domains are connected to the catalytic domain through suitable connection sequences (CS) in correspondence to positions P1 and P5

Twister ribozymes present a conserved consensus secondary structure which was first predicted by the group of Breaker and later confirmed and updated by three different groups trough crystallography (Fig. 1a) [8–10]. The conserved secondary structure includes three essential stems (P1, P2, P4) and eventually three optional stems (P0, P3, P5). Importantly the fold of the twister relies on the presence of two pseudoknots (Pk1, Pk2) whose topology results to be completely different from the one previously observed in the HDV and *glmS* ribozymes. According to the position of the termini, naturally occurring twister ribozymes are classified into three classes (Type P1, P3, P5).

Like the other small self-cleaving ribozymes, the twister motif also undergoes a sequence-specific internal transesterification reaction wherein the 2′-hydroxyl group of a ribose attacks the adjacent 3′-phosphate through an S_N2-like reaction mechanism. The cleavage products contain a 2′,3′-cyclic phosphate and a 5′-OH, respectively. The self-cleavage rate constant of a twister in a bimolecular construct under optimum conditions is estimated to be comparable to kinetics of the hammerhead ribozymes showing k_{obs} values

around 1000 per minute. In particular the twister bimolecular construct described by Roth et al. exhibited a maximum cleavage rate at 1 mM Mg^{2+} and pH 7.4 [7]. The enhanced rate of catalysis of twister is due to (1) the orientation of the reactive groups for the in-line attack, (2) the neutralization of the negative charge on the non-bridging oxygen atoms of the cleavage site phosphate (transition-state stabilization), and (3) the deprotonation of the 2′ oxygen nucleophile (general base catalysis). Until now no good hydrogen bond donor candidates involved in the neutralization of the developing negative charge on the 5′ oxygen leaving group (general acid catalysis) have been identified [9].

The discovery of this new catalytic motif opens new frontiers in the field of RNA biology. In addition, it offers new tools for the construction of artificial genetic circuits to the synthetic biologists. In fact, taking advantage of the modular and hierarchical organization of many regulatory RNAs, ribozymes can be used in association with aptamers (generating the so-called aptazymes), to create artificial riboswitches able to control gene expression in vivo. In such an approach the ribozyme works as the expression platform of the riboswitch, whereas the aptamer domain makes the catalytic activity of the ribozyme dependent on the concentration of small molecules and metabolites [11–13]. The use of ribozymes as expression platform in artificial RNA switches reveals to be highly versatile with respect to the ligand specificity, the type of regulated RNA, as well as the host organism [14–20].

Twister ribozymes are formidable flexible tools for the development of artificial riboswitches for a variety of reasons. As aforesaid, twister ribozymes show to have an activity, at least in vitro, comparable to the one of hammerhead ribozymes and they are 100- to 500-fold faster than other small self-cleaving ribozymes [7]. Moreover, due to the fact that the twister ribozyme is found in both *Eukarya* and *Bacteria* [7], one can assume that this motif can be virtually employed for applications in different organisms. Finally, the twister presents multiple potential sites (P1, P3, P5) which can be modified in order to connect the catalytic domain to the messenger RNA or to aptameric sensor domains (Fig. 1b). The stem P1 is always present with a non-conserved sequence of variable length [7]. Although the nucleotides of the helix P1 are not directly involved in the formation of the active site, the presence of this structure is essential for the stability of catalytic core [8, 9]. The stem P3 is an optional structure that is located at the connection between one strand of Pk1 and the stem P4, whereas the stem P5 is sometimes found in correspondence of a loop, which connects stem P4 to stem P2 (Fig. 1a) [8, 9]. Both structures are accommodated in loops whose nucleotides are not conserved and which are not involved in the formation of the active site. Although the stems P3 and P5 are in general variable in the sequence and in the length, the published crystal structures suggest that their presence could have a

critical role in the maintenance of the overall structure in some twister ribozymes [8]. The presence of multiple sites for the connection of aptameric sensor domains opens the way for developing riboswitches dependent on more than one ligand [21, 22].

Here we present a procedure for developing ligand-dependent twister ribozymes that are enable conditional control of gene expression in *E. coli*. This method is based on an aptazyme design strategy and an in vivo screening approach which have been developed in our group a few years ago to generate artificial hammerhead-based riboswitches [23]. In particular, one of the stems of the ribozyme moiety acts as a molecular scaffold for the sequestration of the ribosome-binding site (RBS) of an eGFP gene located on a suitable plasmid [23]. As an accessible Shine-Dalgarno (SD) sequence within the RBS is essential for efficient initiation of translation, the masking of the SD represent an easy and efficient way of controlling the expression of the mRNA of interest. Addition of ligands to the bacterial growth medium changes the activity of the ligand-dependent self-cleaving ribozyme, which in turn switches gene expression either on, if the RBS is released upon the cleavage, or off, if the cleavage is inhibited and the RBS is masked (Fig. 2). Ligand-dependent masking of the SD sequence is a common

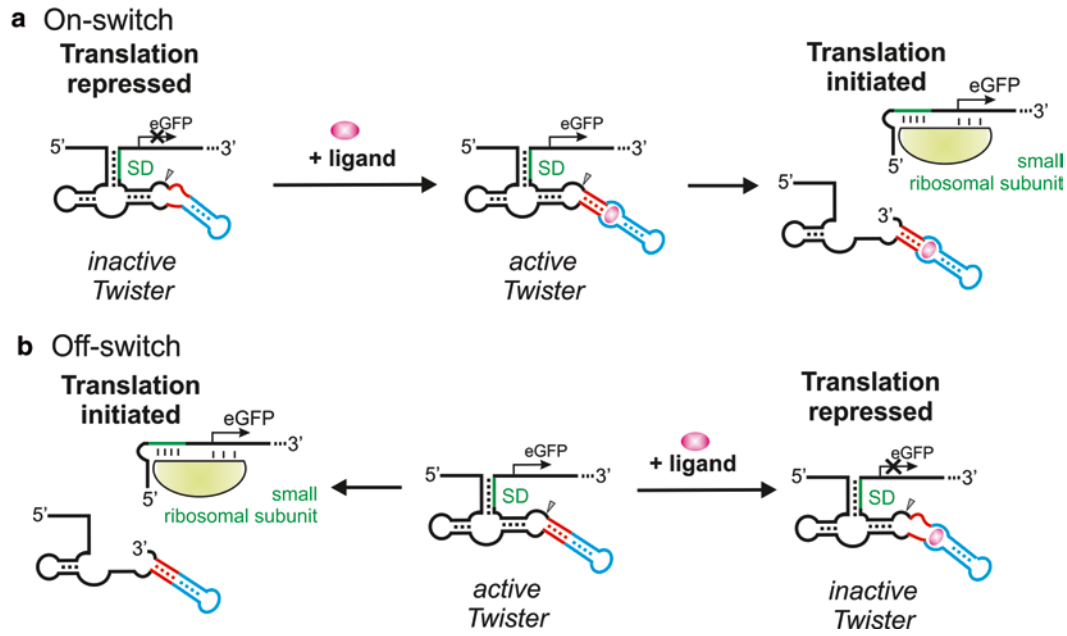

Fig. 2 Mechanisms of the twister-based on- and off-switches. In our approach the twister ribozyme acts as a molecular scaffold for the sequestration of the ribosome-binding site (RBS). Aptamer domains can be attached to the ribozyme as exchangeable ligand-sensing domains. Addition of ligands to the bacterial growth medium changes the activity of the ligand-dependent self-cleaving ribozyme which in turn switches gene expression on (**a**) or off (**b**). The use of eGFP as a reporter gene allowed a fast screening of clones by fluorescence

mechanism to control the expression of downstream genes in naturally occurring riboswitches. Using this strategy the twister can be used to switch on or off the expression of a reporter gene in vivo, combining them with natural occurring (e.g., TPP) or in vitro-selected aptameric domains (e.g., theophylline). The number of nucleotides in the connection sequence can be easily optimized in order to improve the switching activity.

2 Materials

All solutions are prepared using ultrapure water and reagents are of analytical grade quality. Reagents are stored at room temperature, if not otherwise noted.

2.1 PCR, Plasmid Purification, and Ligation

1. 0.5 M EDTA, pH 8.0: Dissolve 73.08 g EDTA and 30 g NaOH pellets in 400 mL H_2O, adjust pH 8.0 using NaOH, adjust volume to 500 mL.
2. 5× TBE buffer: Dissolve 54 g Trizma base, 27.5 g boric acid, 20 mL 0.5 M EDTA (pH 8.0) in 1 L of H_2O. Adjust to pH 8.3, if needed, with concentrated HCl. Dilute to 0.5× in H_2O before use.
3. 6× agarose gel loading buffer: Dissolve 4 mL 50 mM Tris–HCl, pH 7.6, 12 mL 100 % glycerol, 2.4 mL 0.5 M EDTA, 6 mg bromophenol blue, 6 mg xylene cyanol in 20 mL, prepare aliquots of 1 mL. Can be stored at −20 °C for several months.
4. Ready-to-use DNA size standard.
5. Agarose gels. For 0.8 % (w/v) dissolve 0.8 g agarose in 100 mL 0.5× TBE buffer by boiling in the microwave. Dissolved agarose can be stored in an incubator at ≥70 °C for several days. (*see* **Note 1**).
6. DNA oligonucleotides as primers to be used in PCR (*see* Subheading 3.1).
7. DpnI restriction enzyme and NEB buffer 4 (NEB).
8. Ethidium bromide gel staining solution. Dilute 200 μg in 400 mL H_2O. Can be kept in the dark at room temperature and reused (*see* **Note 2**).
9. Destaining solution: 400 mL H_2O. Can be kept at room temperature and reused.
10. PCR cycler.
11. PCR template: e.g., eGFP expression vector (pET16b_eGFP, AG Hartig, University of Konstanz).
12. Phusion Hot Start II High-Fidelity DNA polymerase, 5× HF buffer and 100 % DMSO (NEB). Store at −20 °C.

13. 2 mM dNTP Mix. Store at –20 °C.
14. Quick Ligase, 2× Quick Ligation Reaction Buffer (NEB). Store at –20 °C. Thaw Quick Ligation Reaction Buffer on ice.
15. DNA Gel Extraction Kit (e.g., Gel DNA Recovery Kit (Zymo Research)).
16. DNA Purification Kit (e.g., DNA Clean and Concentrator-5 (Zymo Research)).
17. Tabletop centrifuge.
18. Agarose gel electrophoresis chamber and power supply.
19. Razor blade.
20. UV light table.

2.2 Cell Culture and Screening

1. 96 deep-well plate and 96-well plate incubator (e.g., Heidolph Inkubator 1000 and Titramax 1000) (*see* **Note 3**).
2. Air-permeable adhesive seals for 96-well-plates.
3. 1000× carbenicillin stock solution (100 mg/mL): Dissolve 1 g carbenicillin in 10 mL 50 % (v/v) ethanol. Can be stored at –20 °C for several weeks.
4. Electro-competent *E. coli* (e.g., *E. coli* BL21 (DE3) gold, Stratagene) (*see* **Note 4**). Eighty microliter aliquots frozen at –80 °C.
5. Electroporator (e.g., Eppendorf Electroporator 2510) and electroporation cuvettes (e.g., Electroporation cuvettes, 0.1 cm (Bio-Rad)).
6. Fluorescence plate reader (e.g., TECAN M200).
7. LB-Carb-medium: For LB (Lennox) dissolve 10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 L H_2O, adjust to pH 7.0, if necessary, and autoclave. Supplement with 1 mg/L carbenicillin before use. Can be stored at 4 °C for up to 4 weeks.
8. LB-Carb-agar plates: Dissolve 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 10 g agar agar in 1 L H_2O and autoclave. Let cool until hand-warm, then supplement with 1 mg/L carbenicillin. Pour plates in 15 cm petri dishes. Plates can be stored at 4 °C for up to 6 weeks.
9. Kit for plasmid isolation (e.g., Zyppy Plasmid Miniprep Kit).
10. SOC medium: Dissolve 20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.2 g KCl in 900 mL H_2O. Adjust pH to 6.8–7.0 using NaOH. Adjust volume to 960 mL and autoclave. Add 10 mL 1 M $MgCl_2$, 10 mL 1 M $MgSO_4$ and 20 mL 1 M glucose from sterile filtered stocks. Store 1 mL aliquots at –20 °C for several months.
11. Toothpicks sterilized by autoclaving.
12. Flat, transparent 96-well plates.

3 Methods

3.1 Construction of Randomized Riboswitch Libraries

Whole plasmid PCR is used to subclone a randomized plasmid library encoding eGFP under control of twister aptazymes. A pair of primers is designed to insert the aptamer sequence and a randomized connection sequence bridging the aptamer to the ribozyme (Fig. 3). Unbiased random nucleotides are generated during solid-phase DNA synthesis using a 1:1:1:1 mixture of nucleoside phosphoramidites. One primer needs to be 5′-phosphorylated for ligation, which is a prerequisite for T4 DNA ligase activity. We recommend the purification of primers that exceed a total length of 45 nt by denaturing PAGE (as described in [24]: Preparation of Denaturing Polyacrylamide Gels; Purification of Synthetic Oligonucleotides by Polyacrylamide Gel Electrophoresis).

1. Primers are designed according to following rules (see theophylline twister aptazyme example below):

   Theophylline twister aptazyme sequence:

   - Type P3 twister ribozyme connected to a theophylline aptamer in position P5 (underlined). The anti-Shine-Dalgarno and Shine-Dalgarno sequences are represented in the lower case.

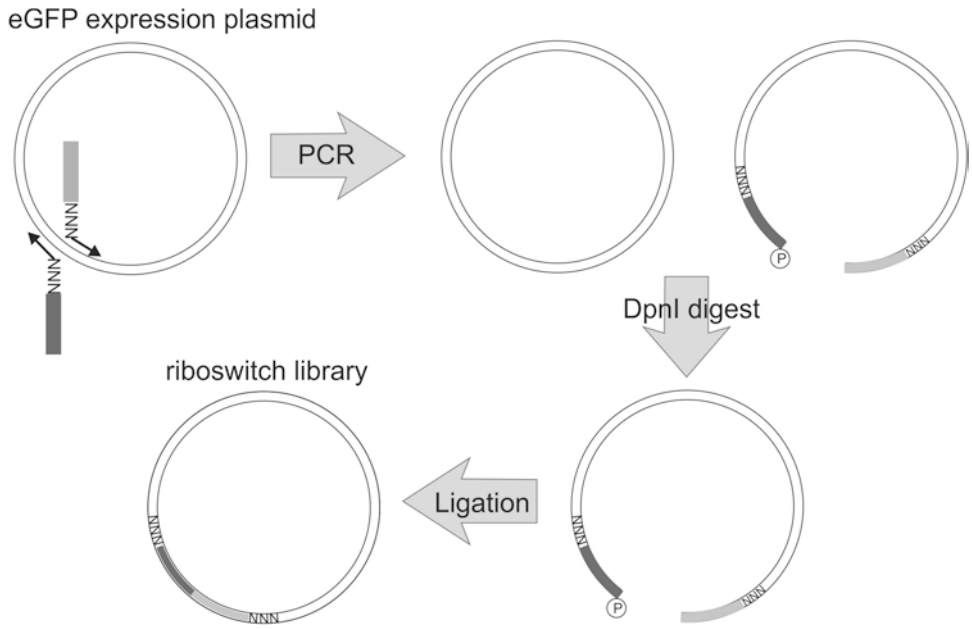

Fig. 3 Library construction: Whole-plasmid PCR is used for incorporation of new aptamer sequences using primers carrying randomized sequences that will constitute the connecting sequence. After PCR the template plasmid is degraded by DpnI digestion. The purified PCR product is ligated yielding a library of randomized riboswitches

Table 1
Protocol for setting up a PCR reaction

Starting concentration	Material	Volume	Final concentration
5×	HF buffer	10	1×
2 mM	dNTP mix	5	200 µM
100 µM	Forward primer	0.3	600 nM
100 µM	Reverse primer	0.3	600 nM
20 ng/µL	Template	0.5	0.2 ng/µL
100 % (v/v)	DMSO	1.5	3 % (v/v)
2 U/µL	Phusion Hot Start DNA polymerase	0.5	0.02 U/µL
	H_2O	31.9	
	Total	50	

- 5'cuccuuUAAAGCGGUUACAAGCCCGCAA **NN** CA UACCAGCCGAAAGGCCCUUGGCAGG **NN** AAU AGCAGAGUAAUGGGAAACCAUUAAUGCAGCU UUAaaggag 3'
  Forward primer:
- Introduce randomized nucleotides at the bridge between the aptamer (underlined) and the twister catalytic core
- Example: theophylline aptazyme
- 5' GGCCCTTGGCAGG **NN** AATAGCAGAGTAAT GGGAAACCATTAATGC3'
  Reverse primer:
- Introduce randomized nucleotides at the bridge between the aptamer (underlined) and the twister catalytic core
- Example: theophylline aptazyme
- 5' TTTCGGCTGGTATG **NN** TTGCGGGCTTGTAA CCGCT3'

2. Prepare the PCR reaction mixture as follows and split the mixture into three PCR tubes. The reaction can be scaled up, if necessary. We recommend using no more than 30 µL per tube (*see* Table 1).
3. Use the following conditions for the PCR with Phusion Hot Start II High-Fidelity DNA polymerase (*see* **Note 5**) (*see* Table 2).
4. Pool the PCR reaction and transfer into one 1.5 mL reaction tube.

Table 2
Description of thermal cycles during PCR

Step	Stage	Temperature (°C)	Time	Go to step	Repeat
1	Initial denaturation	98	30 s		
2	Denaturation	98	10 s		
3	Annealing	58	30 s		
4	Extension	72	20 s/kb	2	24
5	Final extension	72	7 min		
6	Cooling	4	Pause		

5. Purify PCR reaction mixture using DNA purification kit according to the manufacturer's recommendations. Elute DNA with 44 μL H_2O.
6. For removal of the template vector add 5 μL NEB buffer 4 and 1 μL DpnI. Mix thoroughly and incubate at 37 °C for 50 min. Heat inactive restriction endonuclease by 10-min incubation at 80 °C.
7. In the meantime, prepare a 0.8 % (w/v) TBE-agarose gel. A small pocket for the size standard and a large one for the PCR product is required. After DpnI digestion, add 10 μL of 6× agarose gel loading buffer and mix. Load the PCR sample and 2.5 μL DNA size standard. Run the gel at 10 V cm^{-1} for 75–90 min.
8. After the gel electrophoresis, stain the gel into ethidium bromide staining solution. After 10 min transfer the gel into destaining solution for additional 10 min. UV light may cause mutations to DNA. To avoid exposure of the DNA sample to UV light, use the razor blade to excise the marker and a small part of the band with your sample. Use the UV light table for visualization of the DNA bands.
9. The correctly sized band is marked best by excision. Align the two gel fragments and excise the corresponding band in the non-irradiated gel fragment. Use the DNA gel extraction kit according to the manufacturer's protocol for purification. Elute DNA with 20 μL H_2O.
10. Measure DNA concentration based on A_{260} value of the sample.
11. Use the Quick Ligation Kit for the ligation of the PCR product. Mix 50 ng of the DNA sample in 9 μL of water with 10 μL 2× Quick Ligation Reaction buffer and adjust with H_2O to 19 μL. Add 1 μL Quick Ligase, and mix by pipetting. Incubate at 25 °C for 15 min.

12. Purify the DNA sample by using a DNA purification kit according to the manufacturer's instructions. Elute ligated DNA using 7 μL H_2O. Removal of the ligation buffer results in higher transformation efficiency.

3.2 Screening for Functional Aptazymes

The randomized Twister aptazyme library is incorporated into *E. coli* by transformation of the randomized plasmid. For library-scale transformation plasmids are transformed by electroporation of *E. coli* BL21 (DE3) gold ensuring high transformation efficiency.

1. Thaw 80 μL aliquot of electro-competent *E. coli* BL21 (DE3) gold on ice for 15 min.
2. Meanwhile, place one electroporation cuvette and an 1.5 μL aliquot of ligated DNA sample within a 1.5 mL reaction tube on ice.
3. Preheat 1 mL aliquot of SOC medium and LB-carbenicillin-agar plates at 37 °C.
4. Add 80 μL competent cells into the reaction tube containing the plasmid library and mix carefully. Transfer the mixture into the pre-chilled electroporation cuvette and transform by electroporation. Subsequently resuspend the cells in 1 mL pre-warmed SOC medium. Transfer the cells to an unused 1.5 mL reaction tube. Spread bacteria cells on pre-warmed LB-agar plates and incubate plates overnight at 37 °C (*see* **Note 6**).

Single colonies are individually investigated for switch performance in a high-throughput format. Single colonies are grown in microtiter plates and functional riboswitches are identified based on changes of the eGFP expression levels in the presence and absence of the aptamer ligand.

5. First, a bacterial clonal library is constructed in 96 deep-well, termed "master plates." Therefore, prepare 96 deep-well plates with 400 μL LB medium supplemented with carbenicillin are made. Allocate defined wells of the 96 deep-well plate for control cultures (*see* **Note 7**). Use sterilized toothpicks to pick single colonies from the agar plates. Remove toothpicks and seal plate with air-permeable adhesive cover. Incubate plates at 37 °C overnight in the 96-well plate incubator vigorously shaking.
6. Each master plate serves as template, which is used for final screening procedure. The screening of the clonal library requires the growth of each clonal culture once in the absence and otherwise in the presence of the aptazyme ligand. Therefore, prepare two additional destination plates. Add the medium into the wells so that medium without ligand alternates every column with medium supplemented with ligand (as shown in Fig. 4).

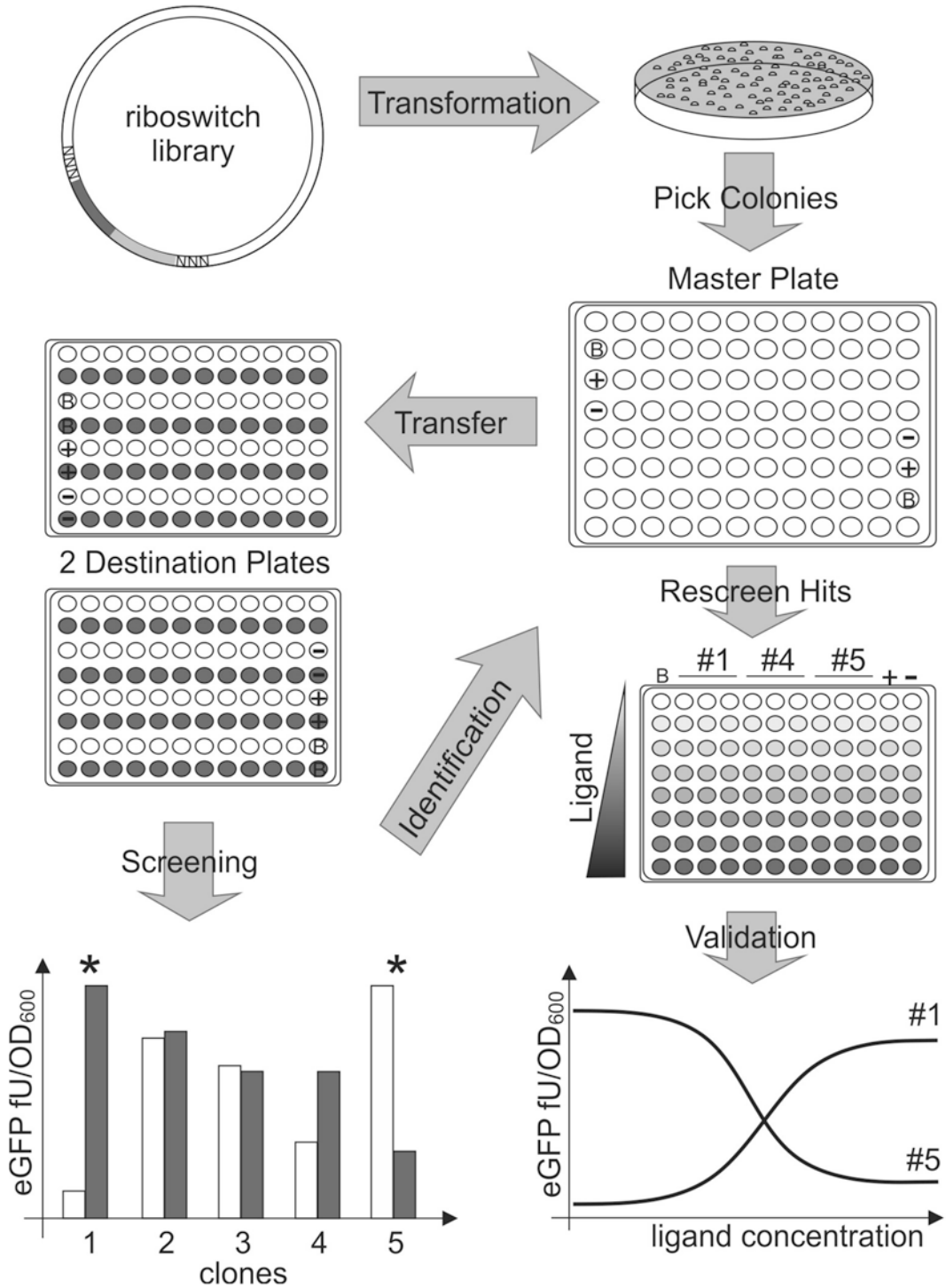

Fig. 4 Screening for functional riboswitches. The plasmid library of randomized riboswitches is transformed into *E. coli*. Individual clones are picked to construct clone libraries, which are screened for eGFP expression in the presence (*white*) or absence (*dark grey*) of the aptamer ligand. Controls for background fluorescence correction (B), positive control (+), and negative control (–) are included in the screening (*see* **Note 7**). Identified hits are further characterized

7. Use bacterial cultures of the master plates for the inoculation of cultures of the destination plates. Make sure that each clone is examined in the presence and absence of the ligand (*see* **Note 8**). Seal destination plates with air-permeable adhesive cover. Incubate the destination plates in the 96-well plate incubator at 37 °C overnight vigorously shaking.
8. Transfer 100 μL of the exponential phase culture into a 96-well microtiter plate and measure expression levels by determining eGFP fluorescence (excitation wavelength $\lambda_{ex} = 488$ nm, emission wavelength $\lambda_{em} = 535$ nm).
9. For analysis and the identification of potential genetic switches first subtract the background fluorescence from each fluorescence value. In addition measure OD_{600} for OD correction. Identify potential hits by comparing the fluorescence of each screened clone in the presence and absence of the ligand.
10. Clones of interest are inoculated in 5 mL LB medium and grown vigorously shaking at 37 °C for 8 h. Grown-out cultures are used for the preparation of glycerol stocks and the isolation of the plasmid encoding the aptazyme switch
11. Plasmids are isolated using a plasmid isolation kit according to the manufacturer's instructions. Sequence plasmids. Perform a sequence analysis to examine the integrity of the artificial riboswitch constructs and the identity of the randomized connecting sequence.
12. For further analysis investigate the riboswitches at increasing concentrations of the ligand. Prepare 96-well plates as detailed in **step 4** using a gradient of increasing ligand concentrations. Conduct experiments as triplicates. Plates are incubated shaking at 37 °C for greater 16 h before measuring eGFP expression.

Pool coverage is calculated by determining the oversampling factor (O_f) which is calculated as

$$O_f = T / V = -\ln(1 - P_i)$$

where T is the number of clones actually screened, V the maximum number of different clones of a randomized sequence (sequence space) and Pi the probability that a particular sequence occurs in the library [25]. A three-time oversampling of the theoretical sequence space results in a pool coverage of approximately 95 %.

4 Notes

1. Separation of 5–10 kb PCR products is best performed with 0.8 % (w/v) agarose gels. Higher percentages of agarose (e.g. 1.5 % (w/v)) result in better resolution for smaller products.

2. Ethidium bromide is toxic and mutagenic. Handle ethidium bromide according to the material safety data sheet (MSDS) and wear nitrile gloves while working with it.
3. For increased library sizes we recommend the use of 384 deep-well plates for cell culture and fluorescence measurements.
4. For the generation of electro-competent *E. coli* a detailed protocol can be found at www.eppendorf.com.
5. There are other polymerases as alternative to Phusion Hot Start polymerase that can be used. Adapt the PCR protocol and apply appropriate buffer and conditions as recommended by the manufacturer's protocol. We recommend the usage of a high fidelity polymerase. Primers should be designed to exhibit an annealing temperature of 60–63 °C. For improved PCR amplification we suggest to determine the optimal annealing temperature by conducting a gradient PCR.
6. The number of colonies depends on the transformation efficiency of the electro-competent cells used and the volume that is plated. Cross-contamination needs to be avoided during the screening process. To yield single colonies plate dilutions of the transformed cells.
7. Always include a positive control, a negative control, and a culture for subtraction of the background fluorescence (*E. coli* transformed with a plasmid not containing the eGFP gene). As positive controls use cells that are transformed with a plasmid expressing eGFP either under control of a catalytically active twister ribozyme or without ribozyme insert. As a negative control transform a plasmid that features a catalytically inactive Twister ribozyme variant (mutation highlighted in yellow in Fig. 1a), unable to free the ribosomal binding site. For determining the background fluorescence is transformed with a plasmid that is not encoding eGFP (e.g., pET16b). Note that all plasmids should transfer the same antibiotic resistance marker. As one master plate will be distributed to two destination plates, we recommend the inclusion of the controls on the top half and on bottom half of each master plate. Thereby, each destination plate will receive a set of controls. We also recommend not using the wells in the corners for screening purposes as growth of the cells will suffer from evaporation of the medium.
8. Minimal medium enables improved control over medium composition. Therefore, when screening for aptazymes that are triggered by natural ligands (e.g., TPP), we recommend the usage of minimal medium, (e.g., M9 or M63 medium). In addition, consider the membrane permeability of you ligand. Not all ligands are readily taken up into the cells; e.g., the screening for TPP riboswitches required the supplementation

with thiamine to the medium, which is processed by the intracellular thiamine kinase and thiamine phosphate kinase to yield TPP. In addition, other ligands might not be stable in the medium. Before applying new ligands or media we recommend measuring growth curves for the bacteria, to rule out any potential toxic effects of the ligand.

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