Engineering Biology

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Preface

This book supports the Synthetic Biology CuRE course at Virginia Tech BSE 4014/5014.

Acknowledgments

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9 Modular Cloning

9.1 Introduction

This chapter introduces the principles of **Modular Cloning (MoClo)**, a standardized method for assembling genetic circuits using reusable DNA parts. MoClo is central to synthetic biology and enables rapid, reliable construction of multigene constructs for functional analysis and synthetic circuit design.

9.2 What is MoClo?

MoClo, or Modular Cloning, is a Golden Gate-based cloning strategy that uses Type IIS restriction enzymes to assemble DNA fragments with predefined overhangs in a single reaction.

9.2.1 Key Features

- **Type IIS restriction enzymes** like **BsaI** and **BsmBI** cut outside their recognition sites, enabling scarless assembly.
- Each DNA part (e.g., promoter, coding sequence, terminator) is cloned into a **Level 0 plasmid** with specific flanking sequences.
- Level 0 parts are assembled into **Level 1 cassettes**, which can then be assembled into **Level 2 multigene constructs**.

  TODO: Insert diagram of MoClo hierarchy (L0 $\rightarrow$ L1 $\rightarrow$ L2).

9.3 Why Use MoClo?

- **Standardization**: Allows parts to be easily shared, reused, and recombined.
- **Speed**: One-pot reactions reduce cloning time.
- **Efficiency**: High-fidelity assembly with minimal hands-on steps.
- **Flexibility**: Easily test multiple combinations of parts (e.g., promoter variants).

9.4 Part Plasmids and Assembly Strategy

Each genetic element is cloned into its own plasmid, called a **part plasmid**. These are stored in **E. coli** for easy amplification and reuse.

9.4.1 Advantages of Using Plasmid Libraries

- Easy to grow and miniprep.
- Stable storage and replication.
- Eliminates need to PCR amplify parts each time.

TODO: Add table of standard part types and corresponding overhangs.

9.5 MoClo Assembly Overview

Step	Description
1	Clone each part into a Level 0 vector with correct overhangs.
2	Use **BsaI** to assemble Level 0 parts into a Level 1 cassette.
3	Use **BsmBI** to assemble multiple Level 1 cassettes into a Level 2 construct.

Junctions between parts are defined by 4 bp overhangs, each unique to a position in the expression cassette (e.g., promoter–CDS, CDS–terminator).

TODO: Include schematic of overhang design and part compatibility.

9.6 Experimental Design Using MoClo

Students used the MoClo system to design constructs testing different auxin-responsive promoters and ARF (Auxin Response Factor) activators.

9.6.1 Example Experimental Goals

- Compare activity of full-length ARF6 with truncated variants.
- Test reporter expression from different auxin-responsive promoters.
- Include negative controls using mutant promoters lacking ARF binding sites.

9.6.2 Promoters and Controls

Promoter	Description
PIA19	Auxin-responsive promoter
PIA19mut	Mutated version, lacks ARF binding sites (negative control)
PER7	Alternative auxin-responsive promoter
P32x	Promoter with moderate activation potential

9.7 Best Practices in MoClo

- Avoid reuse of identical sequences (e.g., same terminator) across constructs to reduce recombination.
- Use **unique promoters and terminators** in multigene constructs.
- Maintain **codon frame** at junctions (especially for CDS fusions).
- Add **GG** or **stop codons** where appropriate.

9.8 Advanced Tools and Resources

9.8.1 Toolkits

- **Yeast Toolkit (YTK)**: Includes a wide variety of parts for use in *S. cerevisiae*.
- **Auxin Toolkit**: Custom parts developed for studying auxin signaling.

9.8.2 Hardware

- Protocols for **robotic minipreps** are being developed to automate DNA extraction from plasmid libraries stored in 96-well plates.

  TODO: Link to available toolkit maps and protocols.

9.9 Application: Combinatorial Design

Students designed and tested modular constructs combining:

- Reporters (e.g., **mRuby2** with **UBM** degradation tags)
- Activators (e.g., **ARF19**, **ARF6** variants)

- Responsive promoters (e.g., **PIN18**, **PER7**)

This modular approach allowed rapid testing of hypotheses such as:

- Optimal ARF expression levels for activation
- Promoter responsiveness to auxin
- Reporter signal tuning via degradation

9.10 Summary

MoClo enables students to move from hypothesis to construct in a streamlined and standardized way. By combining software design, part libraries, and efficient cloning, students can explore complex biological questions with scalable, reproducible tools.

TODO: Add student planning worksheet for construct design.

9.11 Next Steps

- Begin primer design and part selection for Level 0 construction.
- Simulate and test assemblies using Benchling or Ape.
- Prepare to begin Golden Gate assembly in lab next week.

10 Hypotheses, Research Questions, and Experimental Design

11 Level 0 Part Plasmid Design

11.1 Overview

This chapter introduces students to experimental design in the context of synthetic biology and the practical construction of Level 0 part plasmids using Modular Cloning (MoClo) and the Yeast Toolkit (YTK).

We build on the previous lecture's discussion of MoClo principles and emphasize hands-on experience with designing and assembling modular DNA parts for use in downstream genetic circuits.

11.2 Review of Modular Cloning (MoClo)

Modular Cloning allows the assembly of genetic parts—promoters, coding sequences, terminators, and other regulatory elements—into standardized expression cassettes. Each part is cloned into a **Level 0 plasmid**, which can be stored, replicated, and reused.

11.2.1 Key Principles

- Uses **Type IIS restriction enzymes** (e.g., BsaI and BsmBI) that cut outside their recognition sites, allowing for **scarless assembly**.
- Each part is flanked by **standardized 4 bp overhangs**, which determine its position and compatibility within an expression cassette.
- Level 0 parts can be assembled into **Level 1 cassettes** (single gene), and multiple Level 1 cassettes can be assembled into **Level 2 constructs** (multigene systems).

  TODO: Insert diagram illustrating MoClo hierarchy (L0, L1, L2).

11.3 Software Tools for Primer and Part Design

11.3.1 Benchling

Benchling is a browser-based molecular biology tool for sequence annotation, primer design, and cloning simulations.

To use Benchling:

1. Import or create your DNA sequence.
2. Annotate regions (promoter, CDS, terminator).
3. Use the *"Primer"* tool to design and label forward and reverse primers.
4. For reverse primers, use the *"Copy Special > Reverse Complement"* option.
5. Simulate Golden Gate assembly using *Assembly Wizards* or manual cloning.

11.3.2 Ape (A Plasmid Editor)

Ape is a downloadable tool that allows simple manipulation of DNA sequences.

- Supports reverse complement operations and editing.
- Useful for flipping 3′ to 5′ primers back into 5′ to 3′ notation.

TODO: Include screenshot of Benchling or Ape with primer annotations.

11.4 Activity Instructions

Students worked from:

- **YTK Assembly Manual (pages 12–26)**: This guide details how to construct each part type using standardized overhangs and restriction sites.
- **YTK Overhangs Primers Sheet (Google Sheets)**: Shared class document for assigning and recording primer sequences and overhangs.

11.4.1 Group Workflow

1. Open the manual and locate your assigned part type (e.g., Type 1, 2, 3A, 3B…).
2. Record the **part name**, **5′ overhang**, **3′ overhang**.
3. Design forward and reverse primers:
    - Forward primer = [GC clamp] + [BsaI site] + [part-specific overhang] + [homology to template]
    - Reverse primer = Same logic, but **reverse complement** of the sequence

TODO: Add primer design template for students to follow.

11.4.2 Primer Design Guidelines

- Primers should always be written 5 $\rightarrow$ 3 .
- Include at least 4 bp **GC clamp** before the restriction site.
- Use **BsaI** recognition site (GGTCTC) for Level 0 assembly.
- Add part-specific overhangs immediately downstream of the enzyme site.
- Add **GG** for fusion proteins (e.g., Type 3B parts) to maintain codon frame.
- Include a **stop codon** and/or **XhoI site** for terminator parts as needed.

TODO: Insert example primer sequence and breakdown of its components.

11.5 Practical Considerations

11.5.1 In-Frame Assembly

- Coding sequences often require special care to preserve the reading frame across part boundaries.
- For example, combining Type 3A and 3B parts (both coding) needs a GG insertion to keep translation in-frame.

11.5.2 Negative Controls

- Use mutant promoters (e.g., PIA19mut) that lack binding sites to demonstrate ARF-specific activation.
- Design primers that omit ARFs or use constitutively inactive variants as negative controls.

TODO: Provide table of part types and expected features (start codon, stop codon, etc.).

11.6 Constructing Level 0 Parts

To make a new Level 0 part:

1. Identify the **source sequence** (synthesized, genomic DNA, etc.).
2. Design primers to amplify the sequence:
    - Add **BsaI site**, overhangs, and homology arms.

3. PCR amplify the part.
4. Digest both the PCR product and **Level 0 entry vector** with BsaI.
5. Ligate the digested insert and vector.
6. Transform into competent **E. coli** and select colonies.
7. Screen clones by colony PCR or sequencing.

TODO: Include diagram showing Level 0 assembly via Golden Gate.

11.7 Troubleshooting Tips

- Double-check primer orientation and melting temperatures (55–72°C).
- Avoid internal BsaI or BsmBI sites within the part sequence.
- Use Benchling to simulate and verify assembly.

11.8 Summary

This activity combined theoretical and practical training in modular cloning. Students learned to:

- Navigate cloning toolkits and design compatible parts
- Use software to annotate sequences and design primers
- Understand the logic of MoClo part assembly

These skills will be applied to build expression cassettes and multigene constructs for synthetic biology experiments in upcoming weeks.

TODO: Link to shared Google Sheet with part assignments and example sequences.

11.9 Next Steps

- Finalize primer designs for assigned part types.
- Submit sequences for synthesis or begin PCR amplification.
- Prepare for Level 0 part cloning and validation.

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