Abandon All Hope, Ye Who PCR: MoClo and the Quest for Genetic Circuit Characterization

Boston University iGEM 2012

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Background

Building

BioBricks™ is the predominant assembly method in iGEM

Characterizing

Measuring functionality of parts at both single cell and population levels

Sharing

The Registry of Standard Biological Parts
The Problems

- BioBricks™ assembly requires multiple cycles of ligations and digestion. Time consuming when constructing large circuits.

- Characterization methods for circuits containing fluorescent proteins vary across synthetic biology groups, making data comparison challenging.

- Parts pages on the Registry often lack a standard format in which characterization information is displayed.
Our Solutions

- Modular Cloning technique (MoClo) (Weber et al., 2011)
  - Type IIS restriction sites allows ligation of up to 6 DNA parts together in one reaction

- A uniform characterization method for future iGEM teams
  - Applied to circuits with fluorescent markers
  - Makes information more easily compared and analyzed

- A common format for the experience page for all parts on the Registry
  - Data sheet to easily collect all information available
Our Results

The MoClo Kit
- Converted 30 BioBrick parts into MoClo parts as the first step in creating a MoClo library for future iGEM competitions

A preliminary characterization workflow is under development
- Protocol based on flow cytometry for genetic circuits with 1-2 fluorescent protein markers

An outline for a MoClo based data sheet
- The data sheet will be generated using information stored in Clotho
Building
Building Summary

Promoters  RBS  Genes  Terminator

Abstract Transcriptional Unit

...
Our Approach

BioBrick Assembly

MoClo Assembly

Time to build and confirm (restriction mapping and sequencing)

9 Days

3 Days
Modular Cloning

- One-pot reaction where digestion and ligation occur together

- Advantages:
  - Up to 6 DNA parts together in one step
  - Highly modular
  - Easily automated
  - Two restriction enzymes and T4 DNA ligase

- Consists of 3 types of Parts:
  - Level 0: Basic part (ex: promoter, RBS, CDS, etc.)
  - Level 1: Transcriptional unit (up to 6 Level 0 Parts)
  - Level 2: Composite of up to 6 Level 1 parts

Weber et al. (2011) *PLoS One*
MoClo: Level 0 to Level 1

Fusion Sites:

GGAG  TACT  TACT  AATG  AATG  AGGT  AGGT  GCTT

Level 0 A-B  Level 0 B-C  Level 0 C-D  Level 0 D-E

One-Pot Restriction Digestion and Ligation

Level 1 A-E

Fusion Sites

A  GGAG  E  GCTT
B  TACT  F  CGCT
C  AATG  G  TGCC
D  AGGT  H  ACTA
MoClo: Level 1 to Level 2

Level 1 A-E

Level 1 F-G

Level 1 E-F

Level 1 G-H

One-Pot Restriction Digestion and Ligation

Level 2 A-H
Fusion Site Generation

5'...GAAGACNNNNNNN...3'
3'...CTTCTGNNNNNNNN...5'

BpiI Recognition Site

5'...GAAGACNN-3'
5'...NNNNN...3'
3'...CTTCTGNNNNN-5'
3'...N...5'

4bp Overhangs → Fusion Sites

5'...N-3'
5'...NNNNNNGCTTCTG...3'
3'...NNNNN-5'
3'...NNNGAAGAC...5'

BpiI Recognition Site

5'...NNNNNNNNCTTCTG...3'
3'...NNNNNNNNGAAGAC...5'

4bp Overhangs → Fusion Sites
BioBricks to MoClo

We converted BioBrick parts into Level 0 MoClo parts using PCR

- **45** parts were chosen
- **156** primers were designed
- **308** PCR reactions were carried out
- **188** PCR reactions yielded correct band sizes

- **79** fusion site variations on the 45 parts were correctly amplified
We have successfully converted 30 BioBrick parts into MoClo Level 0 parts.

- 17 promoters
- 5 RBS
- 7 genes
- 1 terminator
### New MoClo Level 0 Parts

**mmoR and pMmoR**

- Copper sensitive o^{54}-regulatory system from *Methylosinus trichosporium* OB3b

- *mmoR* has C-D and pMmoR has A-B MoClo fusion sites

- Both parts were also made into standard BioBrick parts

**New fluorescent protein markers**

- EBFP2 and iRFP have also been amplified as C-D Level 0 MoClo parts

- iRFP excitation at 690nm and emission at 713nm (Filonov et al., 2011. *Nature Biotechnology*)

- EBFP2 excitation at 383nm and emission at 448nm (Ai et al., 2007. *Biochemistry*)

Inspired by Scanlan et al. (2009) *FEMS Microbiology*
MoClo Kit for iGEM

🌟 BioBricks Parts Converted:
- 17 promoters
- 5 ribosomal binding sites
- 7 genes
- 1 terminator

🌟 4 new parts
- pMmoR, mmoR, EBFP2, and iRFP

🌟 lacZ cloning vectors:
- Level 0: 7 (pSB1C3 backbone)
- Level 1: 4 (pSB1K3 backbone)

All part numbers available at: http://2012.igem.org/Team:BostonU/Parts
Building Summary

18 Promoters  5 RBS  10 Genes  1 Terminator

34 Level 0 MoClo Parts

Abstract Level 1 MoClo Transcriptional Unit

900 Different Level 1 MoClo Combinations Possible

612 2-part Level 2  384 3-part Level 2  210 4-part Level 2
Finish converting the remaining 14 parts (58 in total with fusion site variations for promoters and terminators) to complete the MoClo Kit.

Create all possible Level 2 cloning vectors for our given set of MoClo parts.

Submit all new parts and cloning vectors to Registry once sequenced.
Characterizing
**Characterizing Summary**

**Confirmation**

![DNA Sequencing Result](CGTACCGTAC)

**Single Cell Analysis**

1. **Day 1**
   - Streak out *E. coli*
   - Circuit containing plasmids
   - LB Agar + Antibiotic

2. **Day 2**
   - Cultures are set up in triplicate
   - LB Broth + Antibiotic
   - After growth, culture is diluted into characterization media
   - LB Broth + Antibiotic + Small molecules

3. **Day 3**
   - After growth, culture is diluted for FACS analysis
   - 1 x PBS

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**Analysis**

- **Graph 1**
- **Graph 2**
Our Approach

Flow Cytometry

- Determines function of a genetic circuit based on fluorescent reporters

- Measures single cells of a population in a high throughput way

- Generates quantitative data that can be analyzed to show a variety of information

- Not all teams have flow cytometers, which opens up opportunities for teams to collaborate

www.abcam.com
Our Workflow

**Day 1**
- Streak out *E. coli* containing genetic circuit
- LB agar with antibiotic

**Day 2**
- Cultures are set up in triplicate
- After growth, culture is diluted into characterization media
- LB broth with antibiotic

**Day 3**
- After growth, culture is diluted for flow cytometry analysis
- LB broth with antibiotic and small molecules
- 1 x Phosphate Buffered Saline (PBS)

Current:
- 18-20 hours overnight growth at 37°C
- 6 hrs at 37°C at 300rpm
- 1:200 dilution
- 12-16 hrs at 37°C at 300rpm
- 1:10 dilution
Characterizing Anderson Promoters

*Parts are currently in pSB1A3 and being cloned into pSB1C3 for re-submission*

BBa_K783068-72*
Characterizing Anderson Promoters

*Parts are currently in pSB1A3 and being cloned into pSB1C3 for re-submission*
Characterizing a BioBrick Inverter

BBa_K783067*

*Part is currently in pSB1A3 and is being cloned into pSB1C3 for re-submission
Characterizing New Parts

🌟 It was unknown if pMmoR would function alone in *E. coli*

🌟 Plates showed no color despite positive restriction maps and sequencing

🌟 It was unknown if *mmoR* would function in *E. coli*

🌟 When combined with the pMmoR-XFP devices, no color was seen on the plates

🌟 System may require *mmoG*, a GroEL-like chaperone protein in *M. trichosporium OB3b*

🌟 Cloning of *mmoG* is in the planning stages
Generate a detailed protocol sheet for part characterization using flow cytometry and share it with the iGEM community.

Generate MoClo inverters (Level 2 parts) and compare them to BioBrick inverters built in our lab.

Expand our analysis to include population level measurements (ex: spectrometry and microscopy).

Population Level Analysis
Sharing
MoClo Data Sheet Outline

MoClo Kit submitted to the Registry

RFC for MoClo

RFC Standard documentation in progress for submission to the BioBricks Foundation

Shared PCR Troubleshooting tips for iGEM teams at: http://2012.igem.org/Team:BostonU/Methodology
**Current Data Sheets**

**BBa_I14018, I14033, I14034**
Promoters for ampicillin (I14018 or "PBlb"), chloramphenicol (I14033 or "PKat") and kanamycin (I14034 or "PKat") resistance.

### Promoter activity levels

<table>
<thead>
<tr>
<th>Promoter name</th>
<th>Activity (RPK)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBlb</td>
<td>3.4</td>
<td>0.2</td>
</tr>
<tr>
<td>PKat</td>
<td>0.80</td>
<td>0.04</td>
</tr>
<tr>
<td>PKat</td>
<td>0.10</td>
<td>0.01</td>
</tr>
</tbody>
</table>

GFP (Arbitrary units)

**BBa_F2620**
3OC12HSL → PoPS Receiver

**Mechanism & Function**
A transcription factor (LuxR) that is active in the presence of a cell-cell signaling molecule (3OC12HSL) is controlled by a regulated operator (PpoPS). Device input is 3OC12HSL. Device output is PoPS from a LuxR-regulated operator. It can be contained the first output such as aTc can be used to produce a Boolean AND function.

**Dynamic Performance**

**Input Compatibility**

**Reliability**

**Authors:** Barry Canton
**Updated:** March 2008

**Source:** Canton et al., 2008. *Nature Biotech*

**Source:** BioFab

**Source:** CSynBI
Our data sheet has four major sections:

- **General Information**
- **Part Information**
- **Growth / Measurement Conditions**
- **Data Analysis**

We will pull this information from Clotho, so any Clotho user can generate the same type of data sheet.
As of the Wiki Freeze, we submitted 55 parts:

- The MoClo Kit: 28 parts (including \textit{mмоR} and pMмоR) and 11 cloning vectors
- New BioBrick parts: 1 (pMмоR)
- Characterized BioBrick devices: 15 (BBa\_K783068-BBa\_K783081)

For a more detailed list, please see:
- http://2012.igem.org/Team:BostonU/Parts
Sharing: Future Work

- Generate data sheets for our parts from our Clotho database and share them on the Registry pages associated with those parts.

- Complete the RFC standard documentation for MoClo and submit it to The BioBricks Foundation.

- Submit the remaining MoClo L0 parts and Level 2 cloning vectors to complete the MoClo Kit.
Collaborations

Clotho and Eugene

Human Practices
Sequence data was entered in Clotho using Bull Trowel

- Includes oligos, parts, and vectors

- Other Apps used:
  - Sequence view
  - SpreadIt Oligos
  - SpreadIt Features
Eugene Scripter used the Eugene language to:

- Define MoClo parts
- Identify device function specifications using Eugene rules
- Permute all possible combinations of MoClo parts to generate devices with that function

Wiki Freeze Kit:
- 5 RBS
- 1 Terminator
- 18 Promoters
- 4 Genes

Current Kit:
- 18 Promoters
- 5 RBS
- 10 Genes
- 1 Terminator

Goal Kit:
- 70 Promoters
- 5 RBS
- 13 Genes
- 4 Terminators

Total Number of Level 1 Parts Possible:

- Wiki Freeze Kit: 360
- Current Kit: 900
- Goal Kit: 18,200
Other Software Tools Utilized

Pigeon
- Used for genetic circuit figure generation
- Developed by Dr. Swapnil Bhatia

FinchTV
- Used for its ability to view trace data

Matlab
- Used to analyze flow cytometry data
We helped define the idea for Wellesley’s MoClo Planner tool.

Met several times over the summer to refine and clarify concepts.

Participated in user studies.
NEGEM Regional Meeting

September 15th at BU
MIT, Wellesley, and Brown (Stanford-Brown)
Presentation feedback
Goal: Determine which of four methods is the best way to introduce Synthetic Biology to a new audience

Four methods: Lecture, Video, Internet, Discussion group

Data will be collected from questionnaires given to students before and after students are exposed to one of the methods

Results will be compared to determine which method should be used

Scheduled to visit Somerville High School on October 24th
Our Contributions to iGEM

34 MoClo parts and 11 cloning vectors submitted to the Registry as the MoClo Kit as first step to move iGEM away from BioBricks

Characterization workflow for circuits with fluorescent proteins in development

RFC Standard documentation will be shared for MoClo assembly

Outlined a data sheet for easy sharing of part, assembly, and characterization data
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Our Sponsors