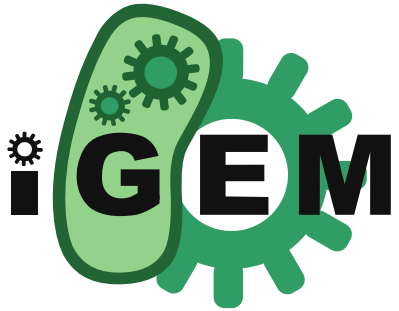
iGEM Measurement Committee



Who Are We?

Iain George

- Molecular Microbiologist @ OSP Microcheck
- 2012-2013 iGEM Calgary
- iGEM Judge 2014 & 2016
- PhD from University of Calgary

Sonja Billerbeck

- Assistant Professor @ University of Groningen, NL
- 2010-1012 iGEM ETH Zurich, CH
- 2014-2018 iGEM Columbia University, USA
- Since 2019 iGEM Uni Groningen, NL

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?

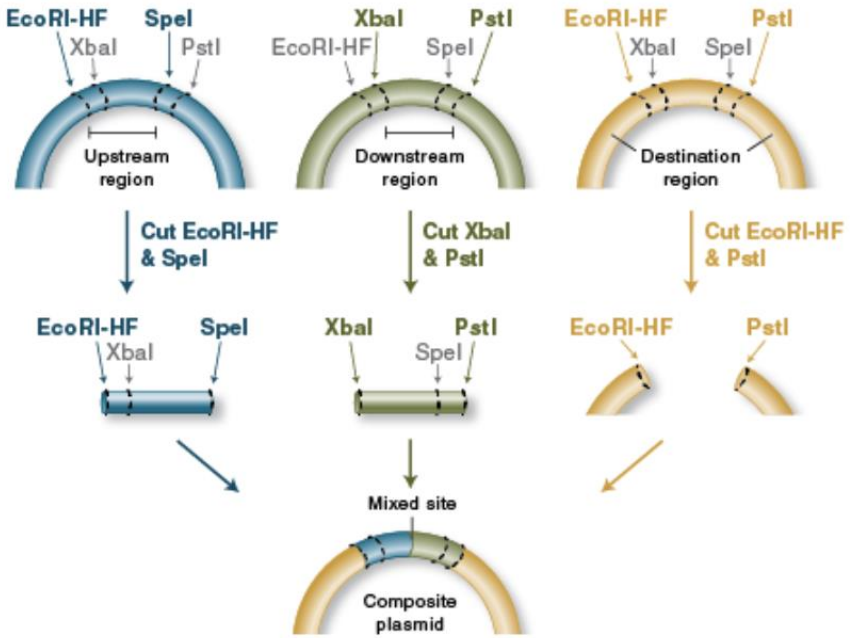
- When you can order the entire sequence from Twist, GenScript or IDT – why would you want to bother with assembling sequences?

- 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

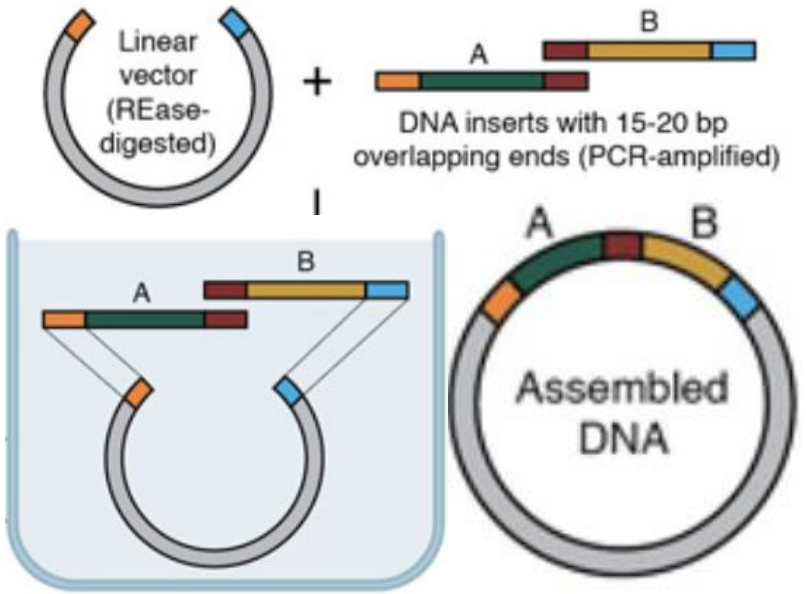
BioBrick Assembly

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



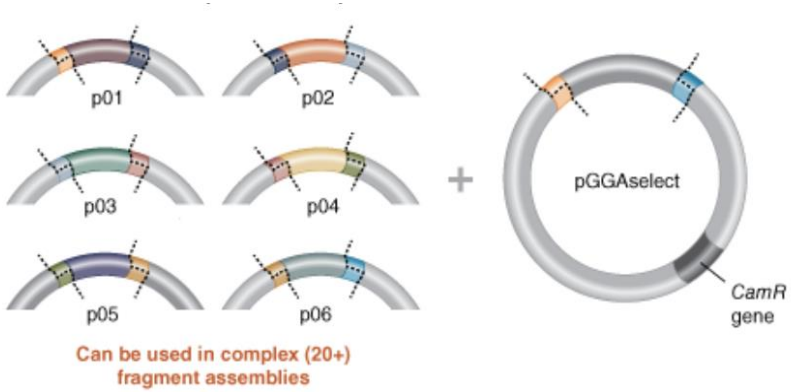
Gibson Assembly

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



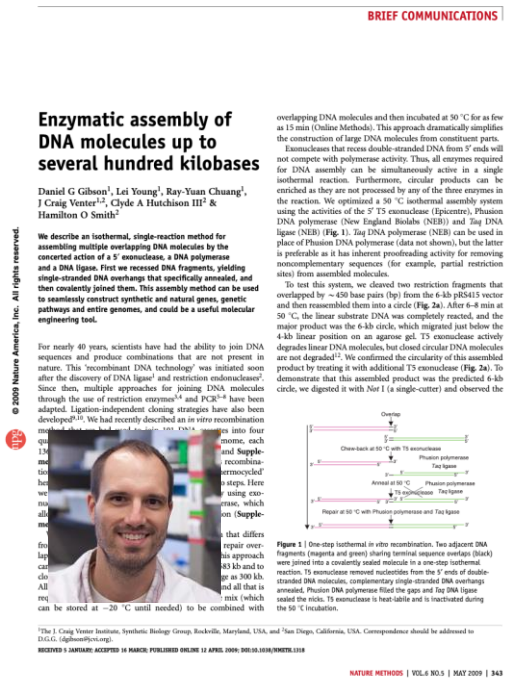
Golden Gate Assembly

- 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.



BRIEF COMMUNICATIONS

Enzymatic assembly of DNA molecules up to several hundred kilobases

Daniel G Gibson[1], Lei Young[1], Ray-Yuan Chuang[1], J Craig Venter[1,2], Clyde A Hutchison III[2] & Hamilton O Smith[2]

We describe an isothermal, single-reaction method for assembling multiple overlapping DNA molecules by the concerted action of a 5′ exonuclease, a DNA polymerase and a DNA ligase. First we recessed DNA fragments, yielding single-stranded DNA overhangs that specifically annealed, and then covalently joined them. This assembly method can be used to seamlessly construct synthetic and natural genes, genetic pathways and entire genomes, and could be a useful molecular engineering tool.

For nearly 40 years, scientists have had the ability to join DNA sequences and produce combinations that are not present in nature. This 'recombinant DNA technology' was initiated soon after the discovery of DNA ligase[1] and restriction endonucleases[2]. Since then, multiple approaches for joining DNA molecules through the use of restriction enzymes[3,4] and PCR[5–8] have been adapted. Ligation-independent cloning strategies have also been developed[9,10]. We had recently described an *in vitro* recombination

overlapping DNA molecules and then incubated at 50 °C for as few as 15 min (Online Methods). This approach dramatically simplifies the construction of large DNA molecules from constituent parts.

Exonucleases that recess double-stranded DNA from 3′ ends will not compete with polymerase activity. Thus, all enzymes required for DNA assembly can be simultaneously active in a single isothermal reaction. Furthermore, circular products can be enriched as they are not processed by any of the three enzymes in the reaction. We optimized a 50 °C isothermal assembly system using the activities of the 5′ T5 exonuclease (Epicentre), Phusion DNA polymerase (New England Biolabs (NEB)) and *Taq* DNA ligase (NEB) (**Fig. 1**). *Taq* DNA polymerase (NEB) can be used in place of Phusion DNA polymerase (data not shown), but the latter is preferable as it has inherent proofreading activity for removing noncomplementary sequences (for example, partial restriction sites) from assembled molecules.

To test this system, we cleaved two restriction fragments that overlapped by ~450 base pairs (bp) from the 6-kb pRS415 vector and then reassembled them into a circle (**Fig. 2a**). After 6–8 min at 50 °C, the linear substrate DNA was completely reacted, and the major product was the 6-kb circle, which migrated just below the 4-kb linear position on an agarose gel. T5 exonuclease actively degrades linear DNA molecules, but closed circular DNA molecules are not degraded[12]. We confirmed the circularity of this assembled product by treating it with additional T5 exonuclease (**Fig. 2a**). To demonstrate that this assembled product was the predicted 6-kb circle, we digested it with *Not* I (a single-cutter) and observed the

Figure 1 | One-step isothermal *in vitro* recombination. Two adjacent DNA fragments (magenta and green) sharing terminal sequence overlaps (black) were joined into a covalently sealed molecule in a one-step isothermal reaction. T5 exonuclease removed nucleotides from the 5′ ends of double-stranded DNA molecules, complementary single-stranded DNA overhangs annealed, Phusion DNA polymerase filled the gaps and *Taq* DNA ligase sealed the nicks. T5 exonuclease is heat-labile and is inactivated during the 50 °C incubation.

[1]The J. Craig Venter Institute, Synthetic Biology Group, Rockville, Maryland, USA, and [2]San Diego, California, USA. Correspondence should be addressed to D.G.G. (dgibson@jcvi.org).

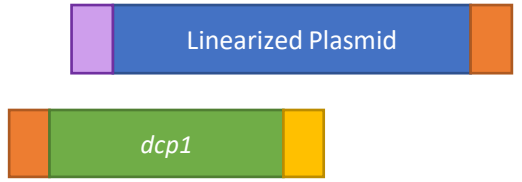
RECEIVED 5 JANUARY; ACCEPTED 16 MARCH; PUBLISHED ONLINE 12 APRIL 2009; DOI:10.1038/NMETH.1318

Gibson et al. 2009. Nature Methods

Steps in Gibson Assembly

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

2. Make fragments either by PCR or DNA synthesis.



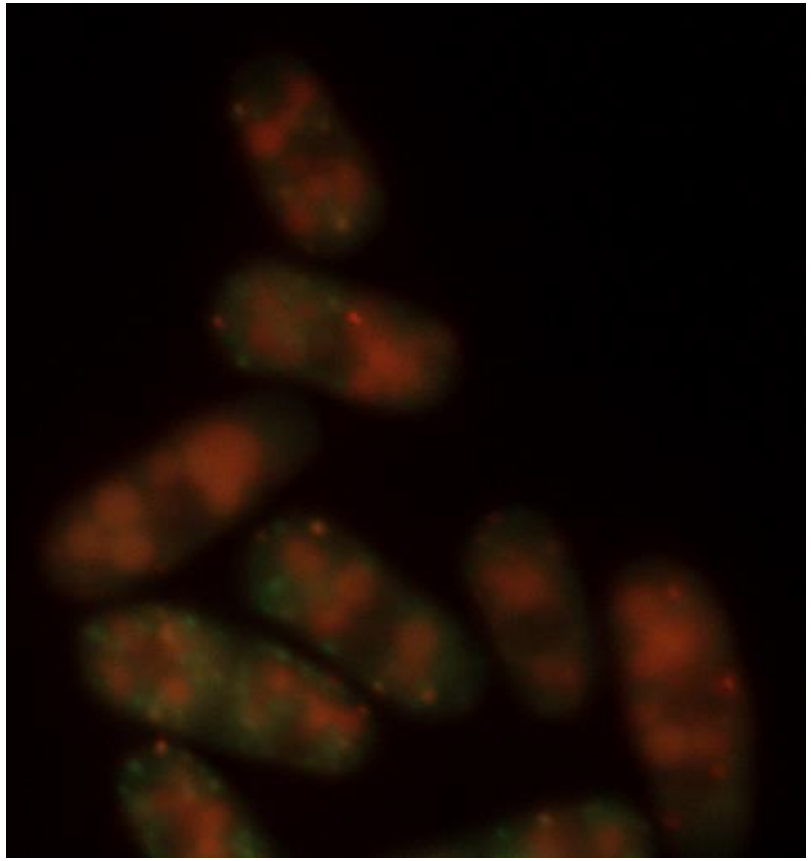
3. Perform Gibson reaction.
    1. T5 Exonuclease – chews the ends of the fragments producing 3’ overhangs.
    2. DNA Polymerase – fills in the gaps after overhang fragments have annealed.
    3. DNA Ligase – covalently links together the filled-in strands.

Example of Gibson Assembly

Example using a Yeast Gene

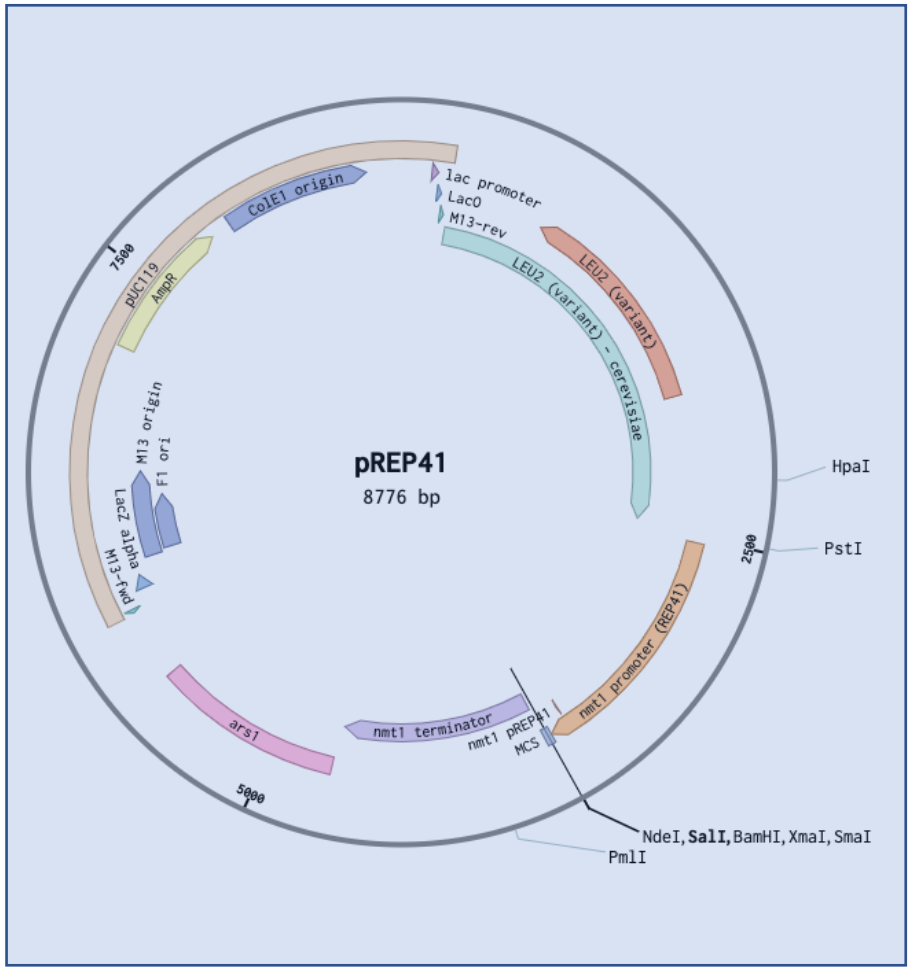
- I am interested in building a biological circuit that has a stress gene (*dcp1*$^{+}$) attached to a fluorescent protein (*RedStar2* aka RFP).

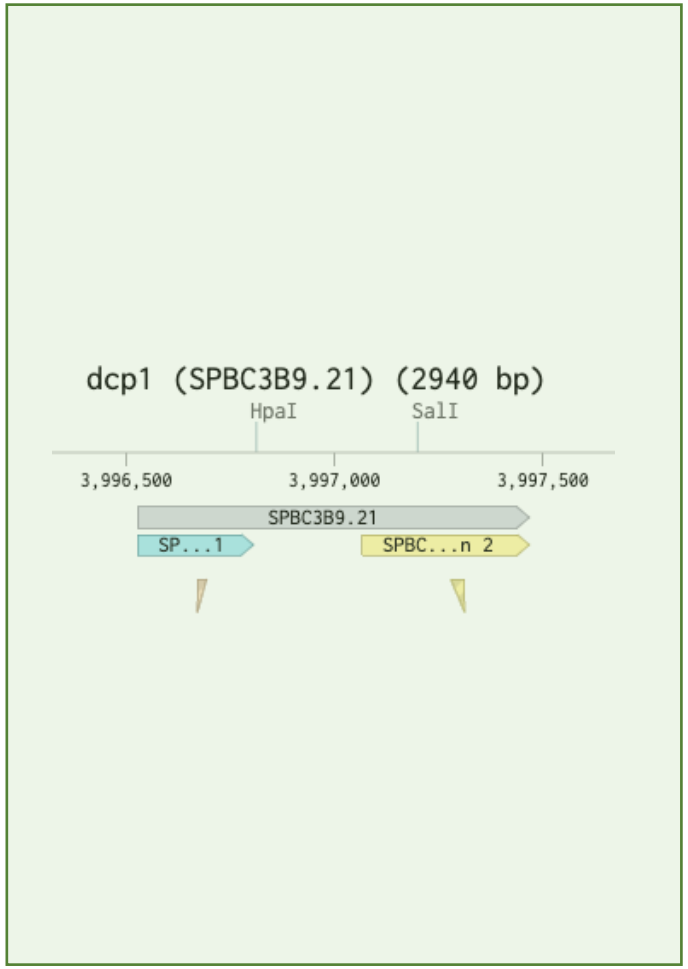
- First step is to get a copy of the gene and other sequences into an editor, I like to use Benchling.

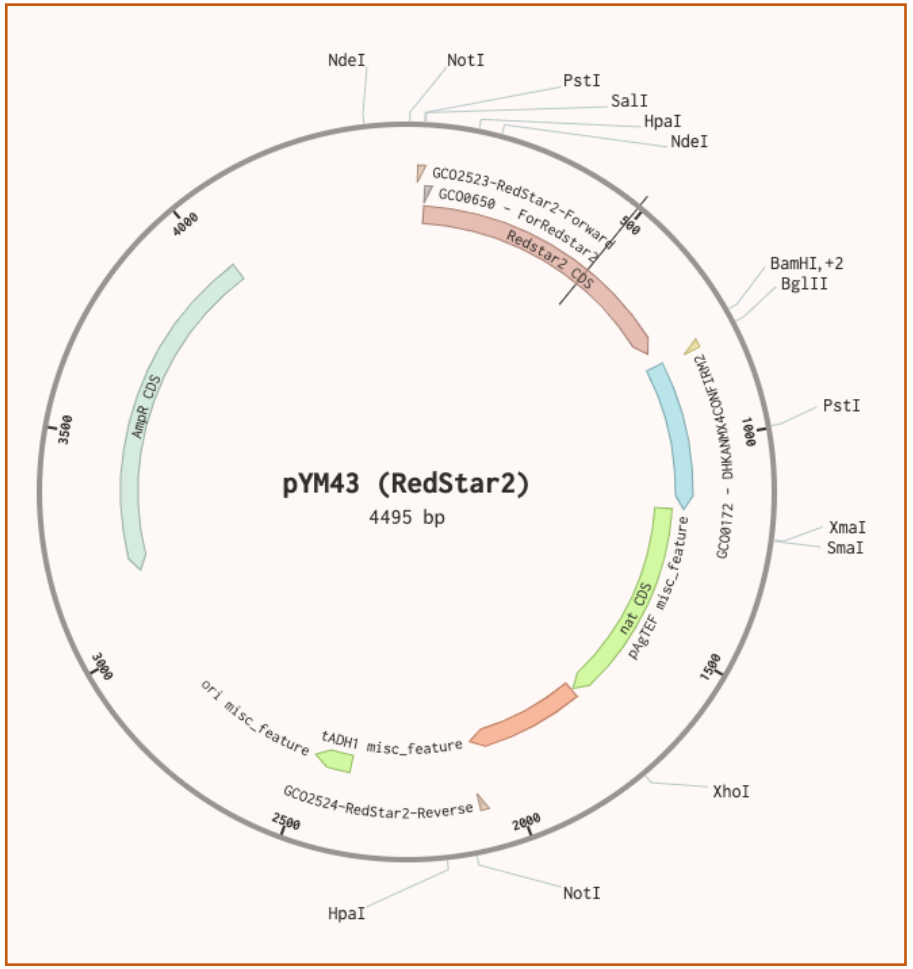


S. pombe cells expressing dcp1-RedStar2 and puf3-eGFP

Obtain the Fragment Sequences







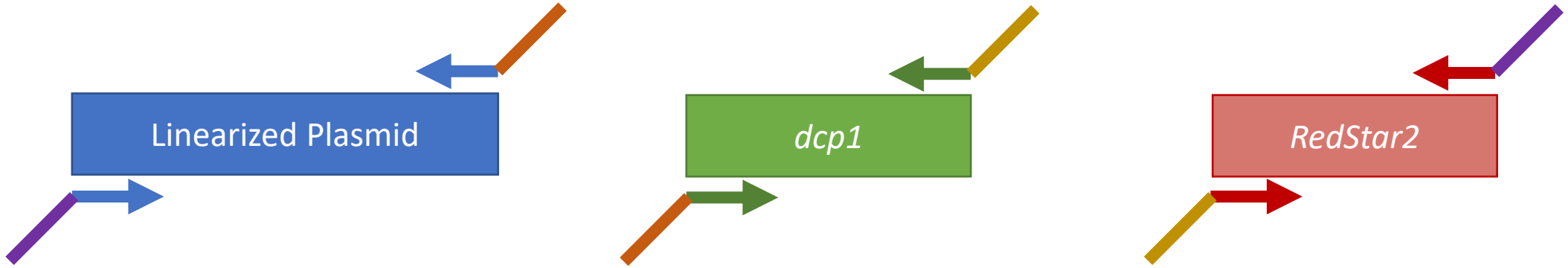
Destination Plasmid

dcp1^{+} 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.”

pREP41 | dcp1 (SPBC3B9.21) | pYM43 (RedStar2)

LINEAR MAP | SEQUENCE MAP

Create | Analyze | Copy | PDF

GGAGAGAAAACAGGGCAAAAGCAAAGCTTAAAGGAATCCGATTGTCATTCGGCAATGTGCAGCGAAACTAAAAACCGGATAATGGACCTGTTAATCGAAACATTG
CCTCTCTTTTGTCCCGTTTTCGTTTCGAATTTCCTTAGGCTAACAGTAAGCCGTTACACGTCGCTTTGATTTTTGGCCTATTACCTGGACAATTAGCTTTGTAAC

nmt1 promoter (REP41)

3,470 3,480 3,490 3,500 3,510 3,520 3,530 3,540 3,550 3,560 3,570

NdeI

AAGATAAAGGAAGAGGAATCCTGGCATATCATCAATTGAATAAGTTGAATTAATTATTTCAATCTCATTCTCACTTTCTGACTTATAGTCGCTTTGTTAAATCAT
TTCTATTTCCTTCTCCTTAGGACCGTATAGTAGTTAACTTATTCAACTTAATTAATAAAGTTAGAGTAAGAGTGAAAGACTGAATATCAGCGAAACAATTTAGTA

nmt1 promoter (REP41) | MCS

3,580 3,590 3,600 3,610 3,620 3,630 3,640 3,650 3,660 3,670

XmaI
SalI BamHI SmaI

ATGTCGACTCTAGAGGATCCCCGGGTAAAAGGAATGTCTCCCTTGCCAGTACTGCTAGGGTTTTTCTTTCAAACTATGGAAGCCCATTCAAGCTGCATATTACGA
TACAGCTGAGATCTCCTAGGGGCCCATTTTCCTTACAGAGGGAACGGTCATGACGATCCCAAAAAGAAAGTTTGATACCTTCGGGTAAGTTCGACGTATAATGCT

MCS | nmt1 terminator

3,680 3,690 3,700 3,710 3,720 3,730 3,740 3,750 3,760 3,770 3,780

TTTTGTTTTTCGCTTTTAGAAAGTGGTTTAGATGAGATAATAGAAAAATTCTTGATCTCCGACAACGAGTACTTTTATTTTTTTTGCTAATCACTTTACTCAATA
AAAACAAAAAGCGAAAATCTTTCACCAAATCTACTCTATTATCTTTTTAAGAACTAGAGGCTGTTGCTCATGAAAATAAAAAAAACGATTAGTGAAATGAGTTAT

nmt1 terminator

3,790 3,800 3,810 3,820 3,830 3,840 3,850 3,860 3,870 3,880

PmlI

TTAGCTCGAAATCGTAGAAACGTAGACGGGTGCGGGATACCGAGTGGTGTAGTTAAGAATTTTTATAAACCACGTGGCCCAAAAATATGAACCCAAAACGTTTAT
AATCGAGCTTTAGCATCTTTGCATCTGCCCACGCCCTATGGCTCACCACATCAATTCTTAAAAATATTTGGTGCACCGGGTTTTTATACTTGGGTTTTGCAAATA

nmt1 terminator

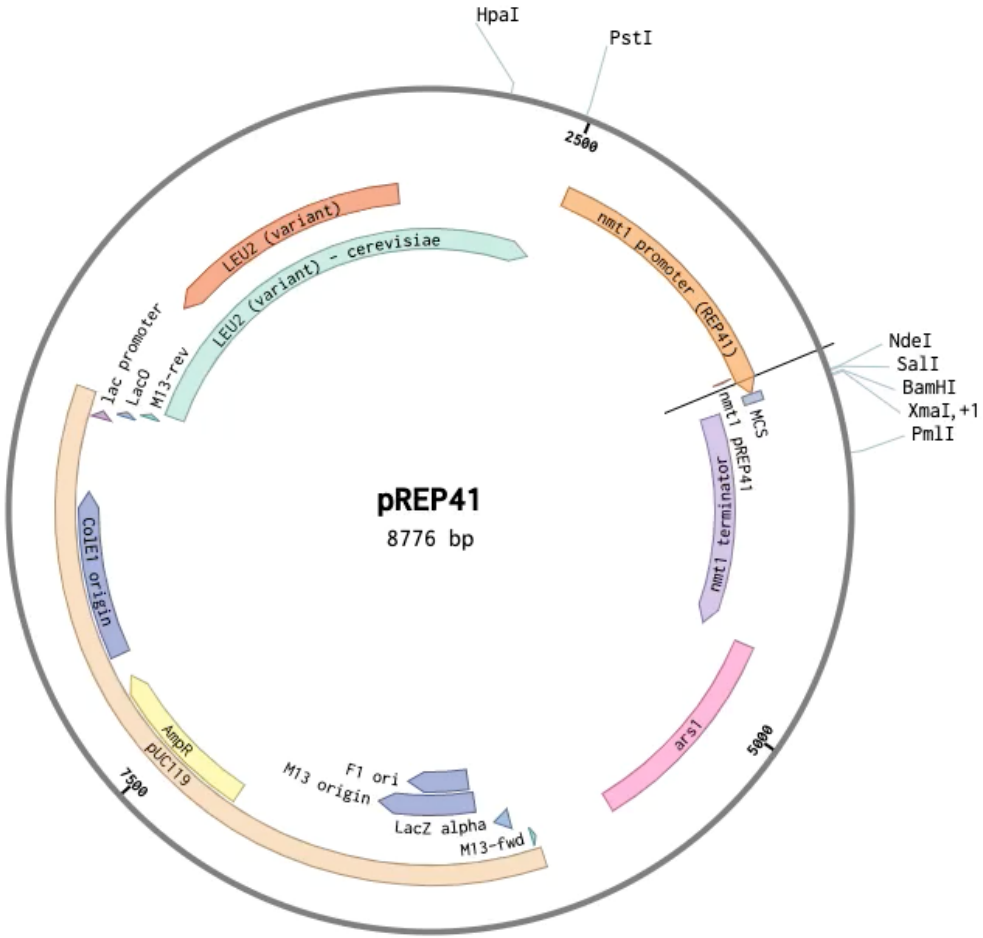
3,890 3,900 3,910 3,920 3,930 3,940 3,950 3,960 3,970 3,980 3,990

ACATGAGTATACTTTAAGAAGGCTATACCCCTTCGTGTTAGATGTAGTTTTAGCTACCCAACCCGAGTCTATGAGCTTGACTTCAGATGTAGAAGGCATTAAATC
TGTACTCATATGAAATTCTTCCGATATGGGGAAGCACAATCTACATCAAAATCGATGGGTTGGGCTCAGATACTCGAACTGAAGTCTACATCTTCCGTAATTTAG

nmt1 terminator

METADATA | DESCRIPTION | PLASMID

PDF



BASES 8776 | INSERT 3602 | ASSEMBLY WIZARD | SPLIT WORKSPACE

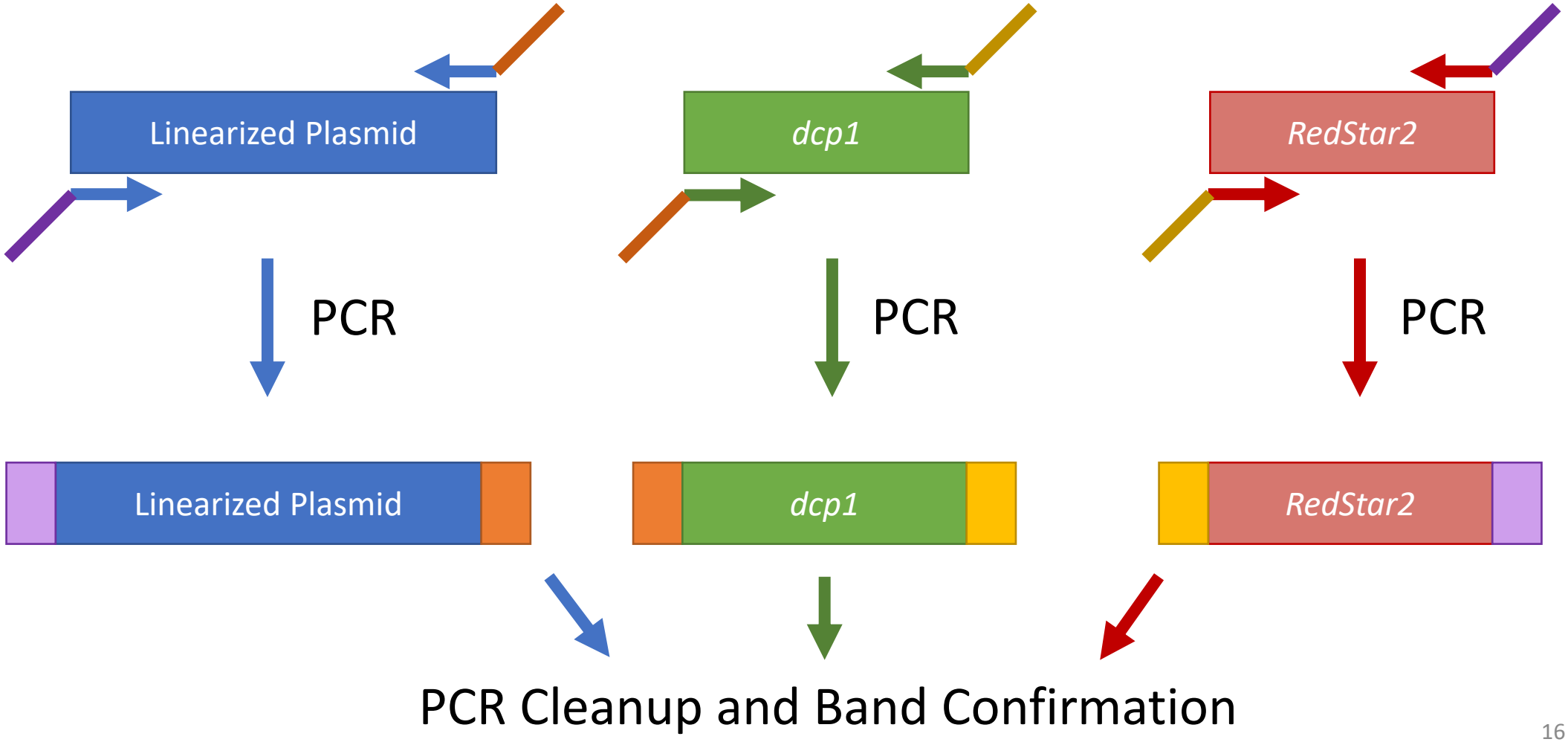
Design Primers in Benchling

- Benchling can do the first pass on designing primers for Gibson using the "Assembly Wizard."

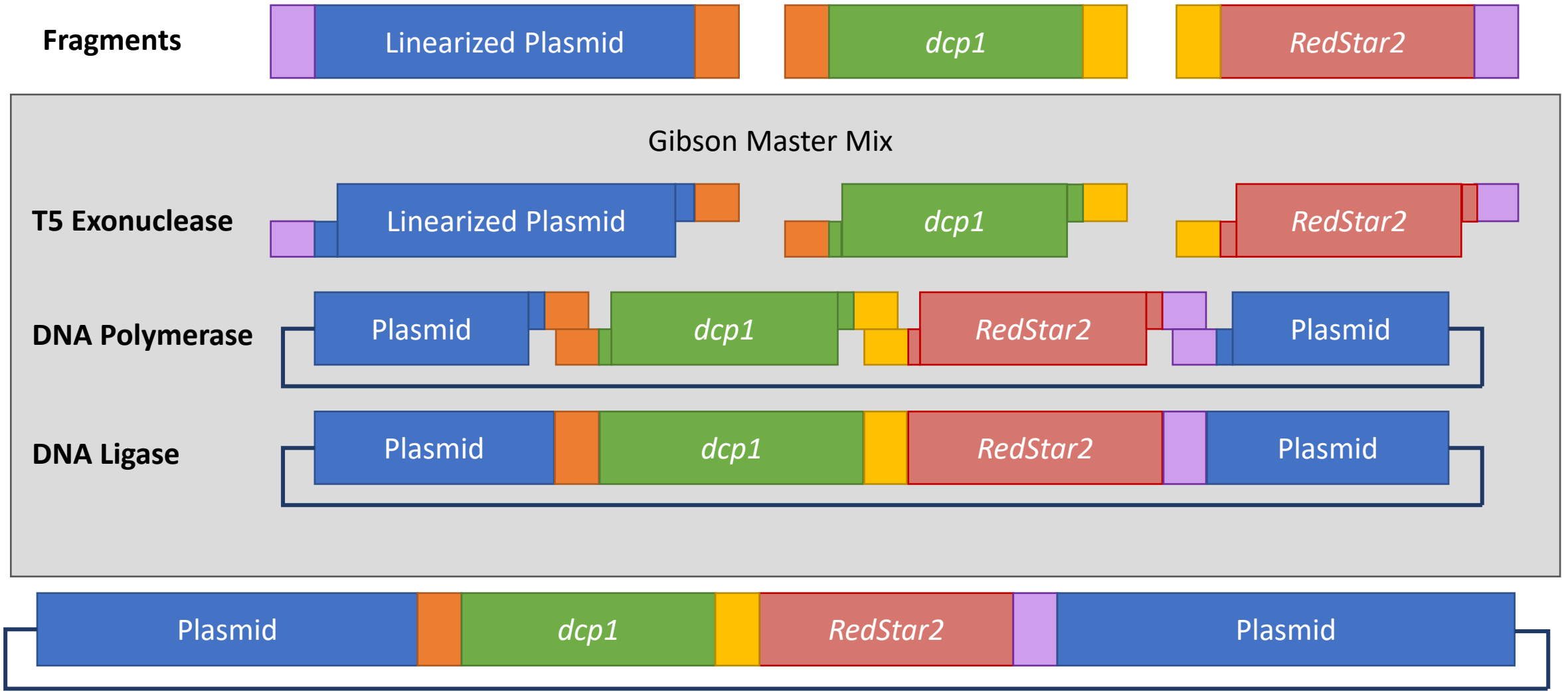
- Benchling provides us with an *in-silico* pre-run of our Gibson assembly reaction and helps us by creating primers that we can use for our PCR.
  - *Note:* you will need to check for melting temps and GC content.

- 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

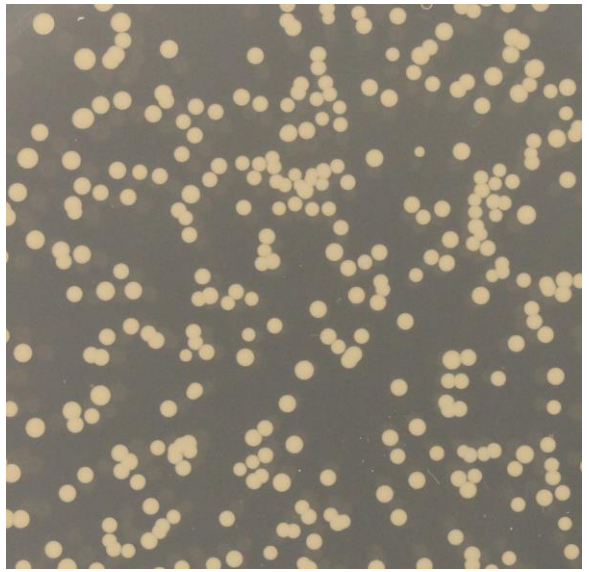


Mix Together Fragments



Transformation

- If you made a plasmid compatible with *E. coli* like we did here, then it can into a strain such as DH5a.
  - 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

Yeast cloning vector

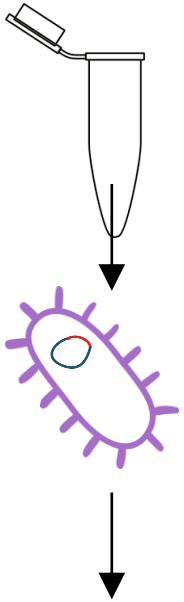
Linearized Plasmid

dcp1

RedStar2

Gibson Assembly

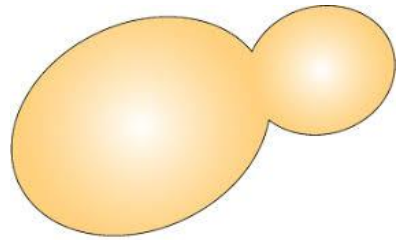
T5 Exonuclease
DNA Polymerase
DNA Ligase



1 hours

Overnight incubation

Yeast Assembly

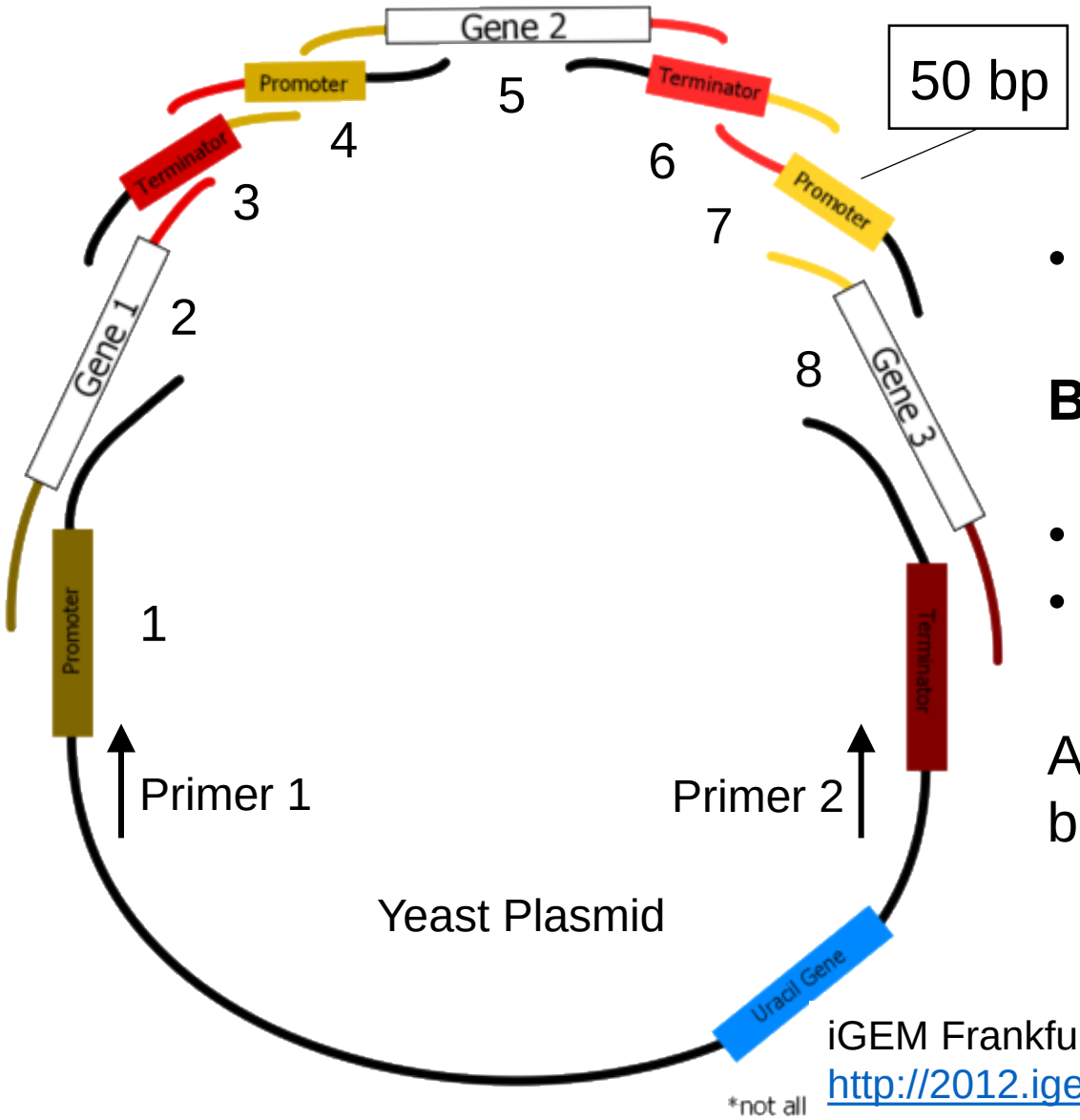


Mix: 30 min

2-3 days incubation

Plasmid | *dcp1* | *RedStar2* | Plasmid

When does it make sense to use Yeast assembly



- When you work with yeast as a final cloning host

But also …

- When you assemble many fragments (more than 3)
- When one or more fragments are short (< 100 bp)

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

iGEM Frankfurt 2012
http://2012.igem.org/Team:Frankfurt/New_Yeast_RFC

Usefulness of yeast homologues recombination

2008: Assembly of Mycoplasma genome

PNAS

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

Daniel G. Gibson[a,1], Gwynedd A. Benders[b], Kevin C. Axelrod[a], Jayshree Zaveri[a], Mikkel A. Algire[a], Monzia Moodie[a], Michael G. Montague[a], J. Craig Venter[a], Hamilton O. Smith[b], and Clyde A. Hutchison III[b,1]

[a]The J. Craig Venter Institute, Synthetic Biology Group, Rockville, MD 20850 and [b]The J. Craig Venter Institute, Synthetic Biology Group, San Diego, CA 92121

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

2018: Synthetic yeast chromosomes

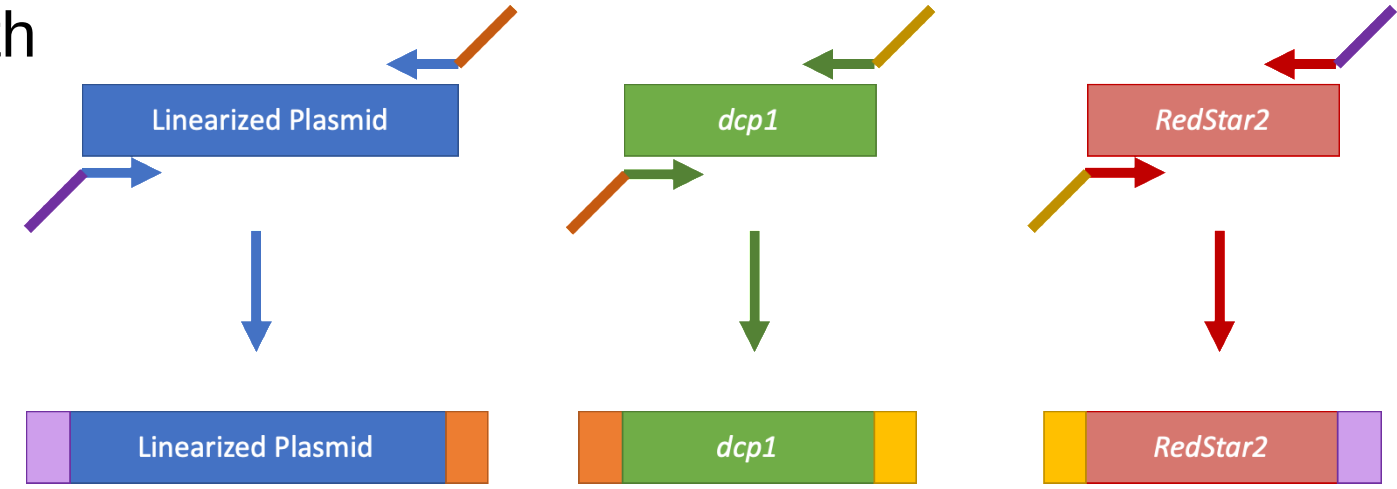
Yeast assembly step-by-step

You need:

- Yeast cloning strain: e.g. BY4741 (ATCC201388)
  Auxotrophic markers: Leucine, Uracil, Histidine, Methionine
  (his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)

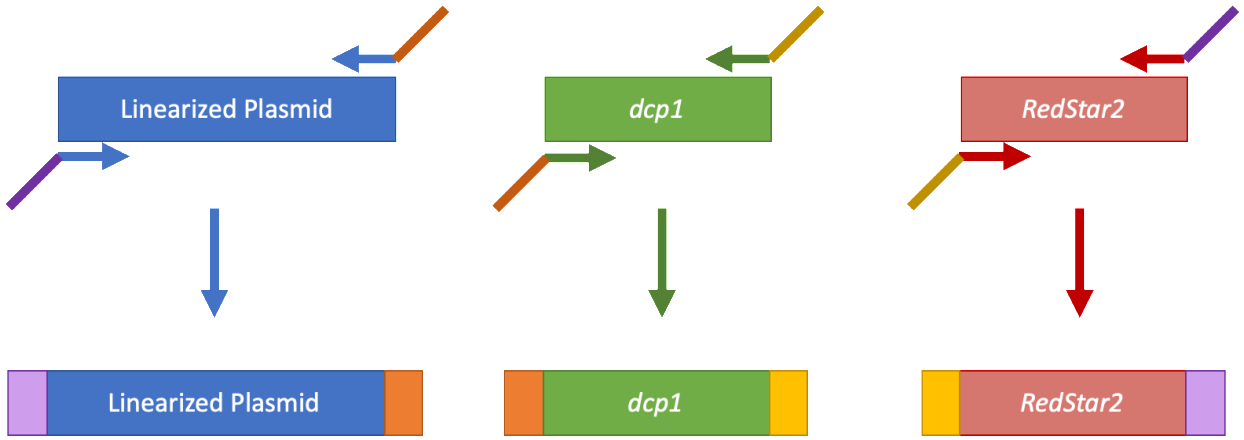
- Yeast plasmid/backbone: e.g. pRS series

- Your PCR amplified fragments with
~40 bp homologues overhangs



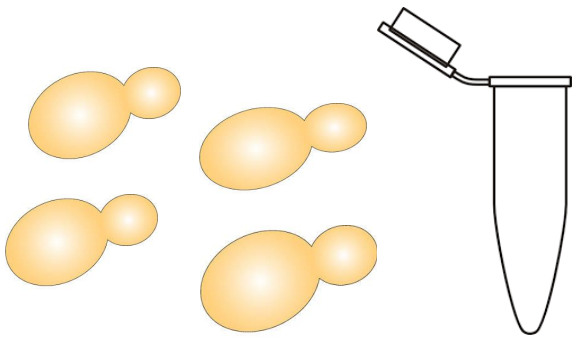
Yeast assembly step-by-step

1. Generation of fragments by PCR



3. Selection of colonies

2. Yeast transformation



- 100 ng of plasmid
- 4-10 molar ratio of each insert

LiOAc/PEG method

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

http://2012.igem.org/Team:Frankfurt

- LiOAc/PEG transformation protocol

https://benchling.com/protocols/QOCa7BoO/yeast-transformation-protocol

- Molecular mechanism of homologues recombination

Recombination Proteins in Yeast
https://www.annualreviews.org/doi/abs/10.1146/annurev.genet.38.072902.091500