iGEM Measurement Committee
Who Are We?

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- 2012-2013 iGEM Calgary
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- 2010-2012 iGEM ETH Zurich, CH
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Basics of Assembly
What is Recombinant DNA?

Recombinant DNA (rDNA) molecules are DNA molecules formed by laboratory methods of genetic recombination to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome.
Why Even Bother with Assembly?

• When you can order the entire sequence from Twist, GenScript or IDT – why would you want to bother with assembling sequences?

• A few reasons:
  • Want to express the same gene with different promoters or tag it differently?
  • Very long sequences can be challenging to get synthesized in one go, it can be faster to order smaller pieces and assemble it yourself.
  • Place your sequence into different plasmids, for instance those that have different antibiotic resistance genes.
Overview of Assembly Methods

**BioBrick Assembly**
- Restriction sites flank every gene fragment, allowing “parts” to be interchanged, but introduces scar sequences

**Gibson Assembly**
- Exonuclease creates large overhangs for annealing fragments, allowing for more accurate assemblies

**Golden Gate Assembly**
- Endonuclease creates fragment-specific overhangs allowing 20+ fragments to be assembled at once in a relatively short time
Introduction to Gibson Assembly

• A method for assembling multiple linear fragments of DNA in a one-pot isothermal reaction.

• Relies on multiple complementary overhanging ends between the sequences, enabling many fragments to be assembled in one go.

• You do not need to rely on specific restriction enzyme cut sites, there are no scar sites, and you can avoid challenges with cut site incompatibilities.

Steps in Gibson Assembly

1. Obtain sequences and design primers to ensure overlaps exist.

2. Make fragments either by PCR or DNA synthesis.

3. Perform Gibson reaction.
   1. T5 Exonuclease – chews the ends of the fragments producing 3’ overhangs.
   2. DNA Polymerase – fills in the gaps after overhang fragments have annealed.
   3. DNA Ligase – covalently links together the filled-in strands.
Example of Gibson Assembly
Example using a Yeast Gene

• I am interested in building a biological circuit that has a stress gene ($dcp1^+$) attached to a fluorescent protein ($RedStar2$ aka RFP).

• First step is to get a copy of the gene and other sequences into an editor, I like to use Benchling.
Obtain the Fragment Sequences

Destination Plasmid

\[ \text{dcp1}^+ \text{ gene} \]

RedStar2
Design Primers

• Overhanging primers are needed at the ends of each fragment.

• These overhangs provide complementary regions for the sequence and this is where the Gibson reaction will work its magic to connect these fragments together.
Design Primers in Benchling

• Benchling can do the first pass on designing primers for Gibson using the “Assembly Wizard.”
Design Primers in Benchling

• Benchling can do the first pass on designing primers for Gibson using the “Assembly Wizard.”
  • Benchling provides us with an in-silico pre-run of our Gibson assembly reaction and helps us by creating primers that we can use for our PCR.  
    • Note: you will need to check for melting temps and GC content.

• Once these primers are vetted, we can order them and proceed to PCR.

• Alternatively, we can use the “Create PCR Product” in Benchling function for each fragment and then order the fragments via DNA synthesis.
Produce Fragments via Synthesis or PCR

Linearized Plasmid → PCR → Linearized Plasmid

PCR

dcp1 → PCR

PCR Cleanup and Band Confirmation

RedStar2 → PCR
Mix Together Fragments

Fragments
- Linearized Plasmid
- dcp1
- RedStar2

Gibson Master Mix
- T5 Exonuclease
  - Linearized Plasmid
  - dcp1
  - RedStar2
  - Plasmid
- DNA Polymerase
  - Plasmid
  - dcp1
  - RedStar2
  - Plasmid
- DNA Ligase
  - Plasmid
  - dcp1
  - RedStar2
  - Plasmid
Transformation

• If you made a plasmid compatible with *E. coli* like we did here, then it can into a strain such as DH5α.
  • This provides us with a place to make many many copies before transforming it into yeast.

• From here you can select a few colonies, perform colony PCR and/or perform a DNA mini-prep to get lots of copies of your DNA.
Confirm Assembly

- Confirmation of assembly should be done by colony PCR/gel and by Sanger sequencing.

- Identify missense and non-sense mutations, such as:
  - A nucleotide substitution that changes an amino acid or changes the promoter sequence.
  - An insertion or deletion (indel), occurred producing a non-sense protein.

- The cost of Sanger sequencing can save you huge amounts of troubleshooting time, just get in the habit of doing it!
Resources

Benchling, the web-based software used in this presentation
• https://www.benchling.com

Resources on Gibson assembly
• https://www.addgene.org/protocols/gibson-assembly/
• https://www.biocat.com/bc/files/Gibson_Guide_V2_101417_web_version_8.5_x_11_FINAL.pdf

A tool like Benchling for planning out a Gibson assembly
• http://nebuilder.neb.com
Questions?
Yeast Assembly
(aka Gap-repair)
DNA assembly in Yeast (*S. cerevisiae*)

- Assembly of DNA parts can be achieved *in vivo* by using the yeast endogenous **homologous recombination** repair mechanism.

- Gibson assembly and yeast assembly are presented together, as the same genetic parts can be used (fragments with 40bp homologues overhangs)

- Yeast assembly is very cheap as it doesn’t require enzymes. But as yeast grows slower, the full process “from fragments to assembled genes” takes about 2-3 days.
Gibson Assembly vs. Yeast Assembly

Fragments

Gibson Assembly

Yeast cloning vector
Linearized Plasmid

T5 Exonuclease
DNA Polymerase
DNA Ligase

1 hours
Overnight incubation

Plasmid
dcp1
RedStar2
Plasmid

Yeast Assembly

Mix: 30 min
2-3 days incubation

dcp1
RedStar2

24
When does it make sense to use Yeast assembly

- When you work with yeast as a final cloning host
- When you assemble many fragments (more than 3)
- When one or more fragments are short (< 100 bp)

After assembly in yeast, gene circuits can be sub-cloned into a user-defined vector

iGEM Frankfurt 2012
Usefulness of yeast homologues recombination

2008: Assembly of Mycoplasma genome

One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic *Mycoplasma genitalium* genome

Daniel G. Gibsona,1, Gwynedd A. Bendersb, Kevin C. Axelrodc, Jayshree Zaveria, Mikkel A. Algirea, Monzia Moodiea, Michael G. Montaguea, J. Craig Ventera, Hamilton O. Smithb, and Clyde A. Hutchison IIIb,1

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Contributed by Clyde A. Hutchison III, October 30, 2008 (sent for review September 11, 2008)

2018: Synthetic yeast chromosomes
You need:
- Yeast cloning strain: e.g. BY4741 (ATCC201388) Auxotrophic markers: Leucine, Uracil, Histidine, Methionine (his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)

- Yeast plasmid/backbone: e.g. pRS series

- Your PCR amplified fragments with ~40 bp homologues overhangs
Yeast assembly step-by-step

1. Generation of fragments by PCR
   - Linearized Plasmid
   - dcp1
   - RedStar2
   - LiOAc/PEG method

2. Yeast transformation
   - 100 ng of plasmid
   - 4-10 molar ratio of each insert

3. Selection of colonies

4. Assembly verification
   - Colony PCR → Sanger sequencing
   - Plasmid extraction from yeast → retransformation into *E. coli* → Sanger sequencing
Resources

- Yeast assembly/GAP repair@ iGEM: Team Frankfurt 2012

- LiOAc/PEG transformation protocol
  [https://benchling.com/protocols/QOCa7BoO/yeast-transformation-protocol](https://benchling.com/protocols/QOCa7BoO/yeast-transformation-protocol)

- Molecular mechanism of homologues recombination