Building with DNA: methods and applications

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Today we are going to discuss methods to build with DNA

- Cloning with restriction enzymes
  - Traditional cloning
  - Biobricks
  - Golden Gate
- Cloning without restriction enzymes
  - Gateway cloning
  - Circular polymerase extension cloning (CPEC)
  - Ligation-independent cloning
  - SLiCE
  - Gibson method (*in vitro*)
  - Gibson method (*in vivo*)

What does it mean to clone a gene?

Cloning with restriction enzymes

Tool to find cut sites in DNA sequence (pIMP1 example):
http://tools.neb.com/NEBcutter2/

- What enzymes cut the plasmid where I want my gene?
- Do these enzymes cut elsewhere in the plasmid?
- Do these enzymes cut the gene I want to insert into the plasmid?

restriction enzyme cloning

PCR gene with restriction sites at 5' end of primers

↓

Cut plasmid and PCR product with restriction enzymes (add CIP to plasmid to remove 5' phosphates)

↓

Ligate (ligase is DNA glue) PCR and plasmid: 5' phosphates attack 3' OH

↓

transform into *E. coli*: heat or electrical shock

↓

Select on petri dishes for antibiotic resistance from plasmid

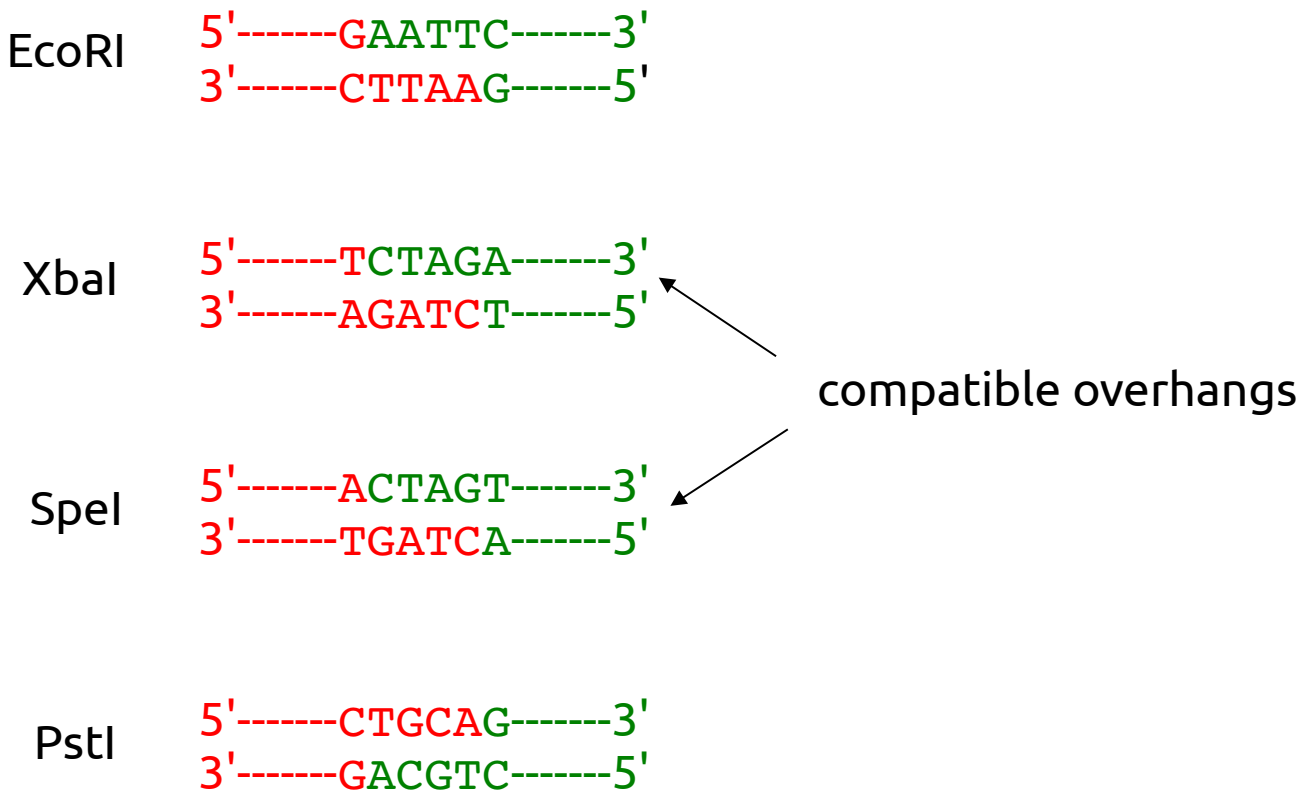
↓

Sequence plasmid to confirm that insert is correct

Biobricks let you assemble genes one-by-one using only 4 restriction enzymes

www.biobricks.org

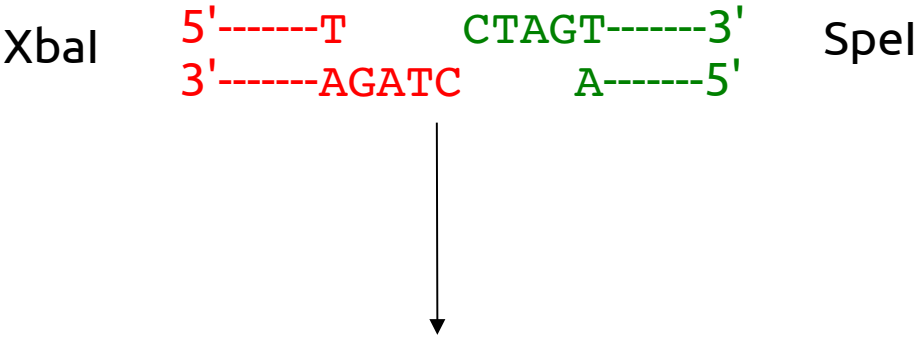
4 restriction enzymes



XbaI and SpeI create compatible overhangs, EcoRI and PstI do not

www.biobricks.org

XbaI and SpeI fragments can be ligated



The resulting DNA is no longer a restriction site (scar)

```
5'-------TCTAGT-------3'
3'-------AGATCA-------5'
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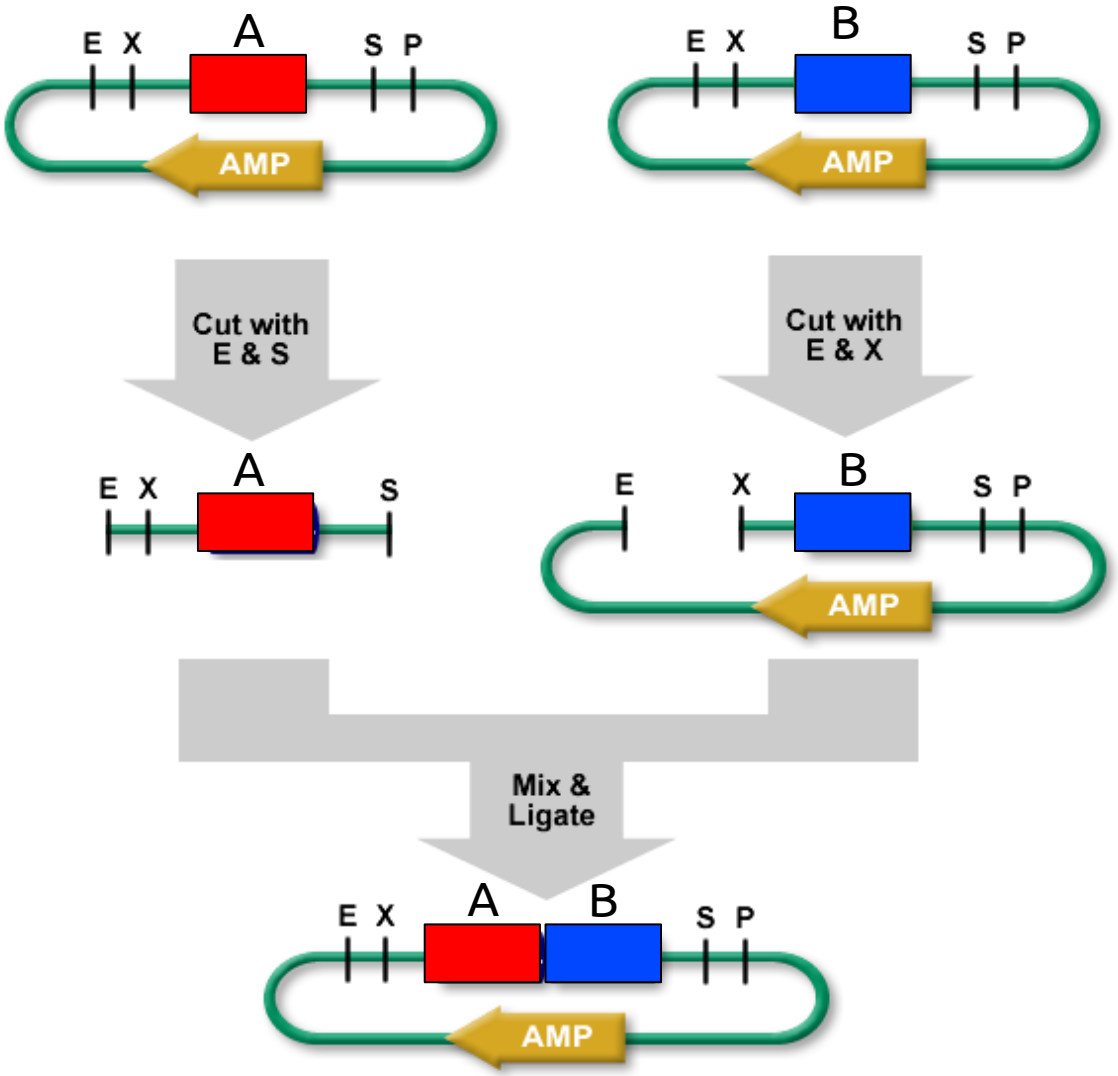
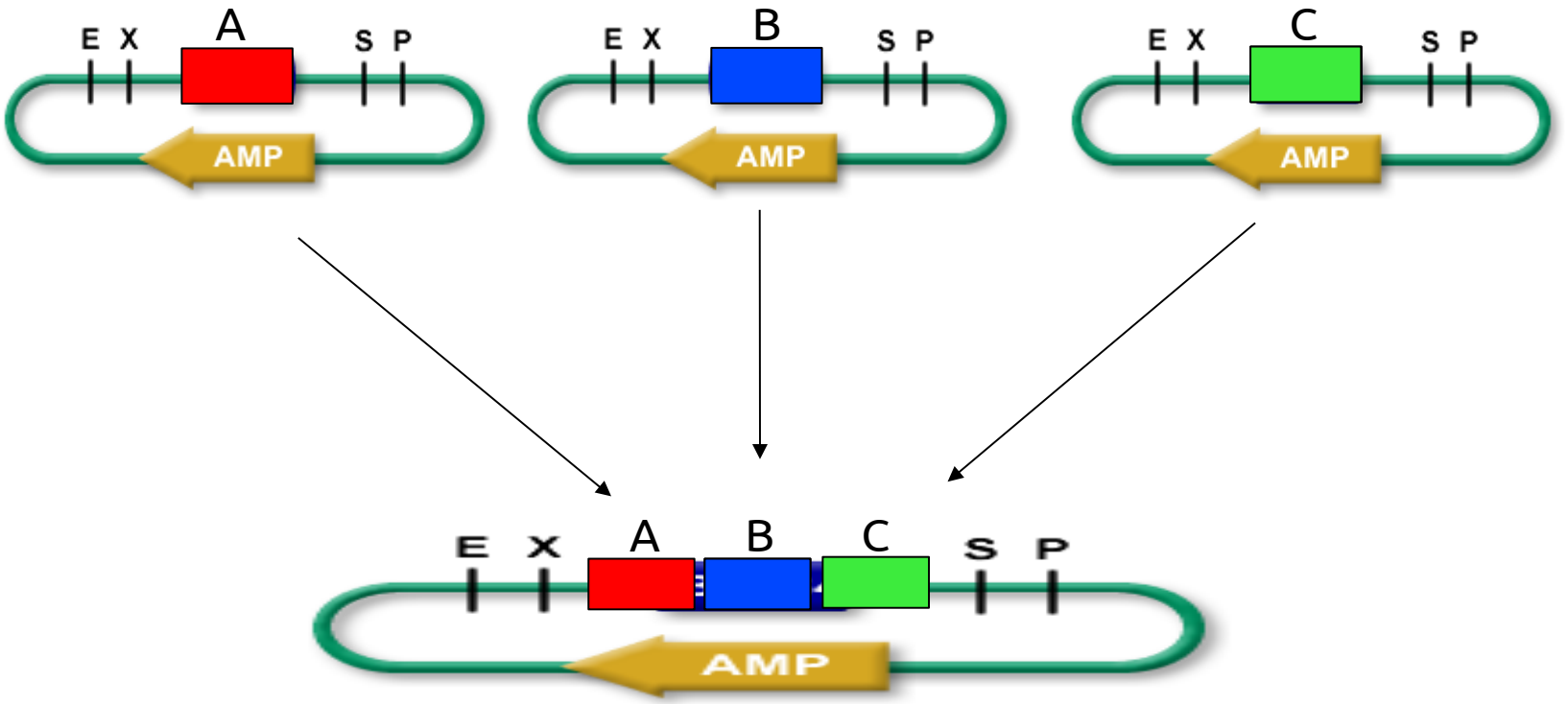
Biobrick assembly



http://partsregistry.org/Assembly:Standard_assembly

How would you assemble 3 Biobricks?



Biobricks conclusions

Advantages

-Only 4 restriction enzymes needed

-Easy one-by-one assembly of genes

Disadvantages

-DNA cannot contain other EcoRI, SpeI, XbaI, PstI sites

-Time consuming (relative to other methods)

-Assembling multiple fragments is difficult

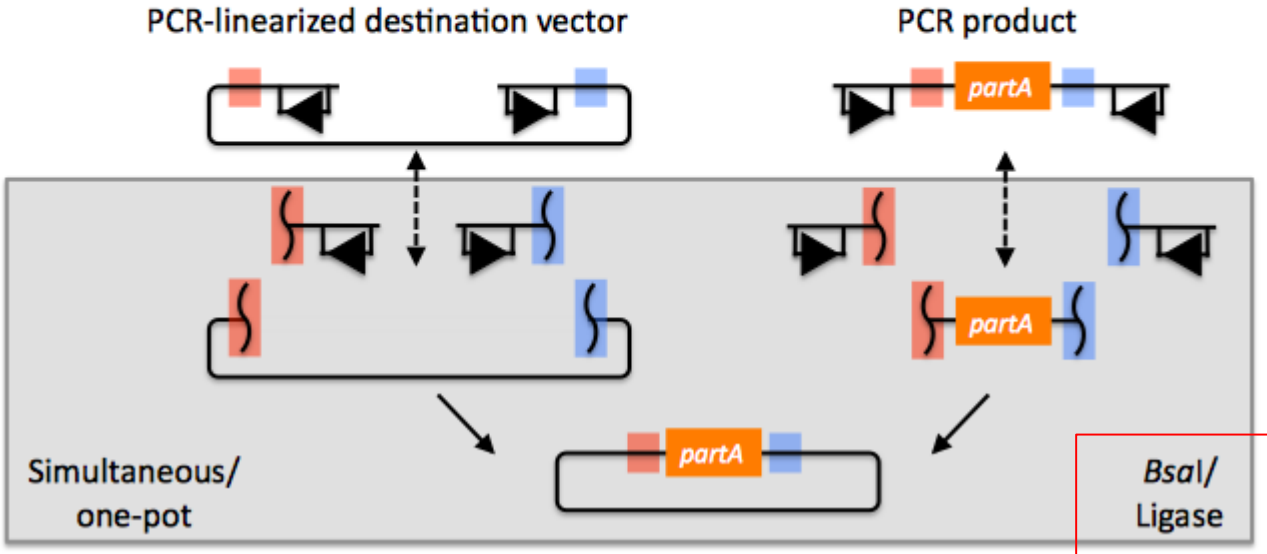
Golden Gate Assembly

Engler et al, 2008 PMID 18985154

Multi-part assembly using type IIS restriction enzymes

-Type IIS cut outside recognition Site

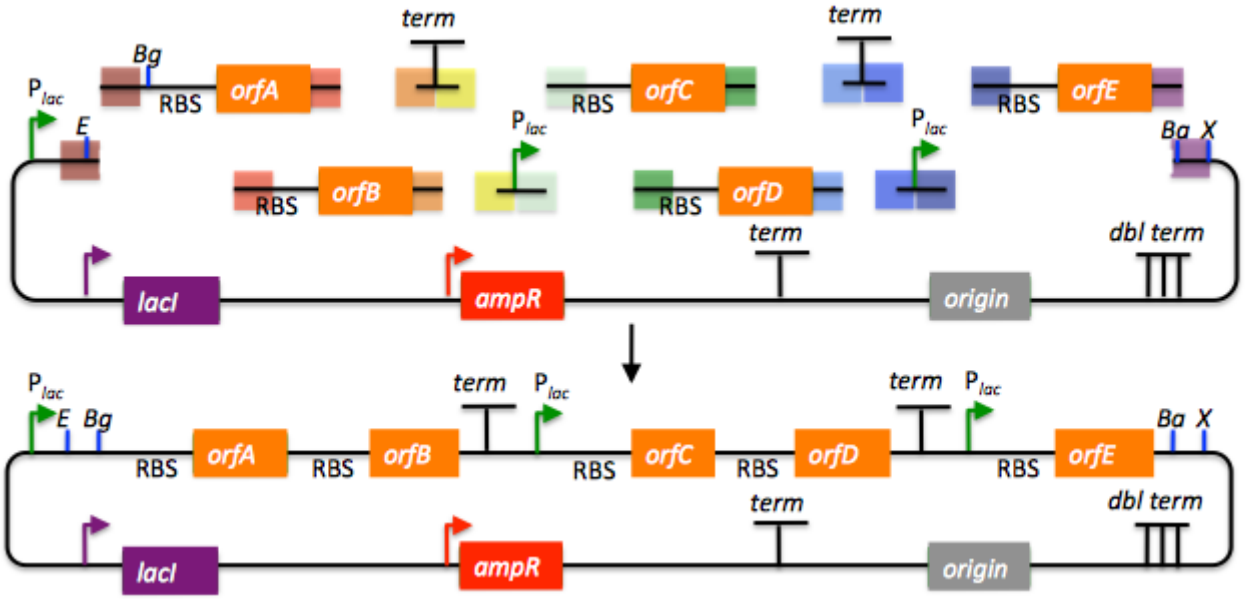
-BsaI site is non-palindrome (directional)



http://j5.jbei.org/j5manual/pages/23.html

1st rxn reversible.
Second is not.

Multi-part Golden Gate assembly



http://j5.jbei.org/j5manual/pages/23.html

Golden Gate conclusions

Advantages

-One pot assembly: 1 restriction enzyme and ligase

-Assemble many parts at the same time

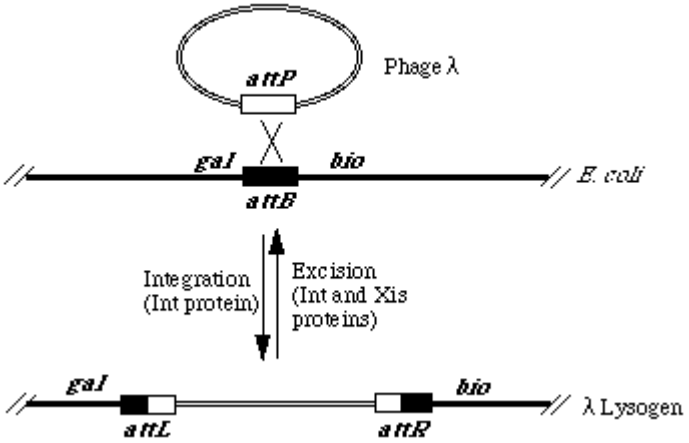
-Scar-less assembly

Disadvantages

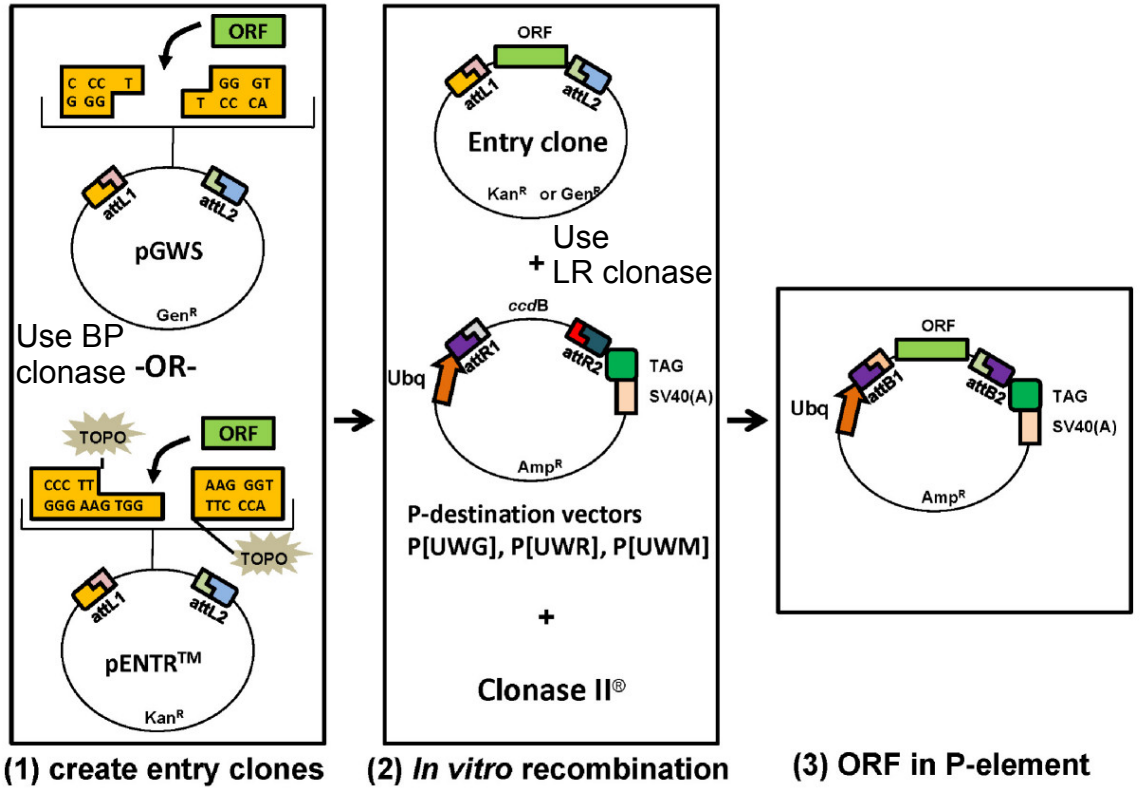
-Restriction enzyme sites cannot be in internal locations

-Sometimes difficult to find compatible 4bp overhangs

Gateway® cloning using phage att sites
(Life Technologies)



http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/phage/lambda-att-sites.html



1 create donor plasmid (attP sites) and PCR product (attB sites).

2 Mix PCR product + donor plasmid + BP clonase → entry vector contains PCR product (attL sites)

3 Mix entry vector+ destination vector (attR sites) + LR clonase → subclone gene into destination vector

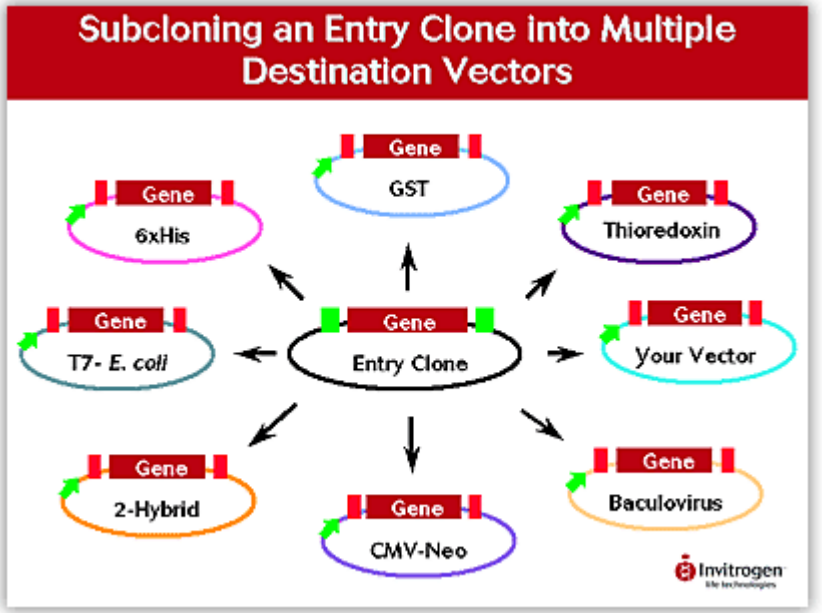
Akbari et al. BMC Cell Biology 2009 10:8 doi:10.1186/1471-2121-10-8

Gateway cloning

Advantages

-no restriction enzymes: everything comes in a kit

-easily subclone gene into many different destination Vectors (different tags, promoters, etc)

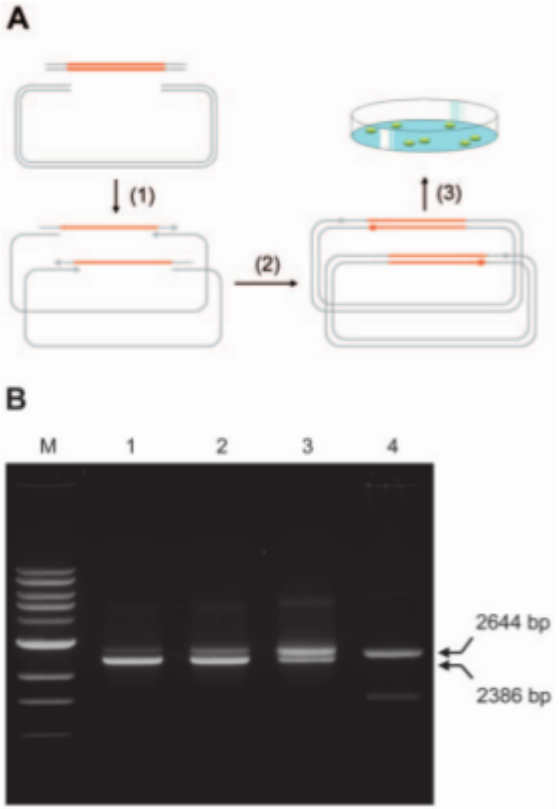


Disadvantages

-need to use specific plasmids

-expensive BP and LR clonase enzymes

Circular polymerase extension cloning (CPEC)



Denature DNA

↓

Reduce temperature to anneal overlapping ends

↓

Use DNA polymerase (5' to 3' extension) and dNTPs to make second strand

Quan and Tian 2009 (PMID 19649325)

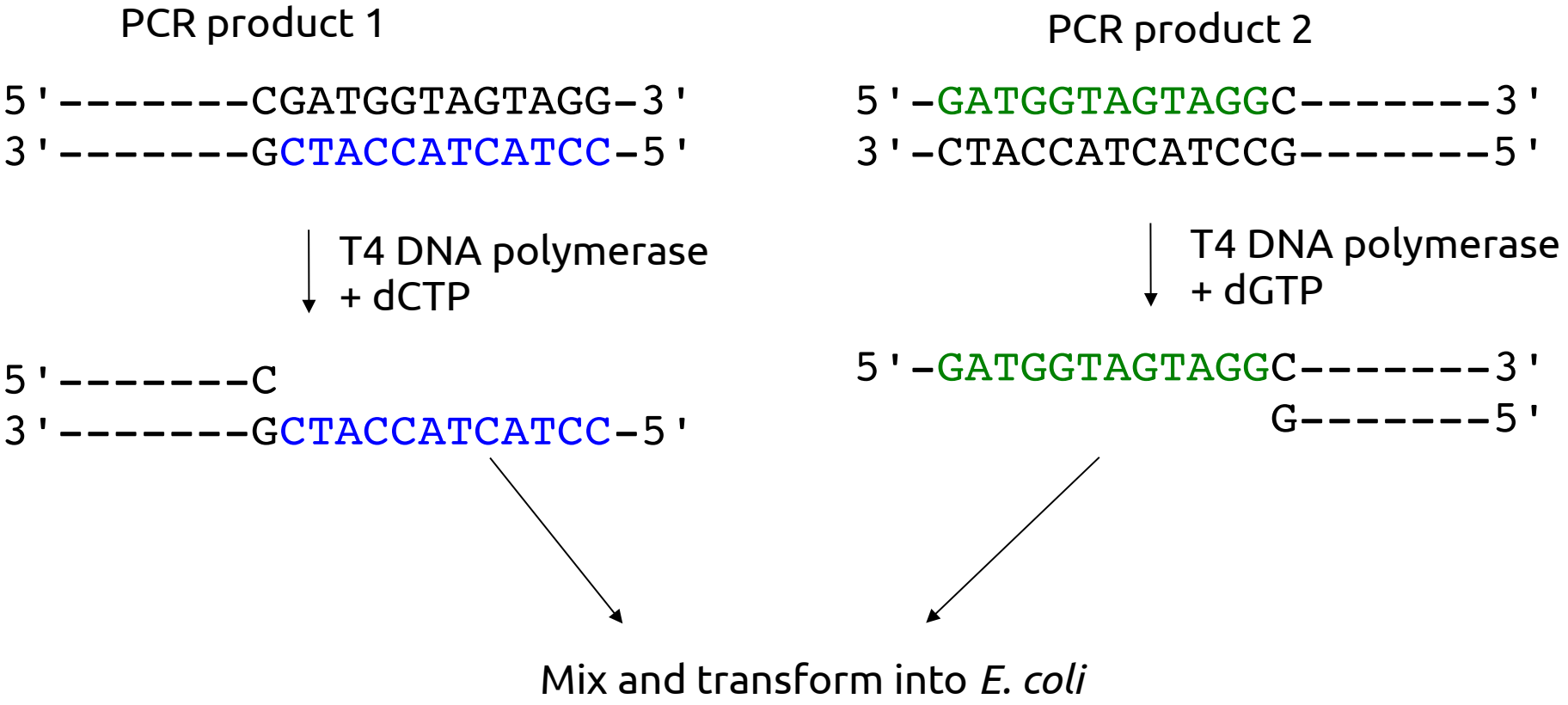
CPEC

- Advantages
  - Fast (no digestion, ligation, or recombination)
  - Simple (DNA polymerase is only enzyme needed)
- Disadvantages
  - Cloned DNA has nick at ends (cannot serve as PCR template)
  - Overlaps must be melting temperature (Tm) optimized so they anneal properly

Ligation-independent cloning (LIC)

Aslanidis and de Jong 1990 PMID 2235490

Use T4 DNA polymerase (extends 5'->3' AND degrades 3'->5') to create complementary overhangs



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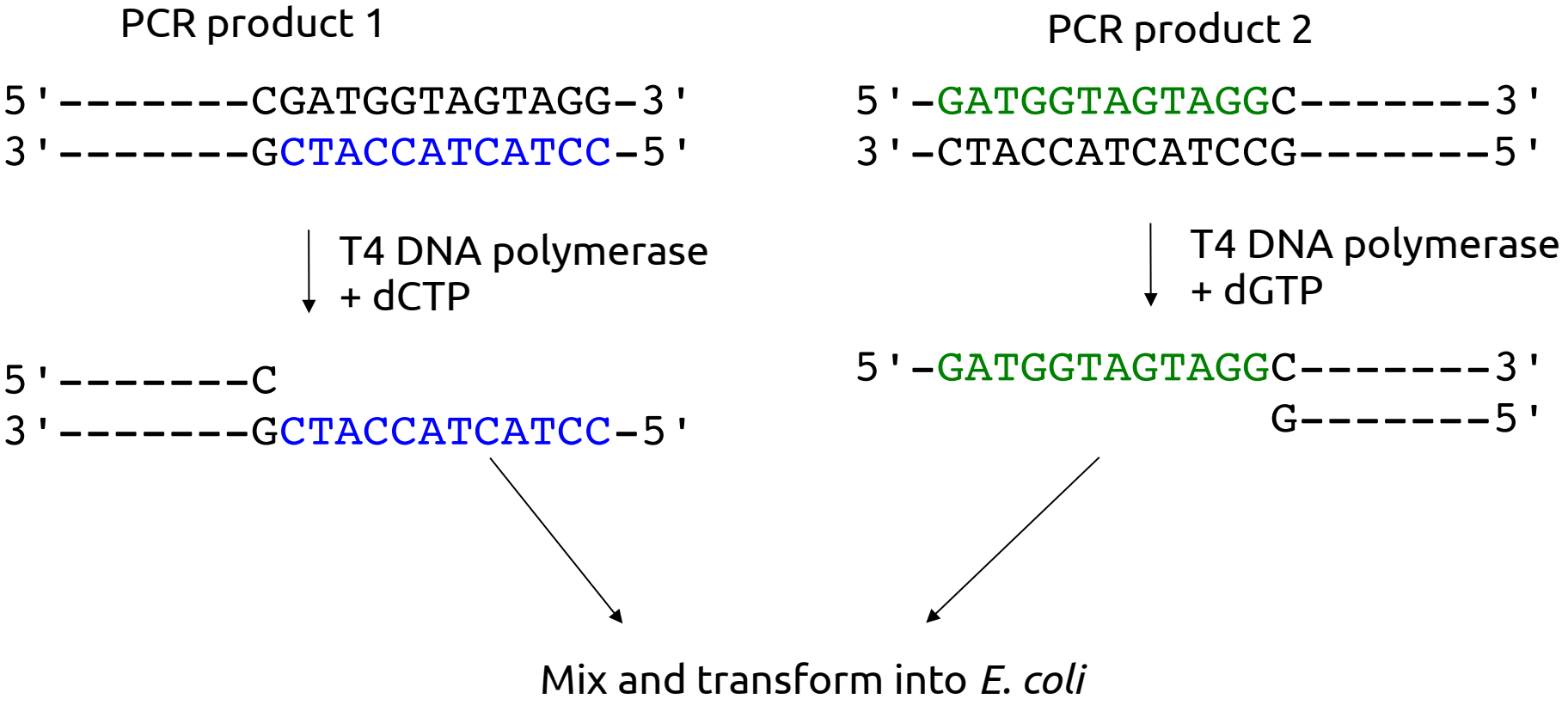


LIC conclusions

Advantages

-No restriction enzymes

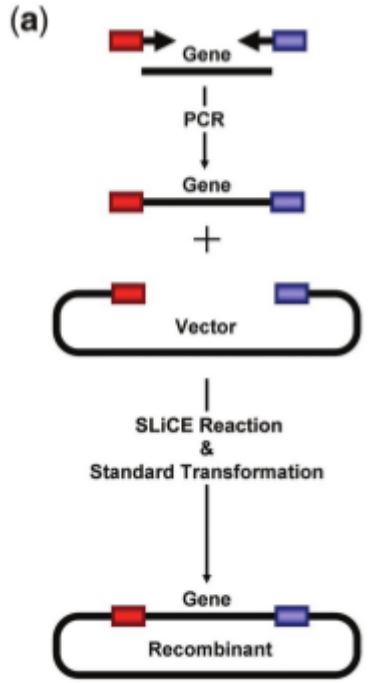
-Very fast and efficient

Disadvantages

-products likely contain gaps (cannot be used for PCR)

-need compatible overhangs missing 1 nucleotide type

SliCE: use bacterial cell extract to assemble DNA fragments



Enzymes from bacterial cell extract enable one-step cloning

Zhang 2012 (PMID 22241772)

Bacterial cell extracts contain all necessary enzymes for cloning (exonuclease, polymerase, ligase)

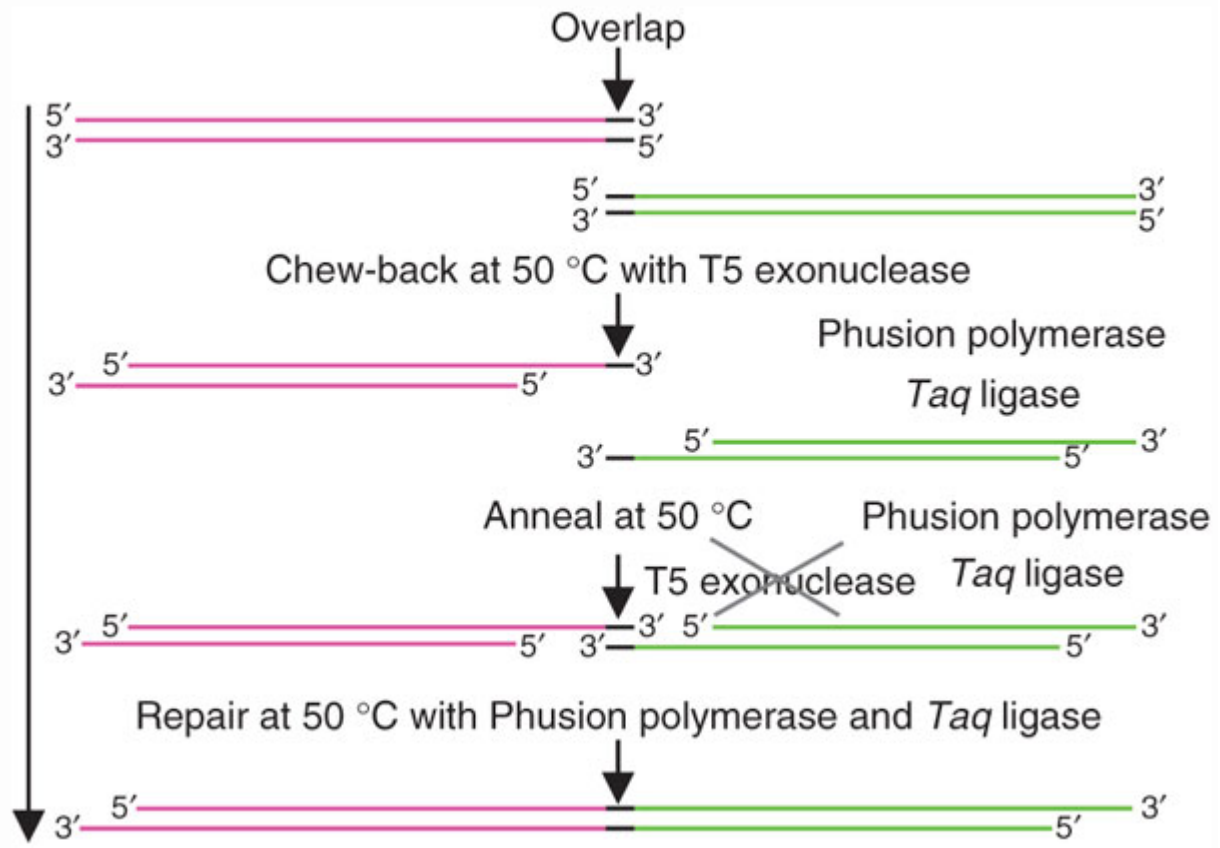
SLiCE

- Advantages
  - Inexpensive: all you need is bacterial cell extracts
  - Further improved by adding lambda red recombination to bacteria
- Disadvantages
  - Potential variability between cell extracts

Gibson assembly

Isothermal assembly (50C) of multiple molecules using 3 enzymes

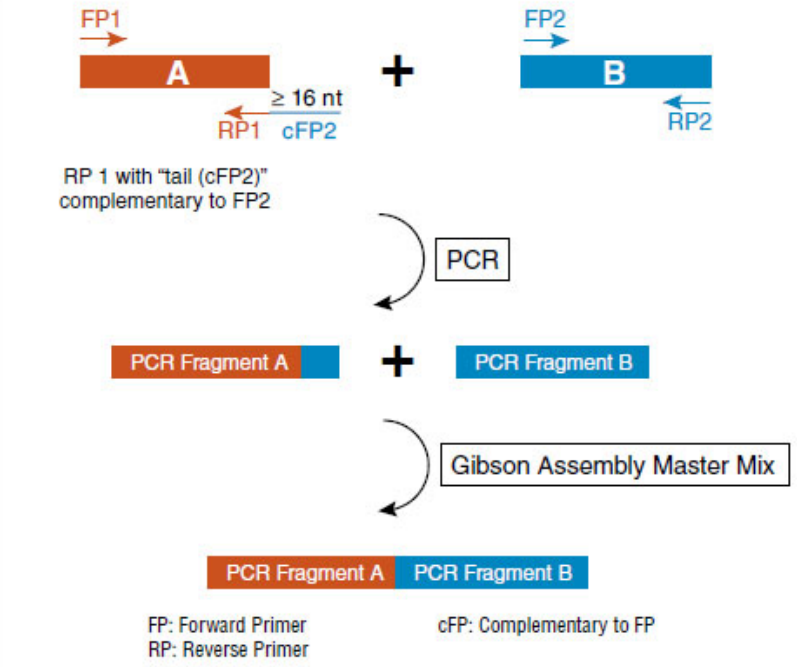
1. T5 exonuclease: 5'->3' digestion (3' overhangs)
2. DNA polymerase: 5'->3' extension (fills gaps)
3. Taq DNA ligase: join adjacent nucleotides

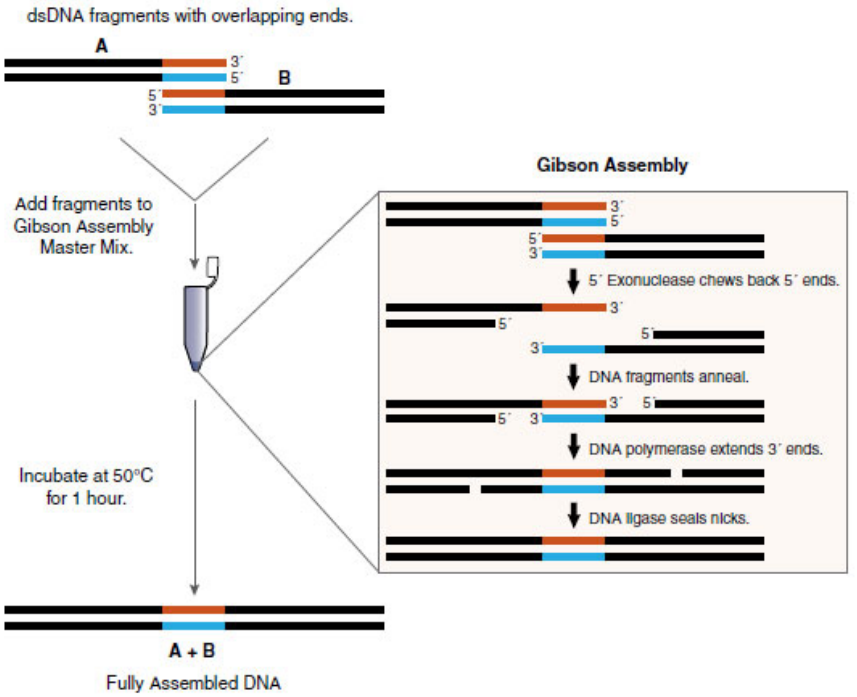


Gibson et al, 2009 PMID 19363495

Gibson assembly

NEB guide to Gibson Assembly of DNA fragments with overlapping ends





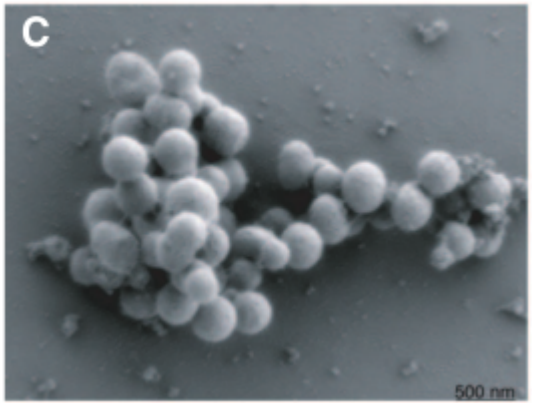
-5' end of primer matches adjacent fragment
-overlap should be 16-40 bp (Tm>48C)

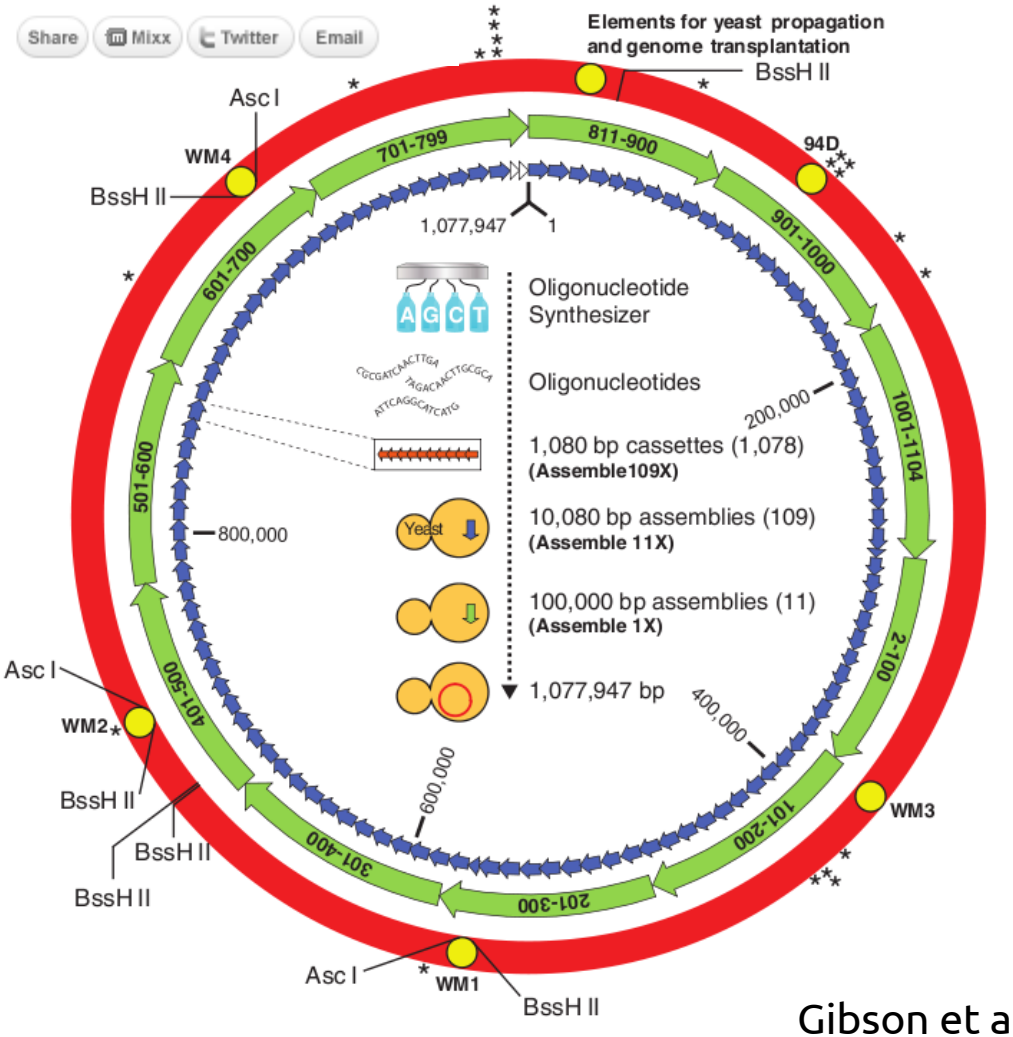
synthesis/transplantation of *Mycoplasma* genome

GENETIC ENGINEERING

Vatican calls synthetic cell creation 'interesting'

May 22, 2010 | By the CNN Wire Staff





Gibson et al, 2010

Gibson Assembly conclusions

Advantages

No restriction enzymes

Very fast and efficient

No scars

Assembly many pieces at once

Product can be used as PCR template (unlike LIC)

Disadvantages

Need big overlaps in primers (high cost and mutations

Can occur in primer sequences)

Gibson yeast *in vivo* assembly

- Advantages
  - Build large DNA molecules from short oligos (short oligos are cheap and have a lower error rate) in a single step.
  - No purified enzymes needed.
- Disadvantages
  - Selection of yeast transformants takes 3-4 days (would treating oligos with yeast lysate work?).
  - May be sequences that yeast do not tolerate (ie toxic sequences).

Building with DNA: future directions

Soon, we will buy genes instead of cloning PCR amplifying them, but we will still need to assemble them.

GBlock Price (2016):

http://eu.idtdna.com/pages/products/genes

Fragment 125-500bp: € 79,00

Fragment 501-750bp: € 109,00

Fragment 751-1000bp: € 129,00