L13 Advanced DNA Assembly

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

Fall 2018

Recall…

Mechanism	Assembly Method	Pros	Cons
Digestion + Ligation	Biobricks	- Scalable - Automatable - Robust - Only 4 REs	- Needs formatting - Fixed scar - Can't add between parts
	Golden Gate/MoClo	- Simple one-pot - Scarless - Efficient - Up to 10 parts assembled	- Needs formatting - Unique overhangs must be designed
Polymerization + Anneal	Gibson CBA	- No internal formatting - Up to 5 parts assembled - scarless	- Long primers - expensive
	SLIC	- Cheaper than CBA	- Less efficient
	SOEing/CPEC	- Cheaper than CBA	- Less efficient/challenging
Recombination	Gateway	- Fast - Up to 4 parts assembled - efficient	- Limited vector choices

This lecture….

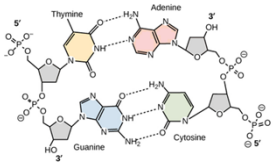
- PCR Review
- Site Directed mutagenesis
- Practice

Polymerase Chain Rxn (PCR)

https://youtu.be/x5yPkxCLads
GTCA - https://youtu.be/ID6KY1QBR5s

PCR amplifies DNA

- Exploits double stranded nature of DNA



- Fixed Watson-Crick pairing rules allow each strand to serve as a template for replication

5

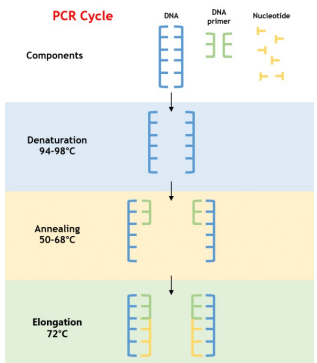
PCR requires 5 components

1. DNA template
   Molecule to be replicated
2. dNTPs
   Building blocks of DNA + energy for replication
3. DNA polymerase
   Enzyme that replicates in 5' → 3' direction only. Adds dNTPs to 3' OH of existing deoxyribose group
4. 2x Primers
   Single stranded DNA that is complementary to region to be amplified
   Targets sequence for replication
5. Buffer components (e.g. Mg^{2+})
   Cofactors needed for enzyme activity

6

PCR amplification relies on temp stability of DNA polymerase

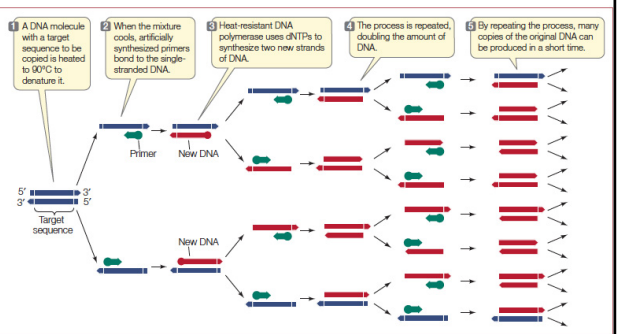
1. **Initialization** (~94-98 °C for 30s - 2 min)
   Activates DNA polymerase and melts DNA
2. **Denaturation** (~94-98 °C for 5 – 30 s)
   First part of the cycle
3. **Annealing** (~50-68 °C for 5 – 30 s)
   First part of the cycle. Anneal temp set by primer melt temperature
4. **Elongation** (72°C for 15s – 60 s/ kb)
   Copies DNA. Makes 2 molecules /cycle
5. **Return to Step 2** 20-40x



7

https://www.clinisciences.com/en/buy/cat-conventional-pcr-3473.html

PCR is exponential and semi-conservative

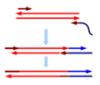


After 30 cycles – 1 molecule → ~10^9 molecules

8

PCR only requires 3' end of primer for binding

Can introduce arbitrary sequence at 5' end to be incorporated in product

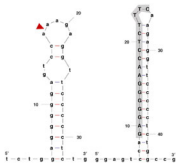


wikipedia.com

e.g. Biobrick prefix/suffix, Gibson homology, etc

There are practical limits to 5' extensions (~50 - 80 bps)

- longer sequences more likely to misprime
- More likely to form stable 2° structures



https://www.researchgate.net

9

Site-directed mutagenesis to remove 'forbidden' sequences

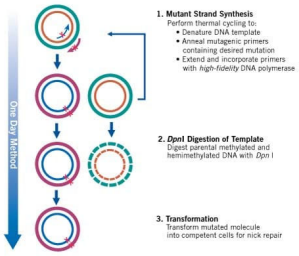
Primers may still work with a handful of mismatches

Primers:

- missing bases → deletion
- extra bases → insertion
- substitutions → point mutation

Staggered mutagenic primers create a SLIC-like product that is repaired in cell

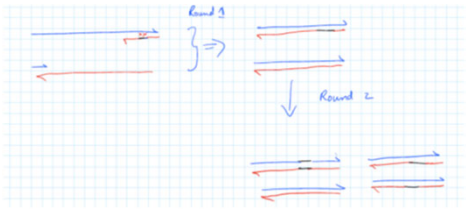
Nominally used for 2-3 simultaneous bp mutations



Agilent.com

10

But what about semi-conservative replication of DNA???



DNA Molecules	Cycle	Original	Hybrid	Mutated
	0	1	0	0
	1	1	1	0
	2	1	2	1
	3	1	3	4

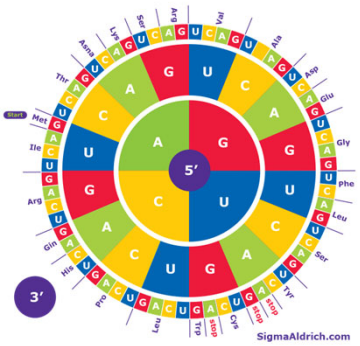
Mutation dominates after only 3 cycles

11

DNA code is redundant

Can alter nucleotide sequence to remove features (e.g. internal RE site) without impacting function

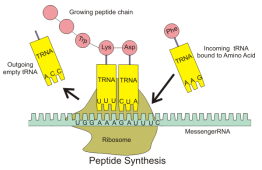
- 4^3= 64 codons for ~ 21 AAs



12

How do we pick b/n codon alternatives?

- Each codon specified by a unique tRNA
- Organisms have **codon bias** dictated by tRNA abundance
  - Proteins with more abundant codons/tRNAs are translated @ higher rates



Peptide Synthesis

wikipedia.com

Codon	Human	Drosophila	E. coli
Arginine:			
AGA	22 %	10 %	1 %
AGG	23 %	6 %	1 %
CGA	10 %	8 %	4 %
CGC	22 %	49 %	39 %
CGG	14 %	9 %	4 %
CGU	9 %	18 %	49%
Total number of arginine codons	2403	506	149
Total number of genes	195	46	149

http://www.blackwellpublishing.com/ridley/

13

Codon optimization essential for expression

- Many rare codons give poor expression
- Using only abundant codons might starve endogenous protein synthesis
- Proteins w/ multiple domains need to fold them independently for function
  - Less common codons might separate domains to introduce "translational pausing" for proper folding
- Metrics such as codon adaptation index (CAI) capture the codon bias and provide targets for optimization

Moving proteins b/n organisms typically requires codon optimization for proper expression

14

PCR is NOT error proof

- Nucleotide addition frequently erroneous and must be corrected
  - Taq = 2.28 x 10^{-5} mutations/bp amplified → **68.4% of 1 kb product** will have at least one mutation after 30 cycles
  - High-fidelity Phusion = 4.4 x 10^{-7} mutations/bp amplified → **1.3% of 1 kb product** will have at least one mutation after 30 cycles

All PCR-based modifications should be sequence verified

15

Next time

- Genome Engineering

16