Multiplex Modular Assembly and Protein Engineering

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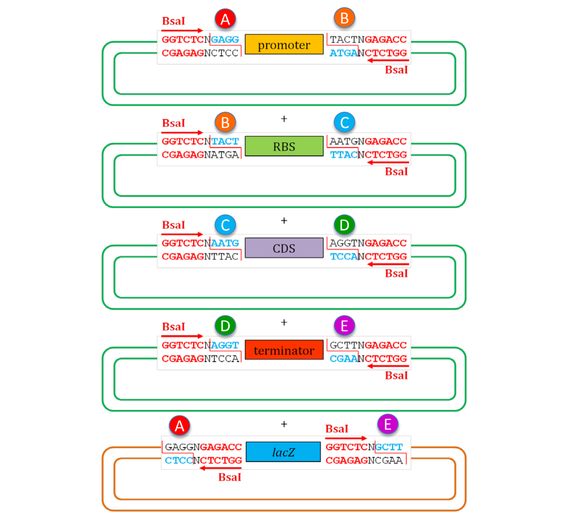
Introduction

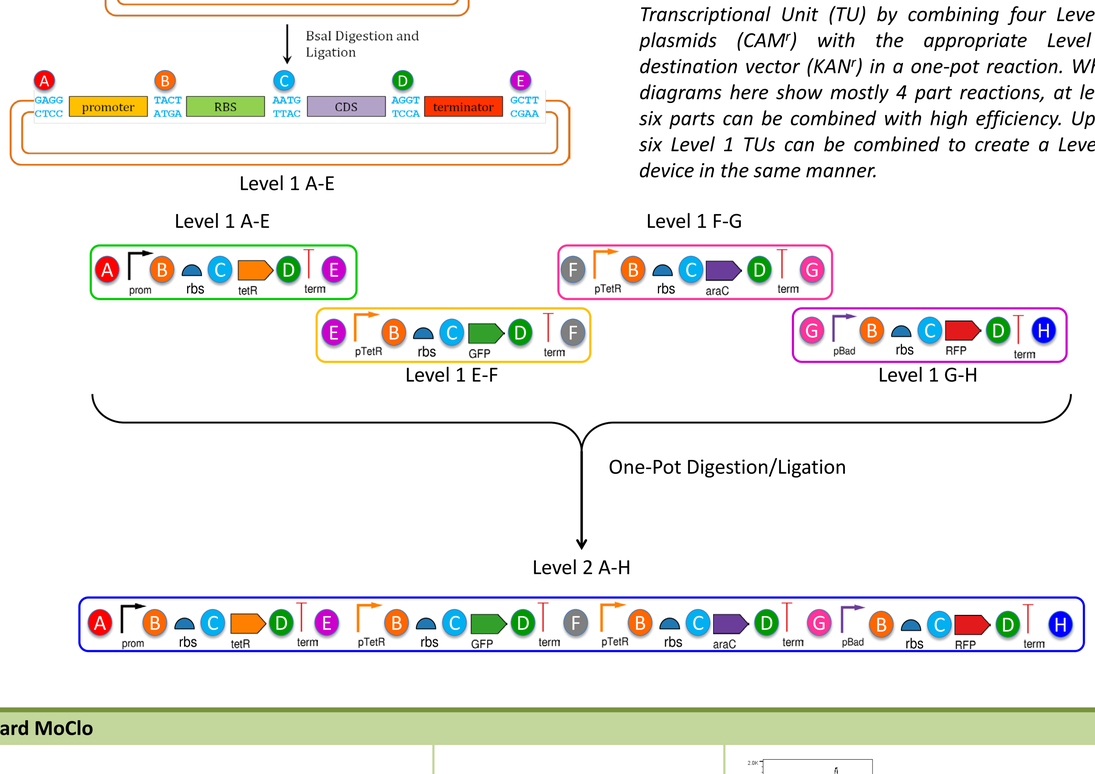
Modular Cloning and Multiplex Assembly

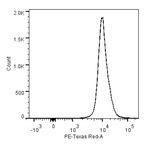
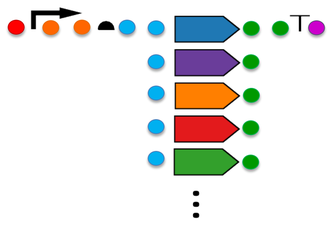
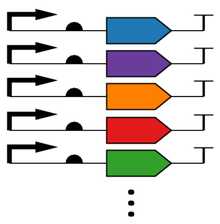
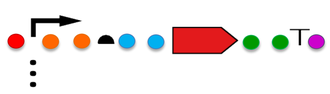
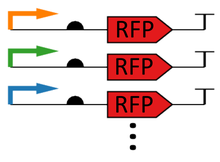
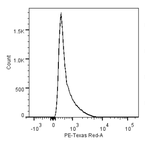
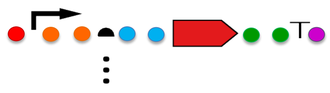
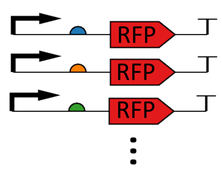
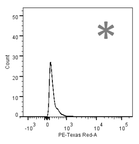
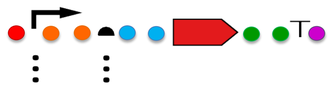
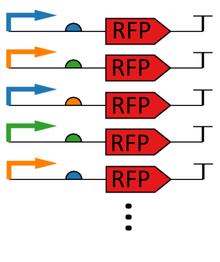
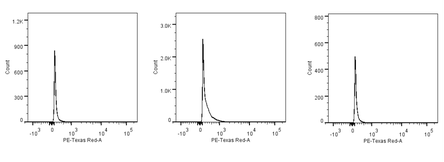
Modular Cloning, or MoClo, is an assembly technique which utilizes restriction Type IIS enzymes to assemble multiple Parts in a one-pot digestion-ligation reaction greatly decreasing the time and cost of constructing large devices (Weber, Engler et al. 2011). Type IIS enzymes cleave DNA at a specific distance from a non-palindromic recognition sequence. MoClo, Golden Gate (Engler, Gruetzner et al. 2009, Werner, Engler et al. 2012), and related assembly methods take advantage of these enzymes to allow for multipart assembly by designating specific 4-bp overhangs at the cut site for these enzymes.



We are developing a suite of multiplex modular assembly methodologies which rapidly increase the efficiency of characterization efforts and construction of complex Devices. Multiplex MoClo provides the ability to construct multiple Devices by adding a library of any Part type or transcriptional unit with no significant decrease in efficiency. By creating a library of level 1 parts, it is possible to tune each transcriptional unit by selecting the variant clone which performs as desired via FACS or other analysis.

MoClo assembly format: *Construction of a Level 1 Transcriptional Unit (TU) by combining four Level 0 plasmids (CAM^r) with the appropriate Level 1 destination vector (KAN^r) in a one-pot reaction. While diagrams here show mostly 4 part reactions, at least six parts can be combined with high efficiency. Up to six Level 1 TUs can be combined to create a Level 2 device in the same manner.*



Standard MoClo		
		 • Standard 4-part TU • Reaction efficiency = 95%
Fusion Protein Assembly with MoClo		
		• 6 part + vector assembly • Reaction efficiency = 93% (% white colonies)
Multiplex MoClo (can be any part type)		
		• Incorporation of each multiplexed part in approximately equal ratios • Parts added as 1/nth molar ratio • Reaction efficiency > 98%
Multiplex MoClo Expression Tuning (promoter)		**Initial Screening: FACS data from mixed population**
		 • Various strength promoters • Single strong 5'UTR • Range of fluorescence • Reaction efficiency = 91% (% white colonies)
Multiplex MoClo Expression Tuning (5'UTR)		**Initial Screening: FACS data from mixed population**
		 • Single strong promoter • Various strength 5'UTR • Range of fluorescence • Reaction efficiency = 90% (% white colonies) • *Fewer transformed cells
Multiplex MoClo Expression Tuning (promoter:5'UTR)		**Initial Screening: FACS data from mixed population**
		 • Various strength promoter and 5'UTRs • Expected trend towards lower expression • Reaction efficiency = 90%

Methods and Results

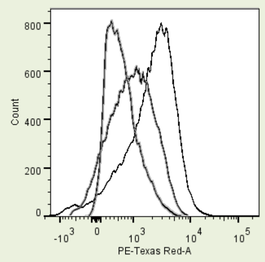
Level 1 (Single TU) Expression Tuning and Selection

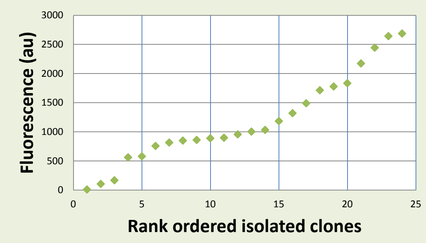
Multiplex MoClo reactions include equimolar volumes of each part type so that multiplexed parts are 1/(n) concentration of each other parts where n=# of parts of that part type. To develop protocols, simple transcriptional units consisting of RFP and a variety of constitutive promoters and 5' UTRs were constructed with standard MoClo assembly reaction conditions and screened by flow cytometry both as a mixed transformation and as isolated colonies.

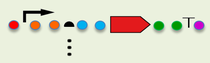
Level 1 Multiplex Expression Tuning Protocol	Level 2 Multiplex Tuning

Single colony FACS analysis of Level 1 Multiplex MoClo Expression Tuning

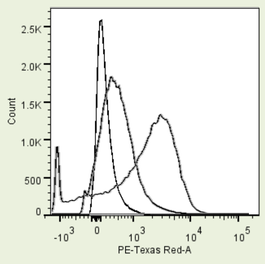
- Single colony analysis of promoter tuning
- Displays large range of expression variation
- Ideal when transcriptional control of expression is sufficient to tune a device

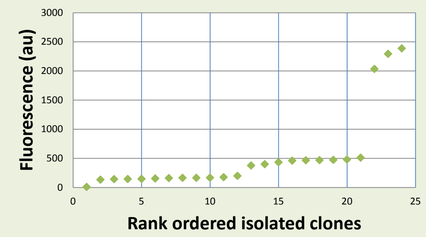


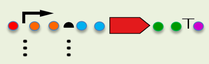




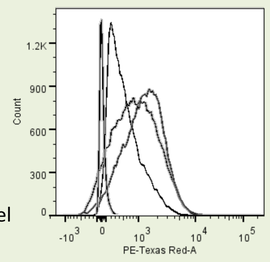
- Single colony analysis of 5'UTR tuning
- Displays uneven expression variation
- Only three 5'UTRs used to test protocol







- Single colony analysis - Promoter:5'UTR tuning
- Displays large smooth range of expression
- Binning by FACS streamlines device tuning
- Alternately, foregoing level 1 selection and performing level 2 multiplexing direct from level 1 MMC reactions provides a greater range of expression variations



Engineered Allosteric Control

Building on the basic multiplex MoClo method, we are developing a high throughput domain exchange protein engineering assembly and screening system wherein a variety of design candidates are assembled and functionally screened for activity. By attaching ligand binding domains (LBD) to the coding sequence of a particular gene of interest (GOI) in a library-based assembly, we can test hundreds of possible fusion proteins in a single reaction and screen for functional clones to rapidly engineer allosteric control of a given enzyme.

Multiplex Modular Assembly and Screening of Engineered Allosteric Control: *By constructing fusion proteins by linking a coding sequence from a library of ligand binding domains (LBD) to the coding sequence for a gene of interest (GOI) likely a trans activation domain, and assembling these transcriptional units in combination with a selectable marker, only correctly functioning clones will be isolated.*

Acknowledgements*: Dr. Swapnil Bhatia for development and maintenance of Pigeon for part and device images, Evan Appleton for assistance with flow cytometry data analysis, Thomas Lozanoski for help with identification of multiplexed devices and, along with Jake Awtry, Pooja Shah, Shawn Jin, Devin Desai for continued expansion and development of the MoClo library.*

*All DNA devices presented were diagramed using PigeonCAD. (*http://pigeoncad.org/).
Bhatia, Densmore. *ACS Synth. Biol.*, **2013**

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