TNT-cloning: a new platform for flexible and universal usage of DNA parts

Henrique C De Paoli[1,2], Gerald A Tuskan[1] and Xiaohan Yang[1]

[1]Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA – Contact: hec@ornl.gov

[2]Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996, USA

Principle

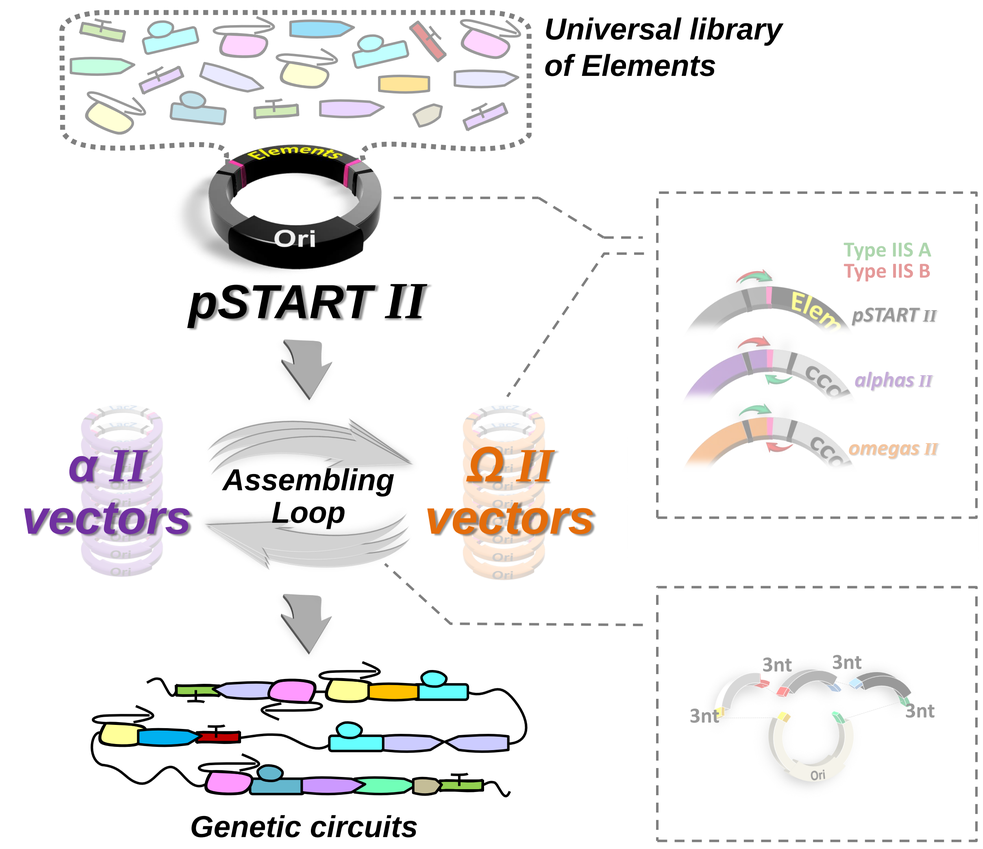

Figure 1 - TNT-cloning overview.
Our system combines all DNA fragments (elements) in one universal library, built in the entry vector pSTART-II (carrying the ccdB reporter). Two different families of vectors, also carrying the ccdB reporter, *alpha-II (α*; purple) and *omega-II* (*Ω*; orange), comprised of 8 vectors each (1A, 2, B, C, 1A-R, 2-R, B-R and C-R, where "R" is the version for anti-sense insertion) can receive any element from the library at any time (arrows). Virtually any regulatory regions (upstream regulatory region, URR; untranslated regions, UTRs; ribozymes; secondary regulatory sequences), CDSs (proteins; localization signals; affinity tags; functional domains), structural sequences (replication origins; repetitive DNA) or engineering scaffolds (interfering RNA, RNAi; artificial microRNA, amiR; guided RNA; recombination sites) can be introduced in the pSTART-II vector. Multi-gene assembly of library elements is performed by a simple mix-and-match step using the *type IIS* enzymes EarI and LguI (top right panel) associated with specific 3 nucleotides signatures that define order and orientation of parts (bottom right panel). Mutation of fragments of interest bearing CTCTTC sequence (domestication) is not necessary for the TNT-system. Up to three fragments can be joined at once and further linked exponentially through a cloning loop (e.g., 27 fragments become one after 4 cloning steps, which can be performed in as little as 5 days). For more details see *De Paoli et al, Sci Rep. 2016 (doi:10.1038/srep19278)*.

Table 1 – Highlights between previous restriction enzyme-dependent methods and the TNT-cloning system.

Characteristics	*Biobricks*	*MoClo*	*GB*	*TNT-cloning*
Require sequence overlap	***no***	no	no	no
Cloning rely on fragment restriction sites	yes	***no***	no	no
Take advantage of type IIS enzymes	no	***yes***	yes	yes
Support endless assembly loop	no	no	***yes***	***yes***[a]
Fragments go from library to any assembling plasmids (α/Ω)	n/a	n/a	***yes***	yes
Signatures allow for both sense or anti-sense orientation of fragments	no	no[b]	***yes***	yes
Assembled fragments can be used directly or employed in new assemblies	***yes***	no	yes	yes
Cloning process leave scars (linkers/adaptors)	yes	yes	yes	***no***
Require domestication for enzymes	yes	yes	yes	***no***
Restrict type of fragments in library	yes	yes	yes	***no***
Require different signatures to clone library fragments	n/a	yes	yes	***no***
Require multiple PCRs for library construction	yes	yes	yes	***no***
Require multiple libraries to carry different fragment types	n/a	yes	yes	***no***
Provide a sharable platform for universal exchange of DNA segments	no	no	no	***yes***
Optimized buffer allow for quick and reliable one-pot digestion/ligation step	no	no[c]	no[c]	***yes***

Limitations firstly surpassed by each method are bold and italic.
[a] TNT-cloning loops up to three fragments at a time; [b] A sub-library is necessary for inverting fragments; [c] One-pot reactions are suggested but takes 6 hours and are unsuited for sub-cloning efficient competent cells (10^7cfu/µg of puc19).
Biobricks, ***MoClo*** and ***GB*** (GoldenBraid) were described previously by Knight, T. (2003), Weber, E. et al. (2011) and Sarrion-Perdigones, A. et al. (2011, 2013), respectively.

Platforms Comparison

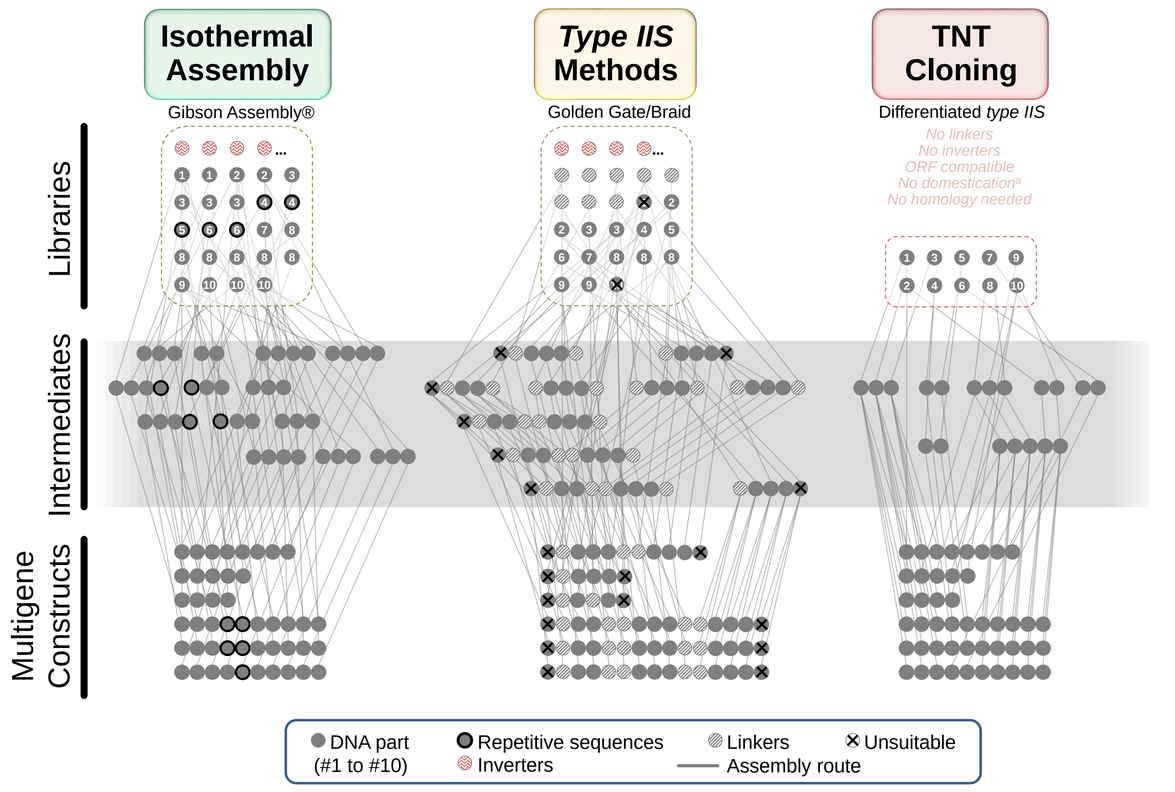

Figure 2 - Schematic representation for building constructs using three different methods.
Isothermal assembly requires sequence homology, making repetitive sequences a hurdle and available type IIS methods require linkers/adaptors and mutation of fragments, making them ORF-incompatible and sometimes unsuitable for multigene assembly (e.g., regulatory regions; crossed elements). Both methods compromise flexibility for reuse of DNA parts and thus require multiplication of individual elements within the library (sub-libraries), use of inverters for sense/anti-sense definition and generate higher number of intermediate constructs (middle panel). The TNT-cloning system bypasses such limitations providing an all-inclusive cloning platform tethering flexibility, fidelity, efficiency and universality. [a]Domestication is not a pre-requisite. Multigene constructs shown carry 4, 5, 8 and 10 DNA parts (elements) and represent the proof-of-concept reported by (*De Paoli et al, Sci Rep. 2016*). Note that inverters will require extra assembly steps and generate other intermediate constructs if isothermal assembly or available type IIS methods are adopted (not shown).

Multiple biological systems

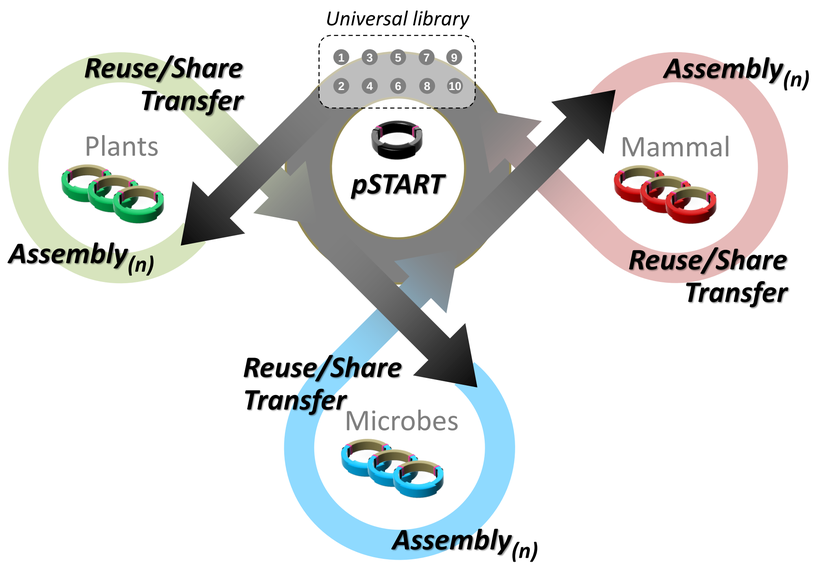

Figure 3 – TNT-cloning is available for multiple biological systems.
Several levels of assembly (n) can be performed for synthetic biology applications in microbes (*E. coli* and yeast), plants and mammalian cells. pSTART can be also used as a destination vector to make new assemblies a new element in the universal library (loop arrows). Library is fully sharable between labs and requires no adjustments (as long as codon usage is not a barrier).

Commercial applications

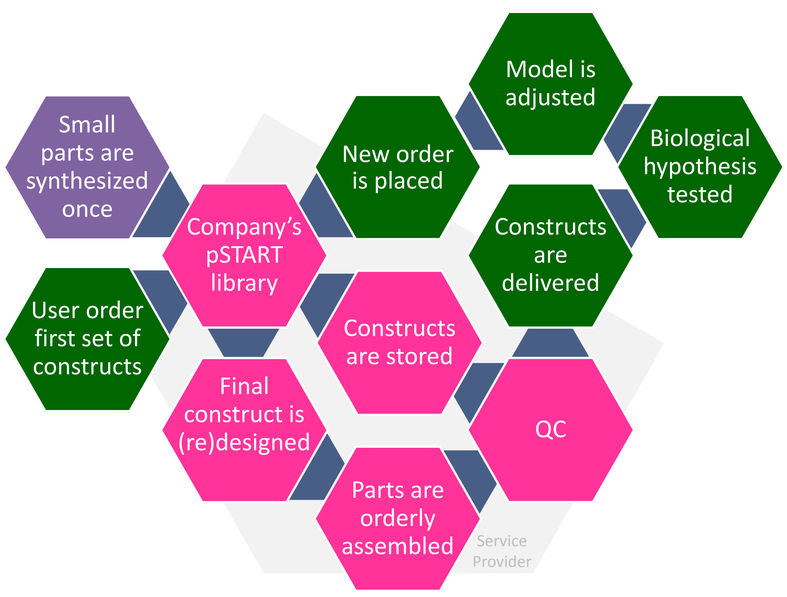

Figure 4 – TNT-cloning as a platform for gene synthesis.
Users submit digital sequences of desired constructs, company order/PCR oligos/small parts once, build library, applies combinatorial assembly for orderly joining multigene constructs, delivers quality material that is also stored, and provide potential refactoring of parts upon user's request. Subscriptions may be offered.

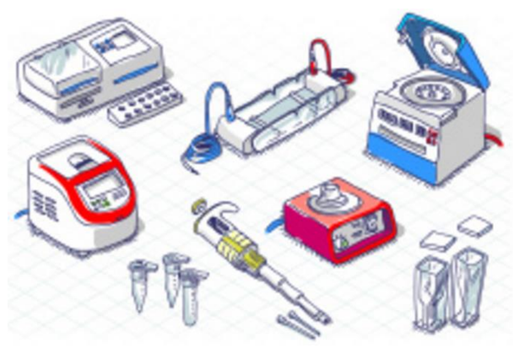

Figure 5 – TNT-cloning as a kit for synthetic biology.
Molecular biology companies may offer the TNT-cloning system as a "do yourself" kit. We have developed Plant, Microbes and Mammalian kits. Materials include pSTART and organism-specific set of assembling vectors (total of 16), 5X Activation Buffer, 5X one-pot high efficiency TNT-Buffer, Enzymes and TFO1-TFO2 Buffers (for cloning of non-domesticated parts). Kit size is expected to be small, medium and large, for 50, 250 and 1000 reactions, respectively.

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