L12 Advanced DNA Assembly

Instructor: Prof. **K. Solomon Ph.D.**
Assistant Professor
Agricultural & Biological Engineering
Laboratory for Renewable Resources Engineering

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Recall…

Required components for DNA delivery

4 mechanisms to assemble DNA

1. Chemical addition
2. Homologous recombination
3. Annealing & Polymerization
4. Digestion & Ligation

BioBrick™ Std

Advantages	Disadvantages
- idempotent/scalable - Easily automatable - Robust - Only 4 REs used	- Need to remove internal Biobrick RE sites from all parts - Have a fixed scar (not good for protein fusions) - Can't add parts between parts

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This lecture….

- Scarless digestion + ligation
- Anneal + polymerization methods
- Recombination–based methods
- Summary

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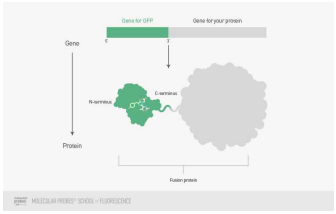
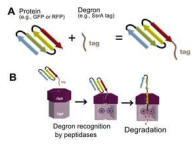
Fusion proteins are common in biotech

Scars between parts may cause frameshift mutations or disrupt folding (function)



thermofisher.com



parts. iGEM.org

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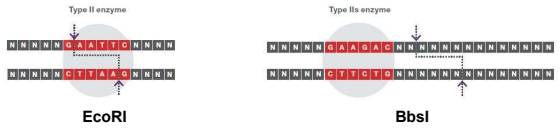
Type IIS restriction enzymes allow you to define overhang (and ligation scars)



Type IIs REs:
- Cleave outside their restriction site
- Are not palindromic
- Can give user-defined sticky ends

Cuts are directional

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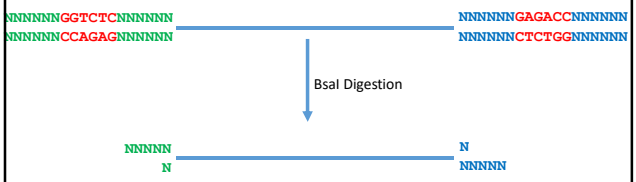
Golden Gate Cloning

GGTCTC NNNNNN
CCAGAGNNNNNN

Uses BsaI



Restriction site removed after digestion

Process is scalable – can be repeated on composite parts without digesting components

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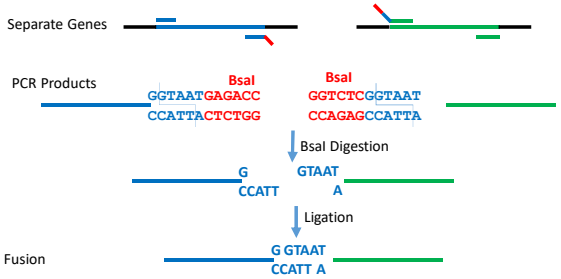
Golden Gate

Primers used to define overhangs for scarless fusions



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Golden Gate

GGTCTC NNNNNN
CCAGAGNNNNNN

Cleavage site is user-defined (specified by position relative to recognition site)
- Can specify linker for fusion proteins
- Can do multi-part assembly with 1 enzyme simultaneously
  - Assembly specified with unique complementary overhangs for each junction

- Cloning is simple and fast
  - Can do digestion + ligation simultaneously (one-pot) ~30min
  - Re-ligation of backbone quickly digested because site is preserved

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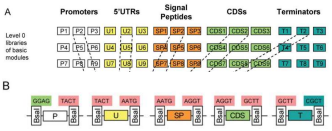
Golden Gate

Real Example: Modular Cloning (MoClo)

- Parts constructed in individual plasmids
- Parks flanked by BsaI site with complementary, user-defined overhangs

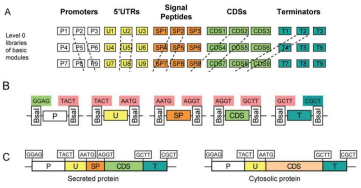


Weber, E. et al. (2011). *PLoS ONE.*

Golden Gate

Real Example: Modular Cloning (MoClo)

- Parts constructed in individual plasmids
- Parks flanked by BsaI site with complementary, user-defined overhangs
- Digestion and ligation result in assembly of multi-part constructs

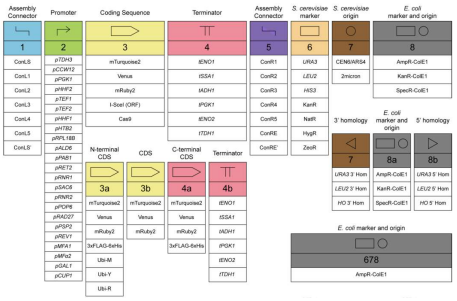


Cloning is simple and fast
- Can do digestion + ligation simultaneously (one-pot) ~30min
- Re-ligation of backbone quickly digested because site is preserved

Weber, E. et al. (2011). *PLoS ONE.*

Golden Gate (MoClo)

Formatted part libraries



Lee, ME. et al. (2015). *ACS Synthetic Biology.*

Golden Gate

Advantages	Disadvantages
• Simple and Fast • Scarless (good for fusions) • Good for libraries (efficient) • Only one RE • **Up to 10 parts** assembled in a single one pot reaction without purification	• Parts need to be in appropriate format + no internal sites • Overhangs must be unique to prevent misassembly

Sequence-independent cloning methods

RE-based assembly requires part formatting that may be difficult

- Overhangs, no internal restriction sites

POLYMERIZATION + ANNEAL

1. Gibson Chew Back Anneal (CBA)
2. Sequence + Ligase Independent Cloning (SLIC)
3. Splicing by Overlap Extension PCR (SOEing)

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Gibson Assembly

Requires:

- Linear DNA with long regions of homology (~20 – 50 bp) @ junctions
- 3 enzymes:

1. T5 Exonuclease
   Chews back 5' end of DNA to make 3' overhang

2. Phusion/Proof-reading DNA polymerase
   Repairs the gaps in overhangs via PCR
3. Taq DNA Ligase
   Forms covalent bonds between parts

Enzymes chosen to work together in one pot at one temperature

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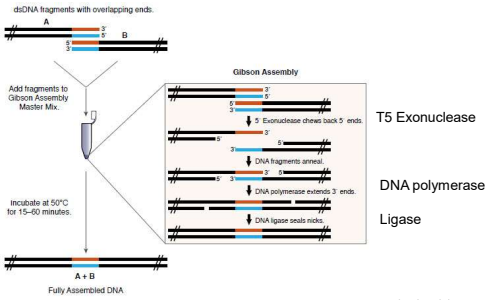
Gibson Assembly

Workflow



New England Biolabs, Inc.

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Gibson Assembly

Advantages	Disadvantages
• Parts don't need to be formatted (sequence independent) • Can make combinatorial libraries (**up to 5 parts**) • One-pot reaction (fast/simple) • Scarless	• Requires long primers that may be challenging • Expensive

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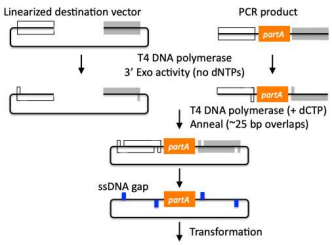
Sequence + Ligation Independent Cloning (SLIC)

- Like Gibson but no ligase or dNTPs to repair chew back
  - T4 DNA polymerase has 3 exonuclease activity in absence of dNTPs
  - dCTPs added to control amount of chew back
- Repair completed in host



https://j5.jbei.org/j5manual/pages/22.html

Advantages	Disadvantages
• Cheaper	• Less efficient (bad for libraries)

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Splicing by Overlap Extension PCR (SOEing)

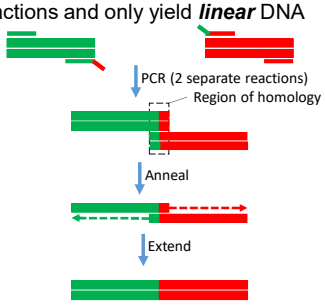
Uses PCR to "stitch" together DNA
- PCR primers generate homology or adjacent parts
- Requires multiple reactions and only yield ***linear*** DNA

1 – Amplify parts separately

2 – Purify and then anneal products (melt + cool slowly)

3 – Products serve as "mega-primers" for each other

Extend/Amplify ~5 cycles to make complete product



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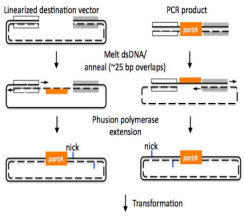
SOEing → CPEC

- Can do similar procedure with a vector + parts (no ligase needed) = Circular Polymerase Extension Cloning (CPEC)
- Using just overlapping primers is how genes are synthesized



JBEI.org

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SOEing Limitations

- Large stretches of ssDNA
  - Must avoid forming stable secondary structures and repeats
    - Can lead to assembly failures or mutations at junctions

But potentially, cheaper → only need primers and polymerase

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Recombination-based methods: Gateway Cloning

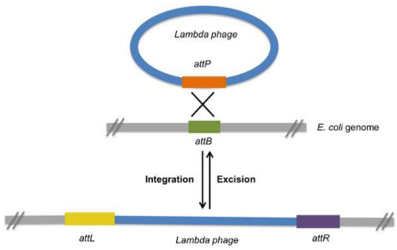

Figure 1: Lambda phage integration and excision reactions. Recombination of attP and attB sites creates attL and attR sites.

Addgene.org

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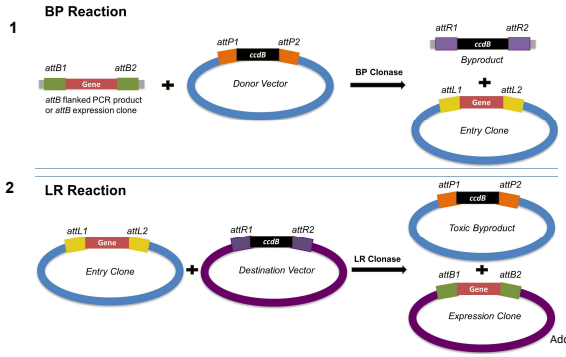
Recombination-based methods: Gateway Cloning



Addgene.org

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Gateway Cloning

Advantages	Disadvantages
• Fast • Can make combinatorial libraries (**up to 4 parts**) • Efficient	• Fixed vector choices

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Next time….

- How to 'format' parts for assembly
- Practice problems

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