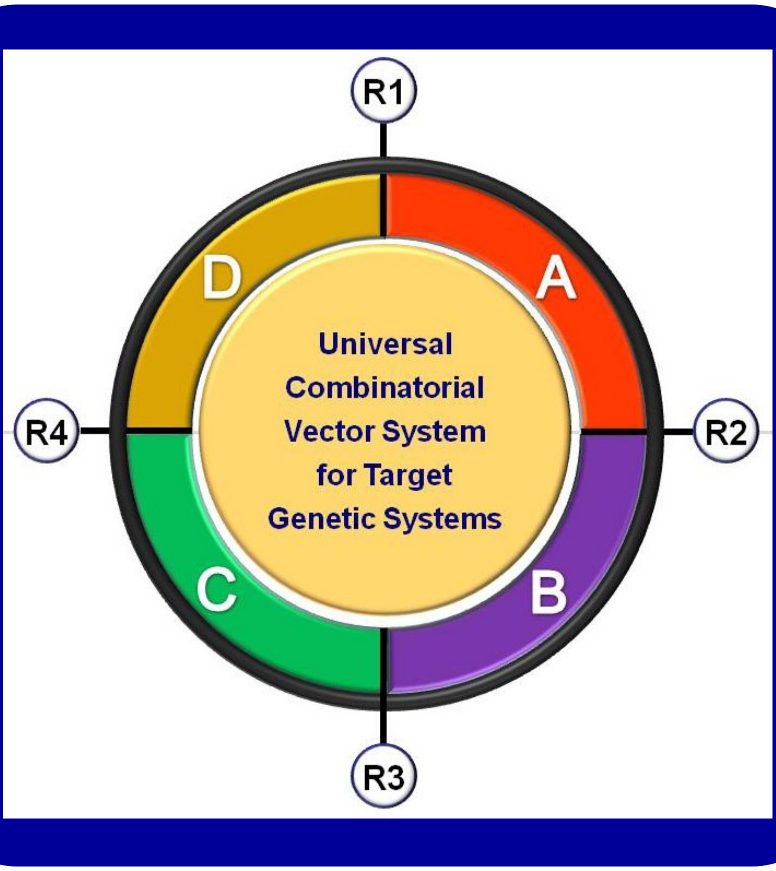
TOGGLE Assembly

A Universal Combinatorial Vector System for Target Genetic Systems



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TOGGLE BASIC *Assembly System*

Researchers can choose from a diversity of cloning and expression systems for building complex multi-gene assemblies. Proprietary designs often run contrary to efforts towards standardizing assembly strategies. Some plasmid lay-outs accomodate compatibility, but truly universal systems are hard to come by.

ATG:biosynthetics GmbH has developed a vector assembly system with standardized modules that adhere to a uniform design logic. This system allows easy modification, exchange, addition, removal and regeneration of components or higher-order gene assemblies. This system has been designed with the idea of an industrial standard in mind and has successfully been implemented for multi-gene assemblies in metabolic engineering of bacteria.

CONCEPT

TOGGLE Assembly Systems adhere to a **standardized unified constructive principle**.

Toggle plasmid vectors generally consist of four modules: applications (**A-Box**), selectors (**S-Box**), as well as target (**T-Box**) and maintenance (**M-Box**) functions (see fig. 1) which are described in detail on the next page.

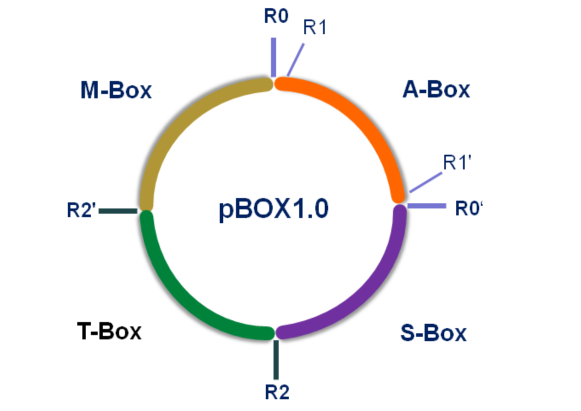


Figure 1: Schematic design of the Toggle Universal Assembly System consisting of four modules.

The system is designed as an **open standard** for the synthetic biology research community but also to researchers in other fields, academic as well as industrial. The standard encourages more intense exchange of parts and modules between researchers.

Modules, especially in the A- and T-Box, can be flexibly exchanged through defined and standardized restriction-ligation cloning. Elements within the individual modules can be added, removed, exchanged in the same way.

The restriction sites in the standard basic vector design are R0=***Abs*I**, R1=***Asc*I**, R1'=***MauB*I**, R0'=***SgrD*I**, R2=***AsiS*I**, R2'=***Pac*I**.

Toggle comes in different flavors: either as **ready-to-use system** (for *E. coli*, insect and mammalian cells) or it can be designed to incorporate **customer-specific** features, e.g. be adapted to your target organism of choice.

Customer genes should be optimized to not interfere with the constructive principles of the system. As the modules are separated by rare-cutting enzymes, most genetic design will be easily implementable.

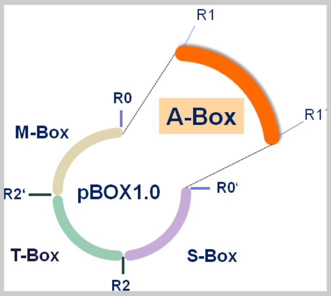
In its basic ready-to-use lay-out, Toggle:

- consists of a pair of **donor** and **acceptor** vectors, respectively
- donors and acceptors come with either of two antibiotic resistance markers and either of two standard promoters; additional antibiotic resistance markers are available on request
- will allow you to build multi-gene constructs using only two antibiotic markers.
- will allow you to add functional elements at a later stage

System Modules

The boxes below gives a schematic overview and brief description of the individual modules that make up the Toggle system.

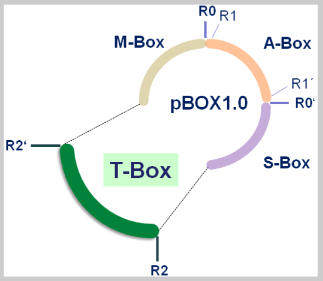
Application functions (A-Box)



Basically anything that makes up a functional gene (cassette)

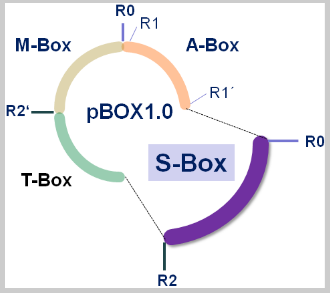
- Cistrons, expression cassettes, gene clusters and operons.
- Peptide or RNAi cassettes
- Reporter genes (fluorescent, luminescent, chromogenic, etc.)
- Multiple cloning sites, promoters (standard, inducible, constitutive,), terminators, control elements, Shine-Dalgarno motifs, etc.
- Recombination, targeting signals, etc. which can be hosted between R0 and R1 and R1' and R0', respectively.
- Or any combination thereof

Target functions for host (T-Box)



- Shuttle oris for target organisms (oriv, yeast oris, etc.)
- Target integration sites (Tn7 any others) if not part of gene cassette

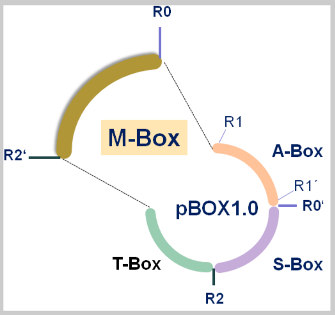
Selector functions (S-Box)



- Antibiotic resistance, auxotrophy and/or lethal factors, etc.
- Chromogenic markers (e.g. β-gal)
- Catalytic enzymes (SEAP, HRP, etc.)
- Toggle-compatible selection marker elements availlable for *E. coli* are:

  Ampicillin (ApR), Chloramphenicol (CmR), Kanamycin (KmR), Spectinomycin (SpR), Tetracycline (TcR)

Maintenance functions (M-Box)



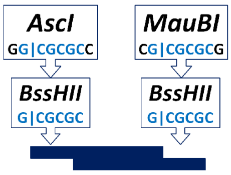
- Oris in the primary host used for building and amplifying gene assemblies, usually *E.coli*
- Single– and any low-, medium– or high-copy oris (F1, pBR322, ColE1, p15, etc.)
- Special-use oris, e.g. R6K γ

Assembly of Gene Cassettes

Design principles

TOGGLE offers a **standardized design for building gene cassettes** in the A-box module that allows introducing, swapping or removing components as required (fig. 2).

The gene cassette is bracketed by *Asc*I (**X** in fig. 2) and *MauB*I (**Y** in fig. 2) in the basic *Toggle* design. The feature of identical *BssHII* core sequences in the *AscI* and *MauBI* recognition motifs allows specific assembly but also simultaneous release of all assembled expression cassettes.



Constructive Elements

A set of control elements, e.g. *cis*-regulatory sequences and promoters, Shine-Dalgarno or Kozak sequences, can be plugged in, removed or exchanged as complete cassettes upstream of the start ATG using *MauB*I and *Nde*I *(CATATG)* restriction sites (purple bracket in fig. 2).

Individual elements can also be swapped around by making use of customer-designed restriction sites (R1, R2, R3) to separate regulatory elements from promoters etc. (red and green double arrows)

R4 can be used to optimize the environment around the start ATG (5'-UTR, 5'-CDS) including the Shine-Dalgarno / Kozak sequence (light blue bracket and arrow).

The R5 restriction site defines the end of the open reading frame. Together with restriction site X it can be used to exchange 5'-UTRs, termination signals, etc. (orange bracket in fig. 2)

It is defined as *BclI* (**TGA**TCA) for eukaryotic and bacterial systems, but for bacterial systems the more common STOP codon TAA may be provided as part of the coding sequence of a synthetic gene and TGA can then serve as an additional STOP codon.

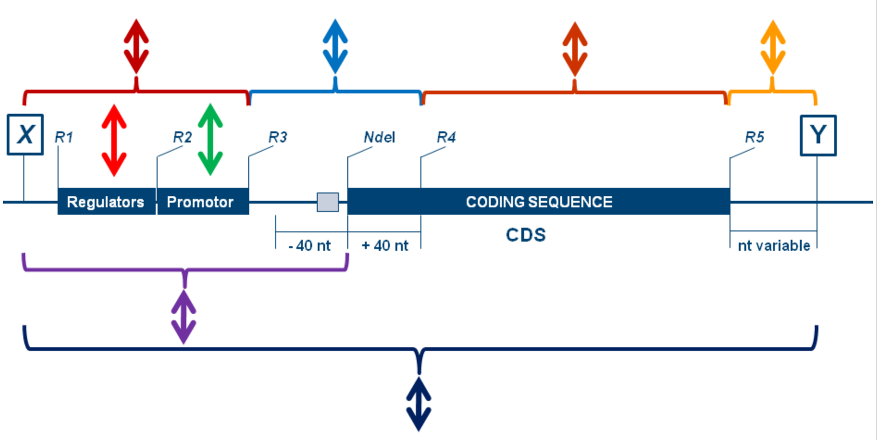


Figure 2: Constructive elements in the Toggle gene cassettes. Restriction sites X (*AscI*) and Y (*MauBI*) can be used to exchange or transfer entire gene cassettes. R1, R2 and R3 can be used to exchange or remove individual components (regulatory elements, promoters, etc.). R4 can be used to introduce sequences optimized for translation initiation. R5 and X allow you to swap terminators, 5'-UTRS, etc.

Donor-Acceptor Toggle Assembly

A smarter approach which exploits the full potential of the system is to toggle the new cassette / element into the recipient as schematically shown in figures 3 through 5.

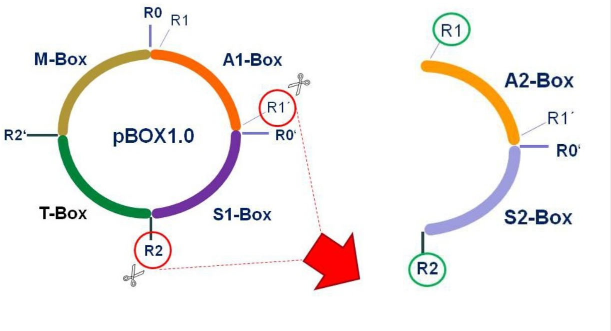


Figure 3: Restricting the recipient vector to allow integration of an A2-S2 box insert.

First, restrict the recipient R1'+R2 while excising the A2 box to be introduced with R1+R2. Keep in mind that R1 and R1' generate fully compatible overhangs.

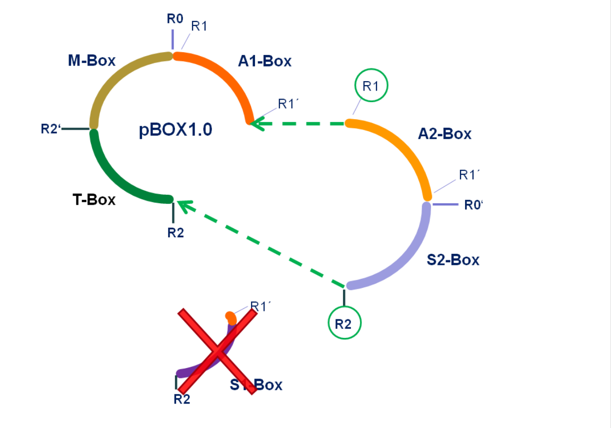


Figure 4: Successful integration events will be selected for by virtue of the introduced selector.

You can mix both reactions and ligate without having to isolate or dephosphorylate any DNA fragments. This is possible as regeneration of the original state as well as re-ligation events are selected against by virtue of the newly introduced S2 selector.

Simultaneously, you select for the newly introduced S2 box and thus the assembly product (fig. 5). By repeating this process cyclically, you can add more and more A boxes by toggling between the two selectors (usually your antibiotic resistance markers).

Joining the two A boxes creates a hybrid restriction site at the junction that can neither be cleaved with R1 nor R1' (circled in figure 5).

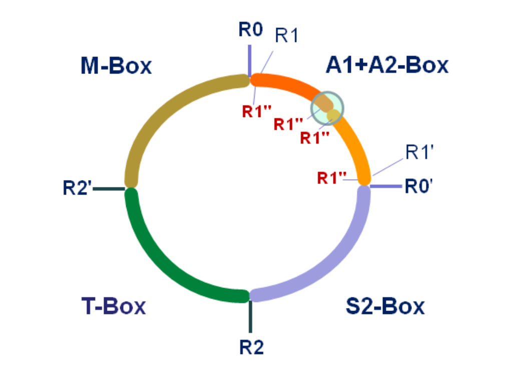


Figure 5: Assembly product after one toggle cycle. Note the new selector S2.

Nonetheless, the individual boxes can be regenerated by cleaving with an enzyme R1'' (*BssHI*I). This design feature can come in handy if you wish to re-use the optimized cassettes for other assembly strategies or multi-gene constructs.

TOGGLE ASSEMBLY: MULTI-GENE CONSTRUCTS

This description focuses on the A– and S-Box. To release gene cassette A2 plus its selector (red solid arrow in the donor cassette of fig. 6), the plasmid is restricted with R1 and R2 (*Asc*I-*AsiS*I).

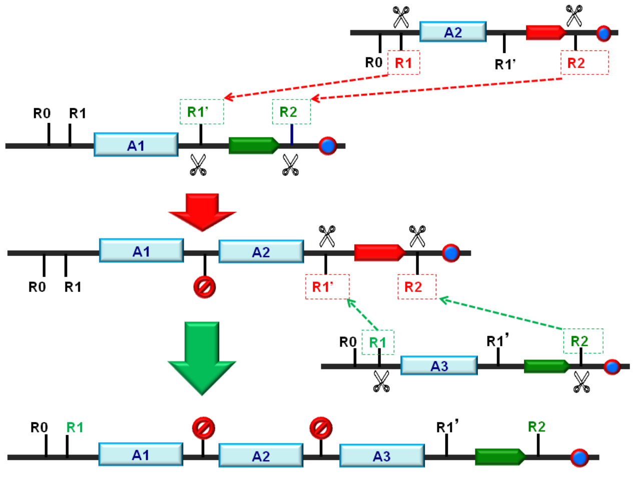


Figure 6: Principle of toggle assembly. A gene cassette A2 carrying one type of selector (red) is added to an existing construct that hosts another selector (green). In the process of adding the new gene cassette, the original selector is replaced and the gene cassette added. Note that the hybrid restriction site (red circle) cannot be cleaved by either R1, R1' or R2 so that elements are added in a specified order.

If the donor cassette comes from a plasmid with a standard ori, you will have to cleave the vector backbone at the ApaLI site and dephosporylate in order to functionally destroy the ColEI ori. This prevents re-circularization and functional reconstitution at later stages. If the insert comes from a plasmid with a conditional ori, (e.g. pir+ dependent) this step can be skipped (see next page) as the recircularized product is selected against.

The accepting vector will be digested with R1' and R2 (MauBI-AsiSI) which generates ends that are compatible with those created by R1 and R2 (AscI-AsiSI) of the fragment donor and also defines an orientation for the inserted fragment.

In the next step, you can simply mix the fragments, ligate, transform and identify the correct fusion events by selecting for the introduced selector (green in fig. 6 and 7). There is no need to purify your inserts although you are free to do so.

Correct products will be selected due to:

- correct integration of insert and subsequent circularization
- as an effect of the above process, the new selector becomes active in the circular plasmid and allows you to eliminate any clones carrying constructs with the old selector.

As you are using only two selectors, you move back and forth (toggle) between the selectors while adding genes or gene cassettes (fig. 7). The serial addition also defines the order in which you want to assemble your multi-gene construct (fig. 7). At the same time you avoid carrying around excess genetic material that is introduced through multiple selectors, thus leaving more space for functional cargo.

The process can be repeated as often as you wish and the process will become limited only by the size of the plasmids one has to handle.

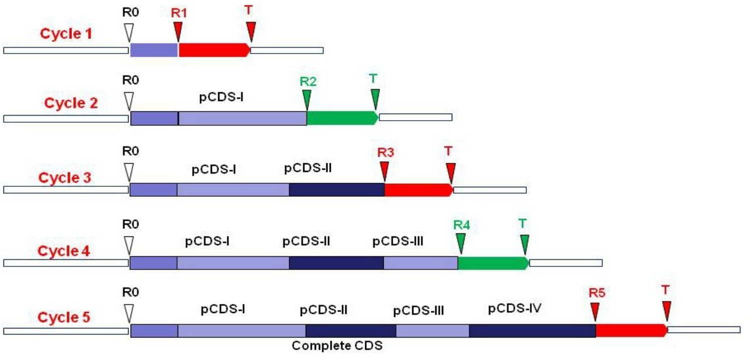


Figure 7: 5 cycles of toggle assembly showing alternating use of selector and serial addition of gene cassettes.

Toggle Assembly: Ditch & Switch

You can completely disassemble *Toggle* multi-gene constructs into individual gene cassettes using *BssH*II, a restriction motif contained in the *MauB*I and *Asc*I sites. This will allow you to re-cycle them for other projects, e.g. to clone them into other plasmid backbones.

By thoroughly predefining suitable specific restriction sites in the design of the gene clusters—preferrably as compatible or identical pairs -you can also remove or replace individual gene cassettes (see fig. 11), e.g. to test a modified gene cassettes.

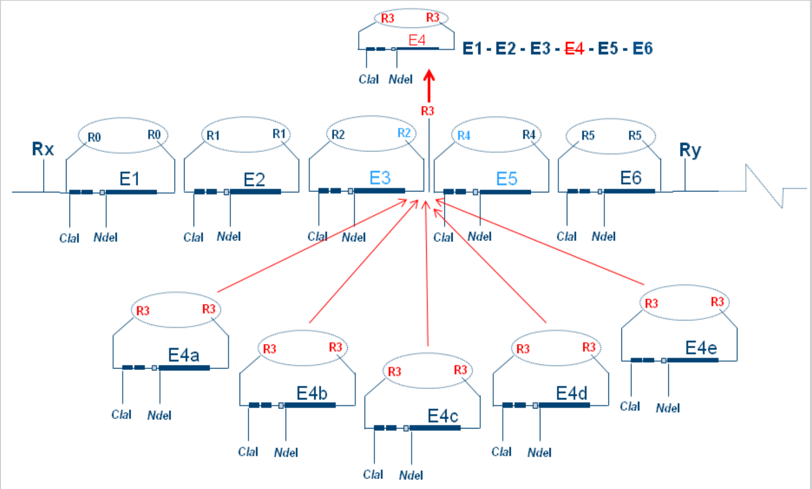
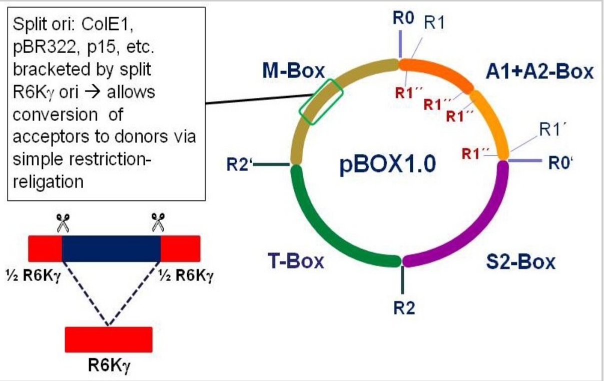


Figure 8: Removing and adding gene cassettes from multi-cassette assemblies

Selector Oris

While you can perform toggle selection at the S-box level alone, Toggle also offers an additional design feature that allows to (a) readily convert acceptor vectors into donor vectors, (b) an additional level of selection.



Split ori: ColE1, pBR322, p15, etc. bracketed by split R6Kγ ori → allows conversion of acceptors to donors via simple restriction-religation
½ R6Kγ
½ R6Kγ
R6Kγ
R0
R1
M-Box
A1+A2-Box
R1´´
R1´´
R1´´
R1´
R1´´
R0´
R2´
pBOX1.0
T-Box
S2-Box
R2

Figure 9: Split-ori concept and principle of converting acceptors (standard ori) into donors (R6Kγ) ori.

In the standard system, plasmid vectors are defined by their bacterial ori: acceptors carry a standard low-/medium-/high-copy ori such as ColE1, pBR322 etc., whereas donors are equipped with the R6Kγ ori that only works in strains that produce the π factor from a *pir*-gene (low-copy or high-copy), e.g. BW23474 or the like.

While toggling, you can perform an extra step that will introduce a conditional ori for which you can then select in *pir*-carrying strains. This step is completely optional but provides an additional level of selection.

You destroy the central standard ori with *ApaLI* and dephosphorylate, then digest with *Sap*I and religate, transform in pir$^+$ *E. coli* strains and plate. This procedure eliminates ColEI oris and reconstitutes the split R6Kγ donor constructs into functional conditional pir$^+$-dependent donor vectors which can be used to propagate the donor fragments before the assembly procedure is performed.

Key Points & Advantages

Meets criteria of a standardized (industrial) vector system

Backbone and inserted elements are fully compatible with one another – due to the design principle and the application of synthetic bioinformatics to optimize hosted synthetic DNA constructs.

Key Points

- Compact, highly flexible
- Highest degree of compatibility for "Synthetic Biology" application designs
- Add-on modules available
- Easily build gene cassettes and gene assemblies

Advantages

- Uniform design principles for generating conceptionally compatible genetic elements
- **Open standard**: use it for all your projects and enjoy the freedom of design
- Easily exchange elements with anybody else using the system
- Can accomodate customer's design wishes
- Can be adapted to any genetic system

Effective selection: no isolation of fragments — no empty vectors!

Additional Services

- **Gene design / optimization / synthesis** to get the best out of your system and your synthetic genes

Bottom-up approach for building systematic constructional DNA-designs

Computational modification and elimination of Toggle restriction sites from your gene design and selection/ deselection of additional restriction sites from your gene cassettes.

We are your experts for DNA sequence design.

- **BioDesigner** repository and platform for offering, requesting and exchanging elements, products, etc.— the one-stop market place for everyone (will open in September/October)

What are you waiting for?

Get started today and see for yourself!

***Toggle*—towards a new standard in constructive synthetic biology**