

# SCIE5508 Synthetic Biology – Solving global challenges

Lecture 4 – Designing synthetic circuits II: DNA assembly  
standards & techniques



THE UNIVERSITY OF  
**WESTERN**  
**AUSTRALIA**

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We are learning on  
**Noongar land**



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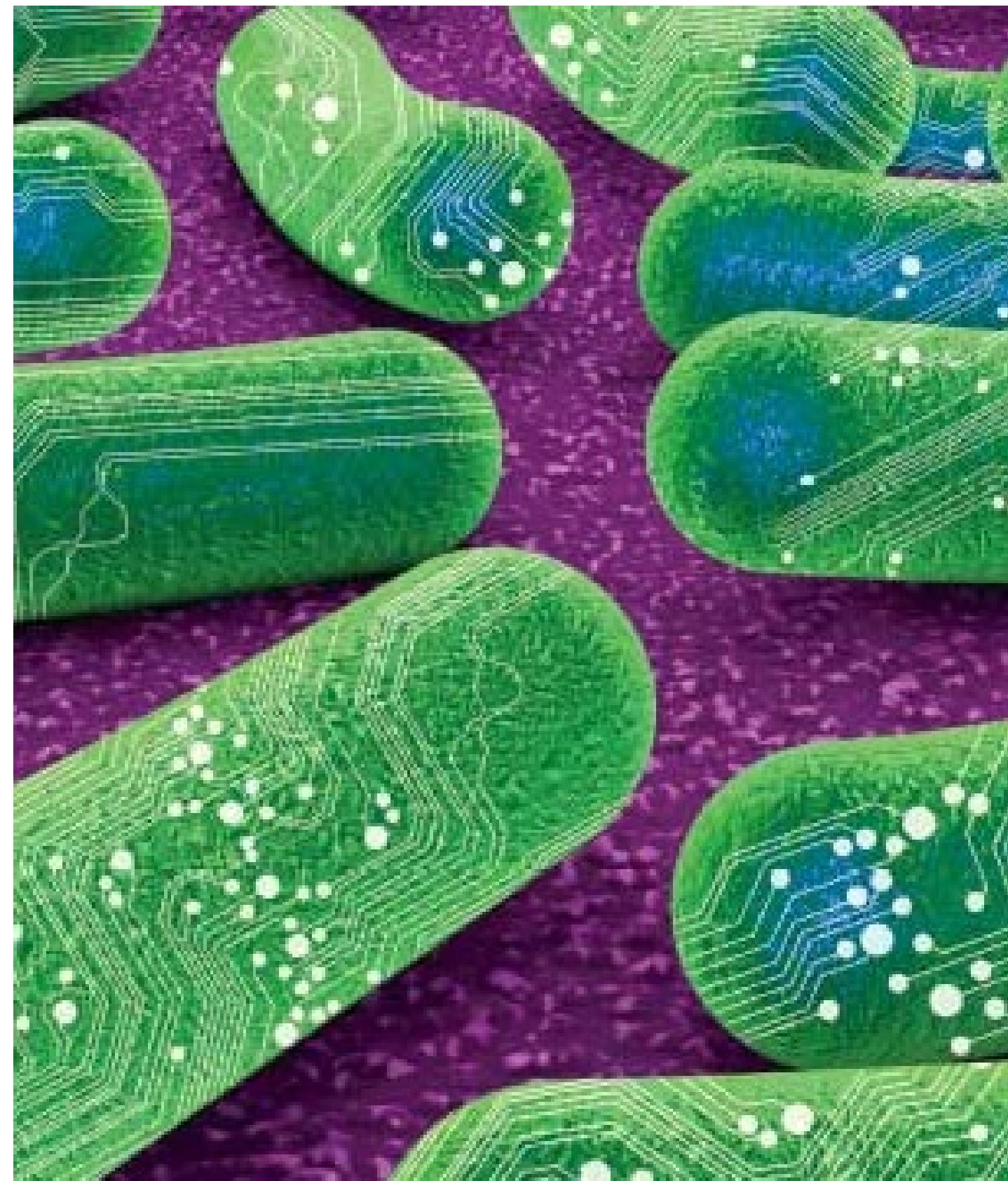


# Designing synthetic circuits II – DNA assembly methods and assembly standards

## Learning outcomes

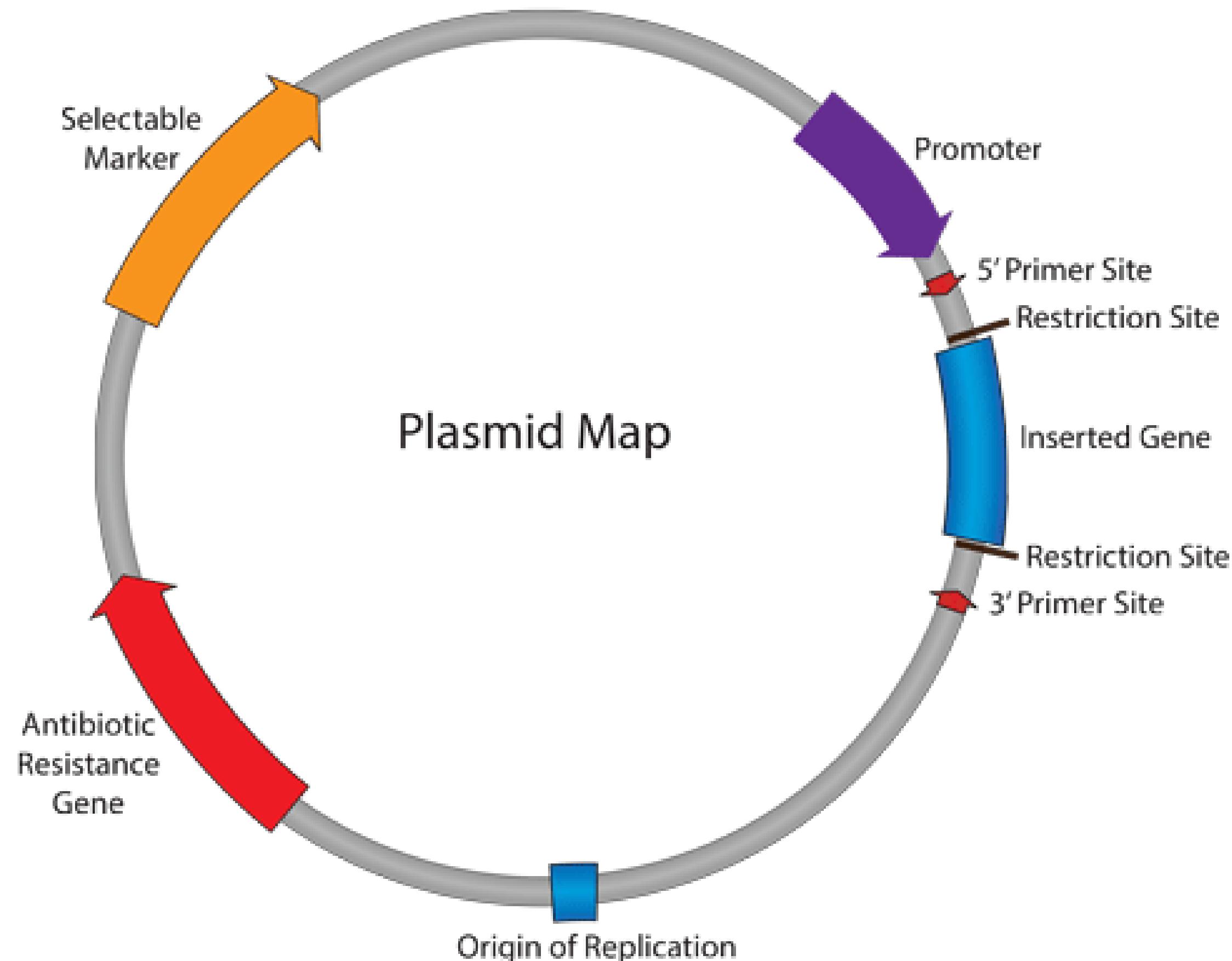
After this lecture, students should understand and be able to describe:

- Why there is a need for standardisation in DNA assembly
- Registry of standard biological parts and the BioBrick standard
- Golden Gate assembly and the Modular Cloning (MoClo) standard
- Gibson assembly
- Ligase Cycling Reaction (LCR) via bridging oligos
- Pros & cons of the methods & standards



# What is a plasmid?

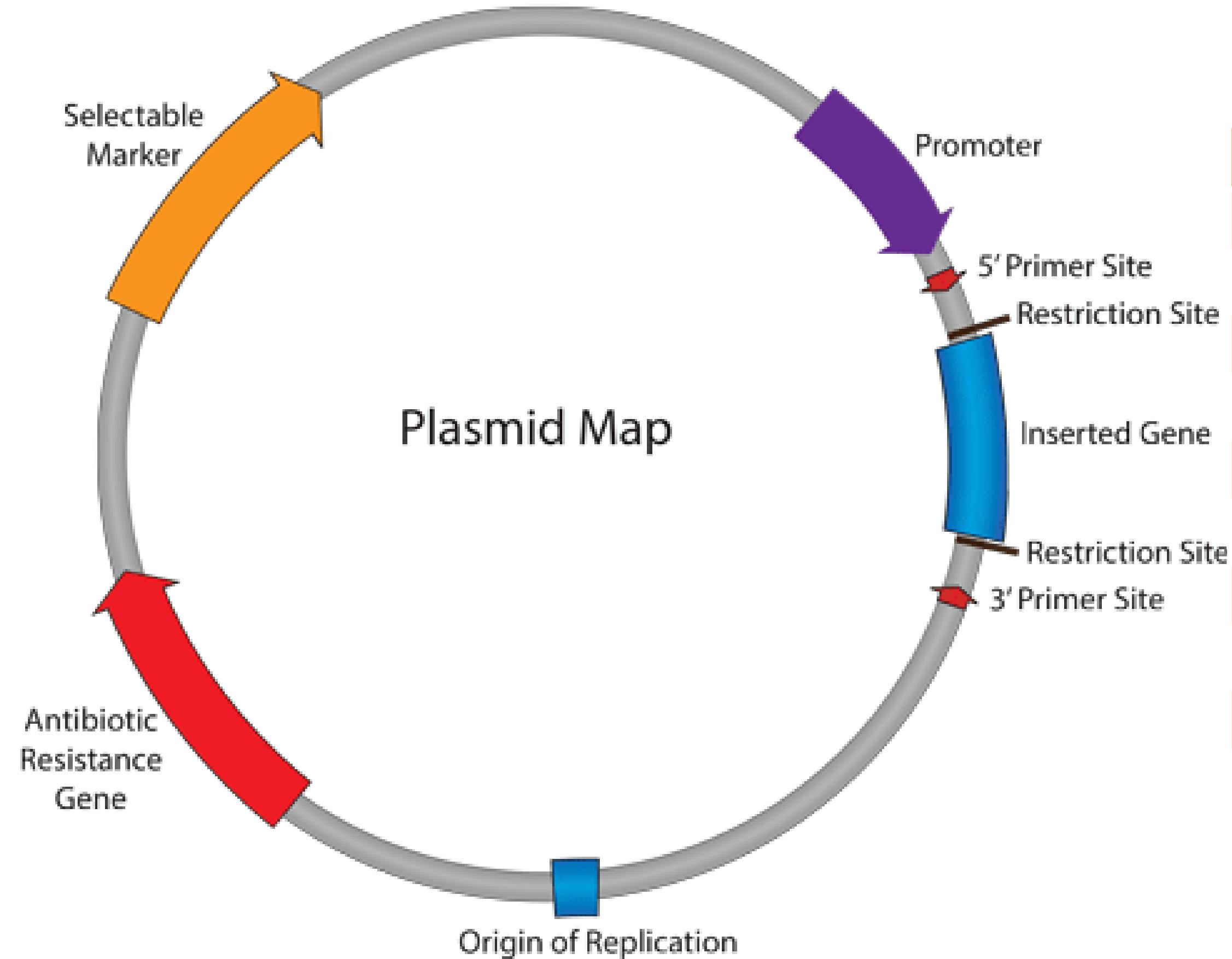
<https://youtu.be/SdqJFA6mOkl>



Vector Element	Description
Origin of Replication (ORI)	DNA sequence which allows initiation of replication within a plasmid by recruiting transcriptional machinery proteins
Antibiotic Resistance Gene	Allows for selection of plasmid-containing bacteria.
Multiple Cloning Site (MCS)	Short segment of DNA which contains several restriction sites allowing for the easy insertion of DNA. In expression plasmids, the MCS is often downstream from a promoter.
Insert	Gene, promoter or other DNA fragment cloned into the MCS for further study.
Promoter Region	Drives transcription of the target gene. Vital component for expression vectors: determines which cell types the gene is expressed in and amount of recombinant protein obtained.
Selectable Marker	The antibiotic resistance gene allows for selection in bacteria. However, many plasmids also have selectable markers for use in other cell types.
Primer Binding Site	A short single-stranded DNA sequence used as an initiation point for PCR amplification or sequencing. Primers can be exploited for sequence verification of plasmids.

<https://blog.addgene.org/plasmids-101-what-is-a-plasmid>

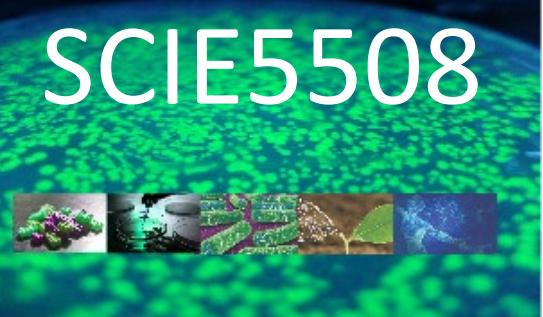
# What is a plasmid?



Plasmids with the same ori are incompatible!

Common Vectors	Copy Number <sup>+</sup>	ori	Incompatibility Group	Control
pUC	~500-700	pMB1 (derivative)	A	Relaxed
pBR322	~15-20	pMB1	A	Relaxed
pET	~15-20	pBR322	A	Relaxed
pGEX	~15-20	pBR322	A	Relaxed
pColE1	~15-20	ColE1	A	Relaxed
pR6K	~15-20	R6K*	C	Stringent
pACYC	~10	p15A	B	Relaxed
pSC101	~5	pSC101	C	Stringent
pBluescript	~300-500	ColE1 (derivative) and F1**	A	Relaxed
pGEM	~300-500	pUC and F1**	A	Relaxed

<https://blog.addgene.org/plasmid-101-origin-of-replication>

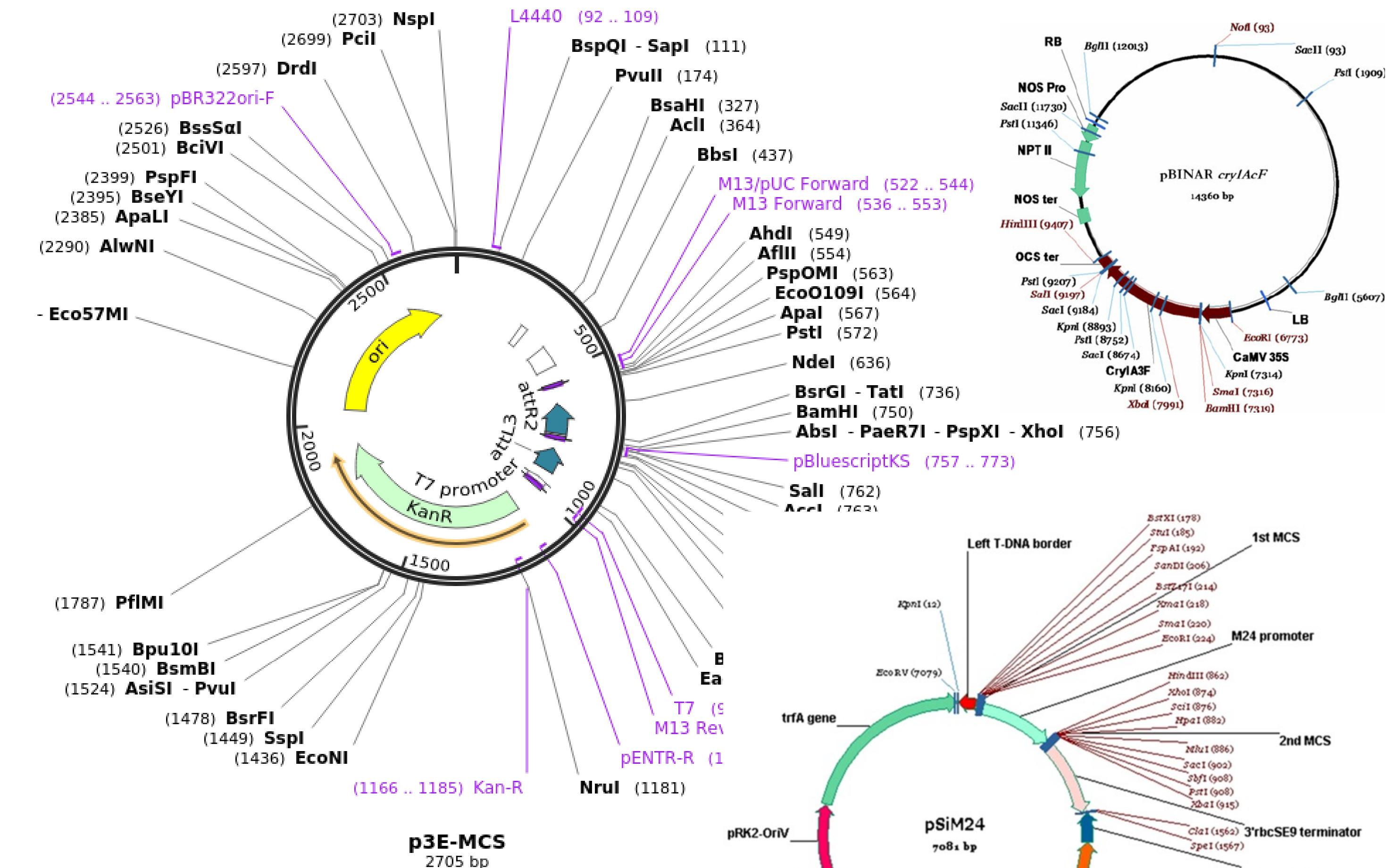
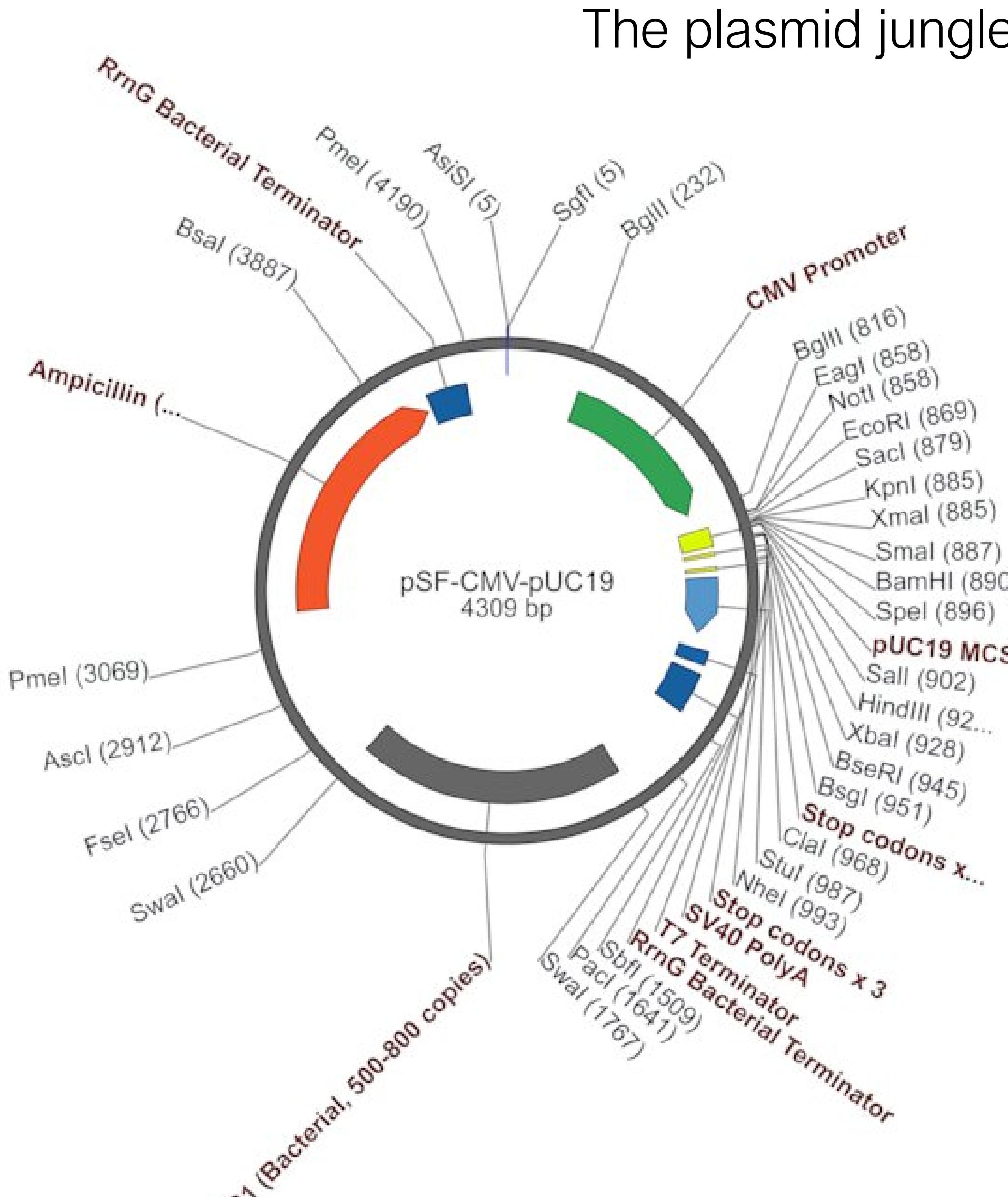


# Re-usability of DNA parts requires standardization!

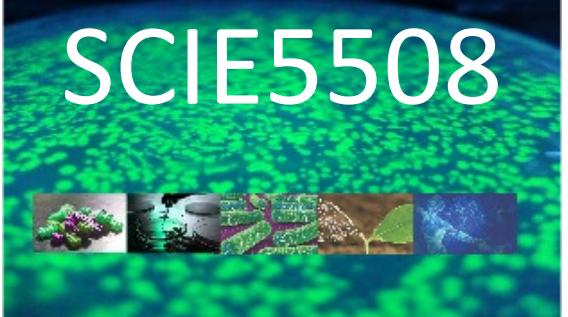


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Created with SnapGene®



Different oris, resistances & enzyme restriction sites!



# Registry of Standard Biological Parts

<http://parts.igem.org>

- Founded in 2003 at the Massachusetts Institute of Technology, as part of a summer competition on Synthetic Biology
- >20,000 standardized genetic parts: DNA, plasmids, plasmid backbones, primers, promoters, protein coding sequences, protein domains, ribosomal binding sites, terminators, translational units, riboregulators, and composite parts
- Part assembly conforms to the BioBrick standard



<https://igem.org>

# BioBrick RFC10 standard (3A assembly method)

## 1. Restriction Digests

- a) The left part is cut out with EcoRI and SpeI.
- b) The right part is cut out with XbaI and PstI.
- c) The linearized plasmid backbone is a linear piece of DNA. It has a few bases beyond the EcoRI and PstI restriction sites. It is cut with EcoRI and PstI.

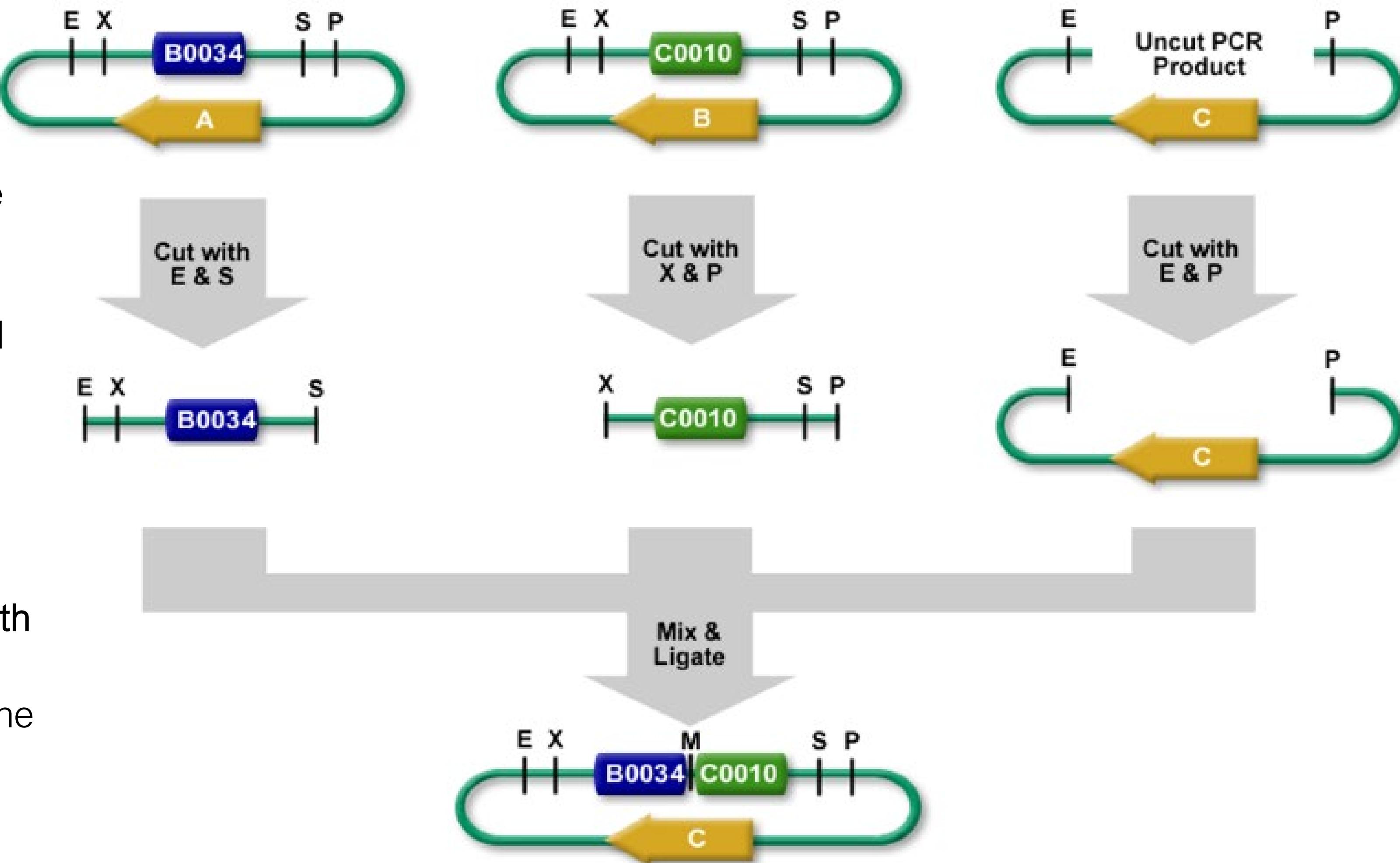
2. All 3 restriction digests are heated to **heat kill all of the restriction enzymes**.

3. An equimolar quantity of all 3 restriction digest products are combined in a **ligation reaction**.

4. The desired result is the left part sample's SpeI overhang ligated with the right part sample's XbaI overhang resulting in a scar that cannot be cut with any of our enzymes.

5. The new composite part sample is ligated into the construction plasmid backbone at the EcoRI and PstI sites.

6. When the ligation is transformed into cells and grown on plates with antibiotic C, only colonies with the correct construction survive.

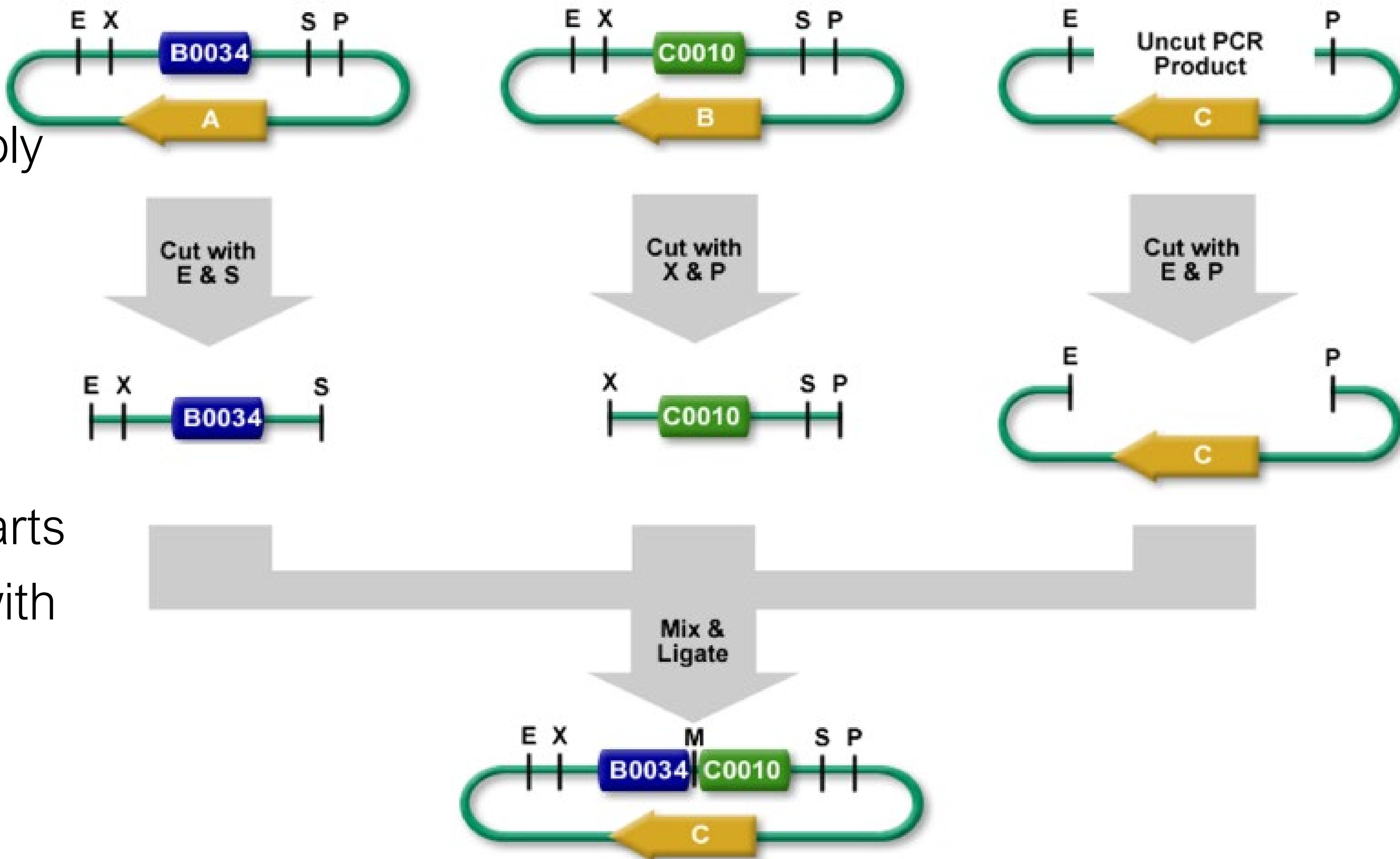


A, B, C = different antibiotic resistance cassettes

# BioBrick RFC10 standard (3A assembly method)

## Limitations:

- 6bp scar (M) from each assembly can have adverse effects on protein, RBS, terminator, etc., function
- Binary assembly: Exchange of parts in complex construct requires re-assembly of most parts
- BioBrick assembly only works with previously BioBrick'd parts

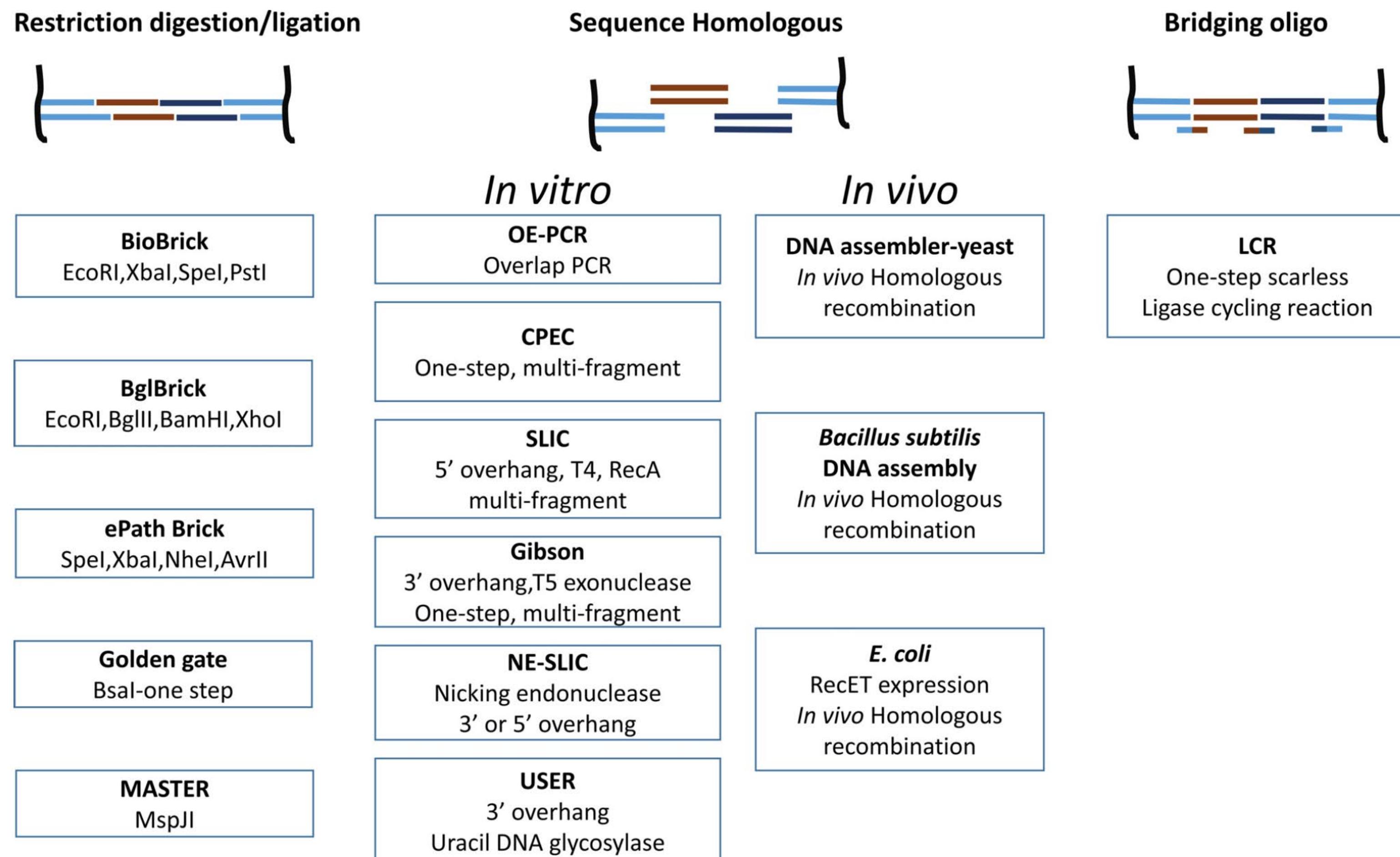


A, B, C = different antibiotic resistance cassettes

# Contemporary DNA assembly technologies & standards

3 broad classes of technologies:

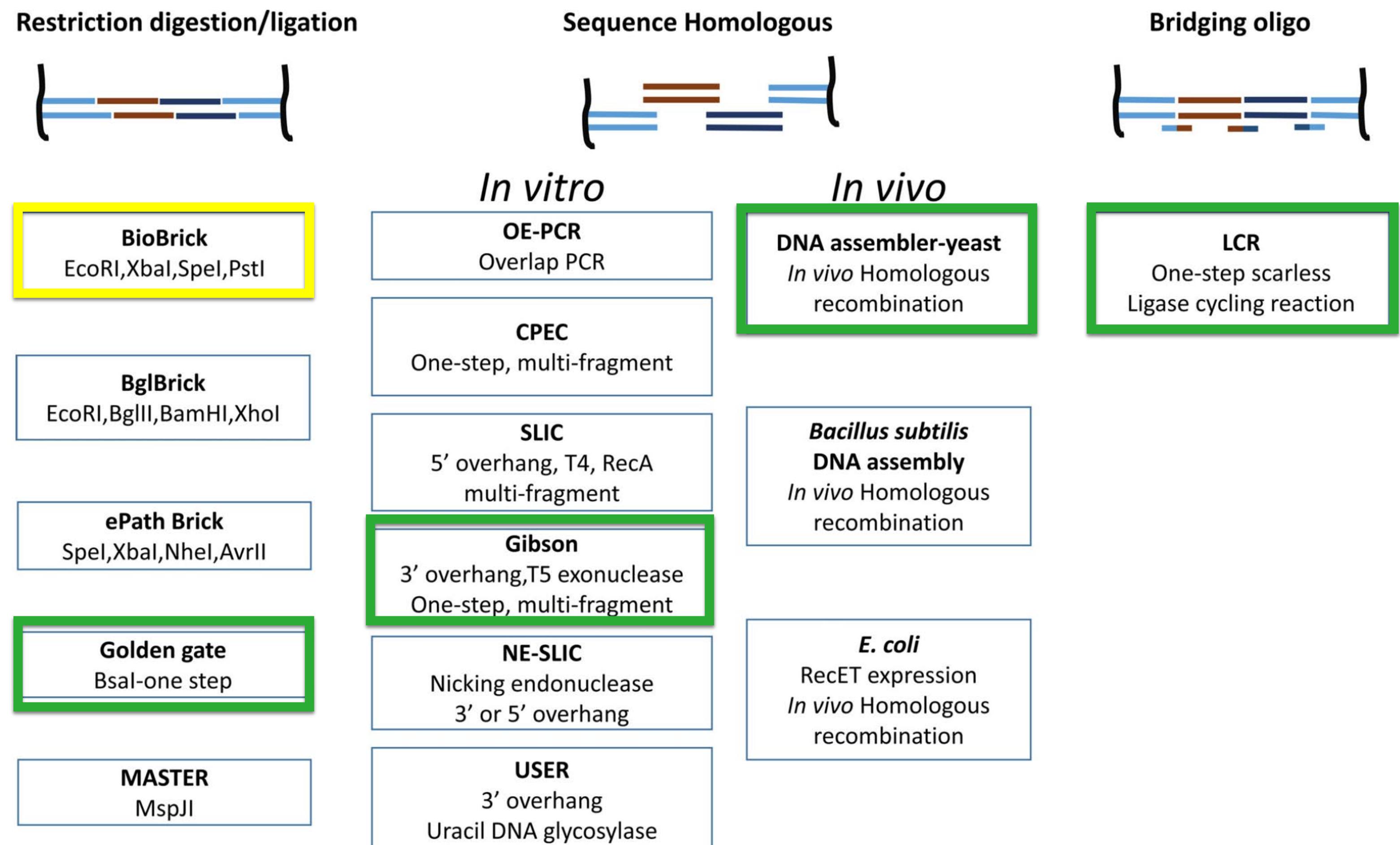
- Restriction digestion/ligation via
  - ✓ Type IIP enzymes (Palindromic specificity), typically cleaving within recognition sequence
  - ✓ Type IIS enzymes (Shifted cleavage)
- Sequence homology-based methods
  - ✓ *In vitro*
  - ✓ *In vivo*
- Bridging oligo-based method



# Contemporary DNA assembly technologies & standards

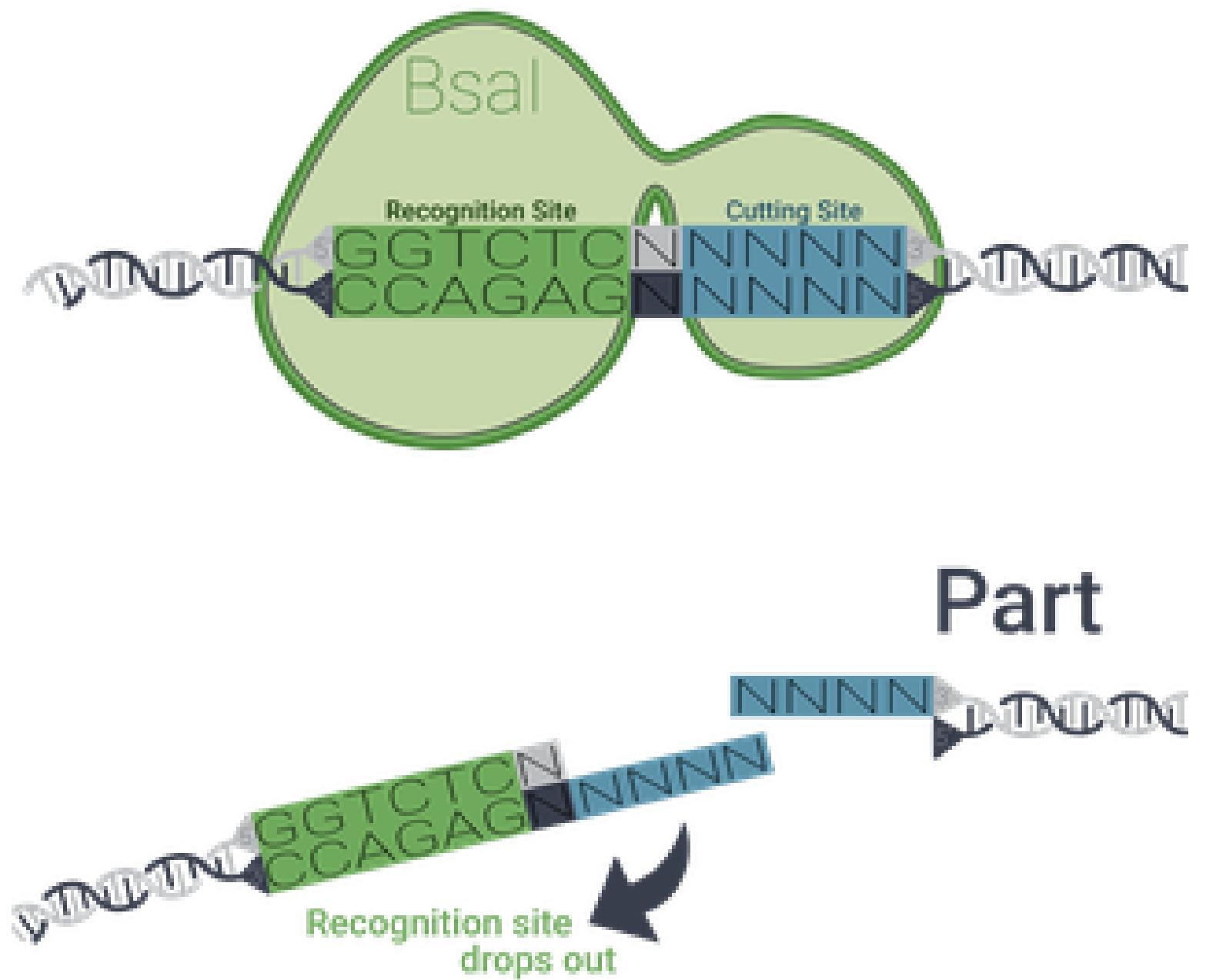
3 broad classes of technologies:

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# Golden Gate assembly

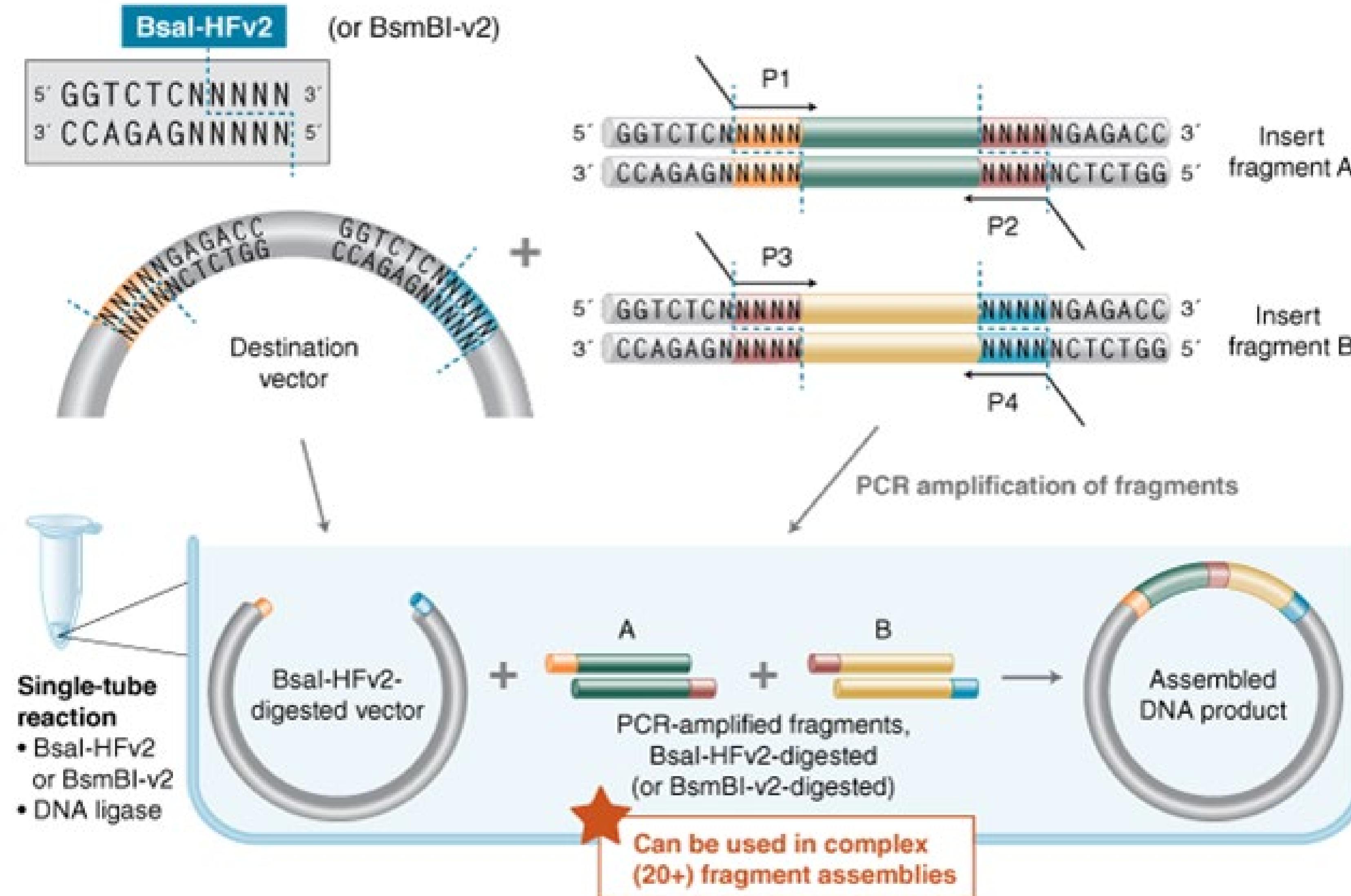
## Type II S



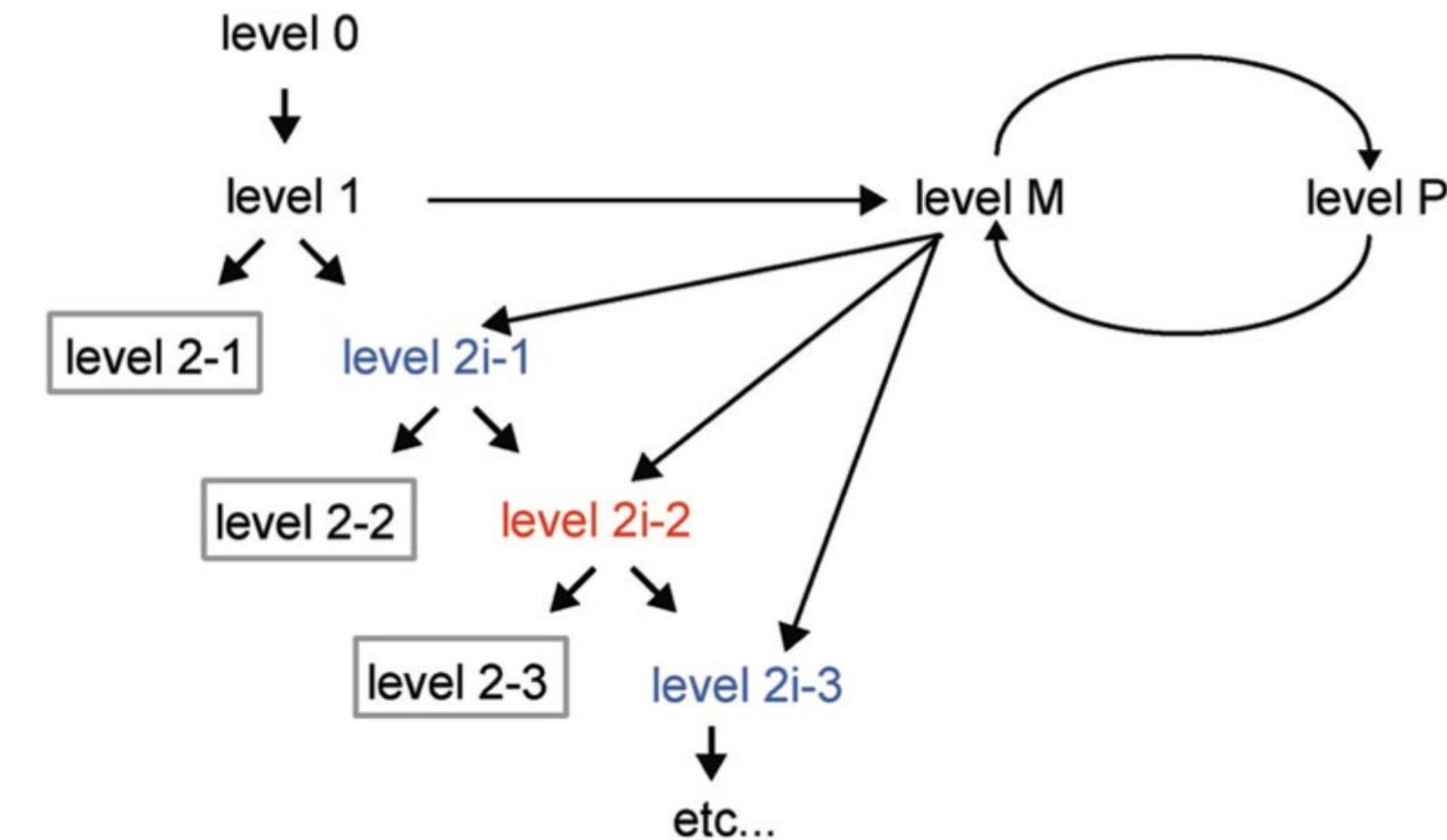
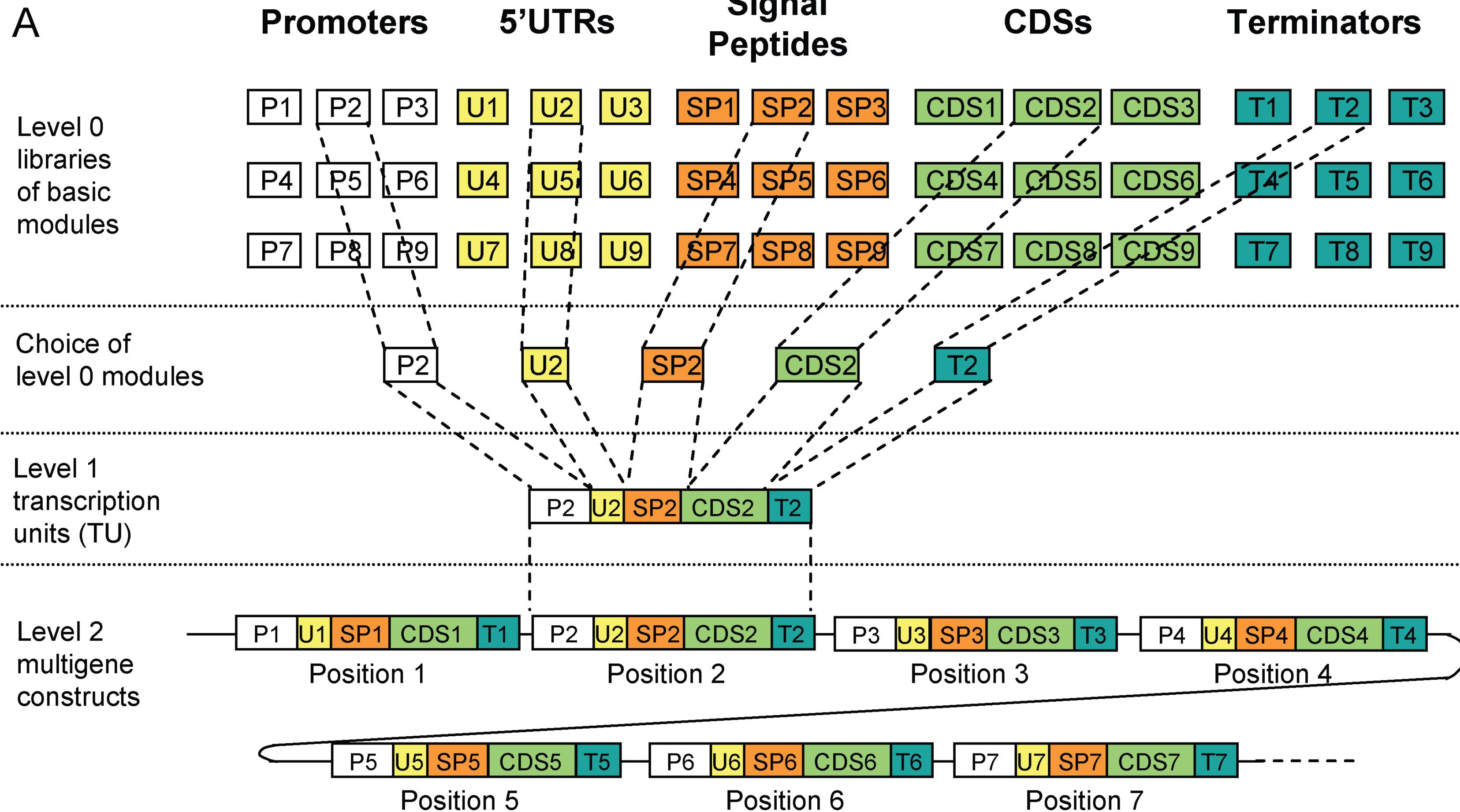
free choice of 4bp  
“fusion site” = bridge



# A one-pot reaction for multi-part assembly



# The modular cloning (MoClo) standard

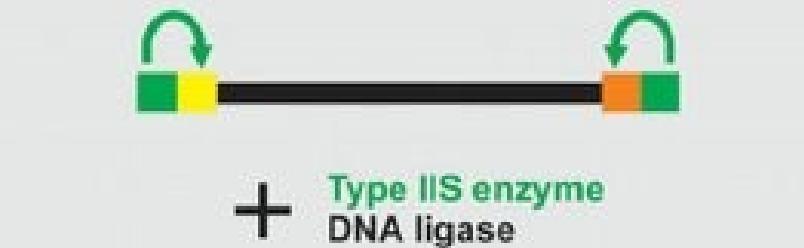


Stefan Werner, Carola Engler, Ernst Weber,  
 Ramona Gruetzner & Sylvestre Marillonnet  
 (2012) Fast track assembly of multigene  
 constructs using Golden Gate cloning and the  
 MoClo system, *Bioengineered*, 3:1, 38-43, DOI:  
 10.4161/bbug.3.1.18223

# Building level 0 parts

PCR product

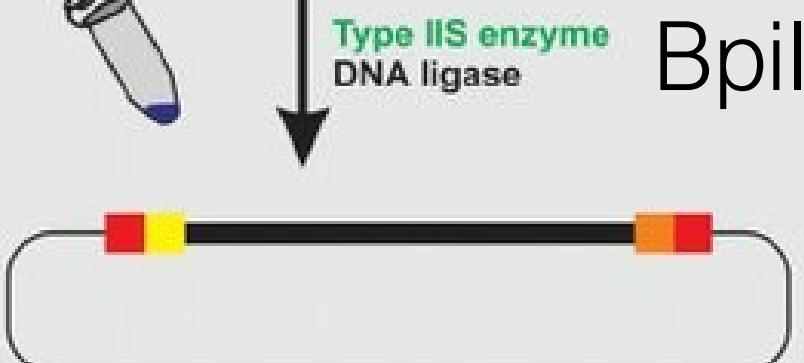
## Type-IIIS-based cloning



Level 0 entry vector



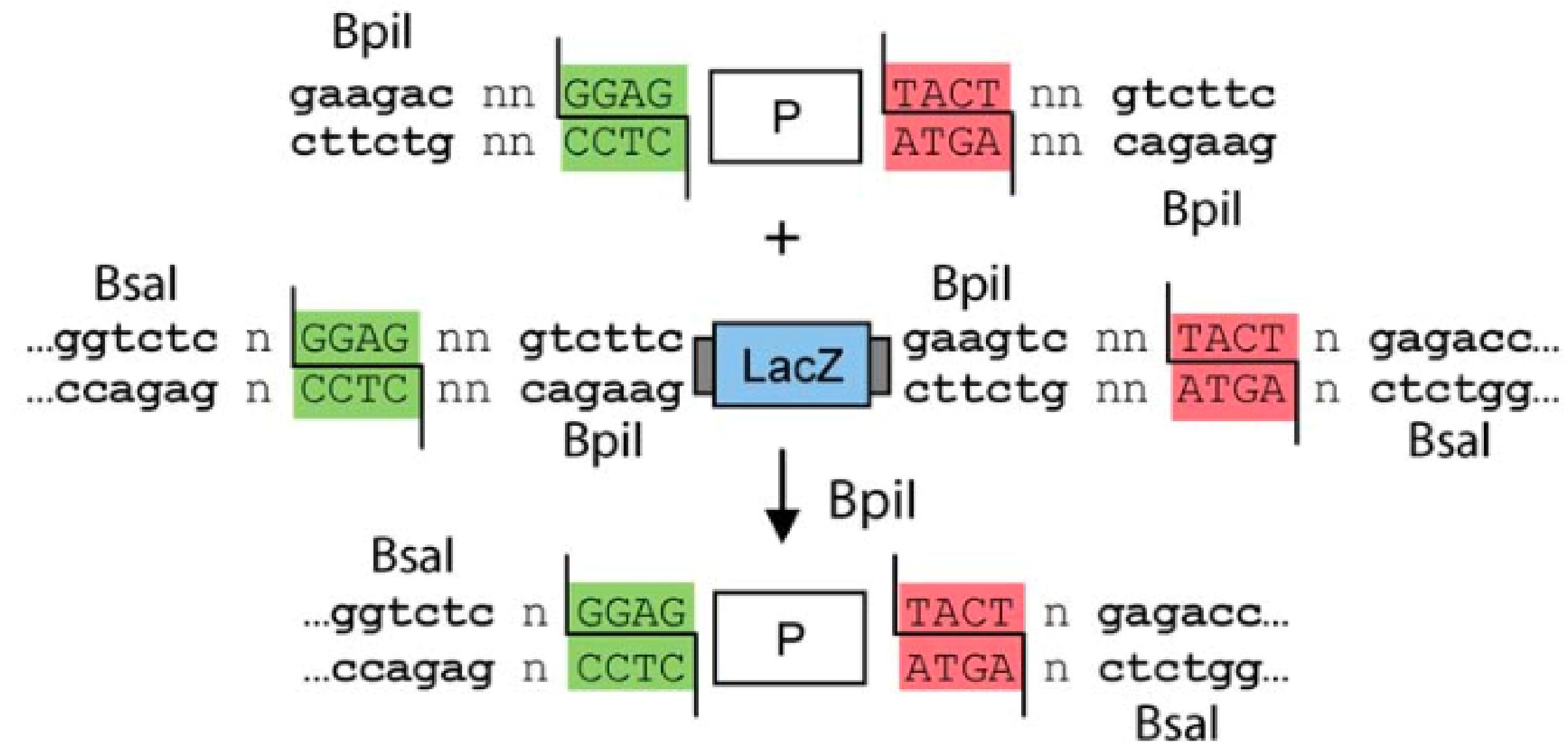
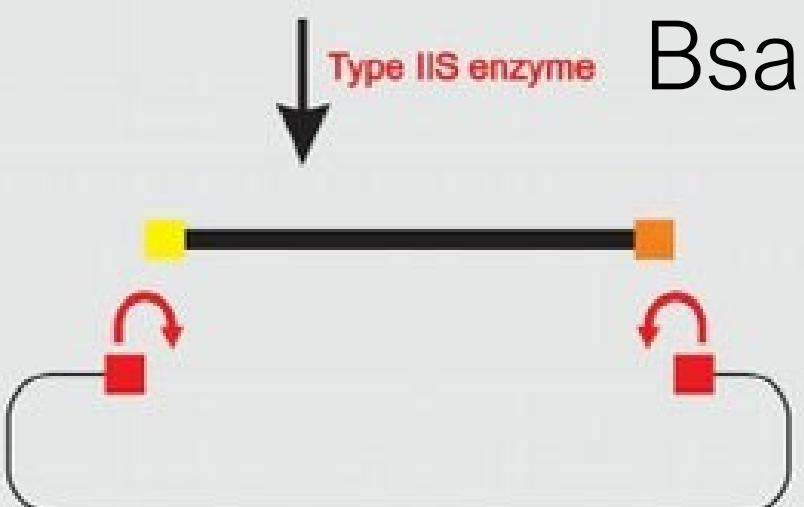
Level 0 module



Re-ligated vector  
= blue colony



Level 0 part release



# Building level 0 parts

PCR product

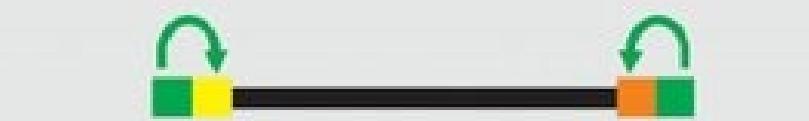
Level 0 entry vector

Level 0 module

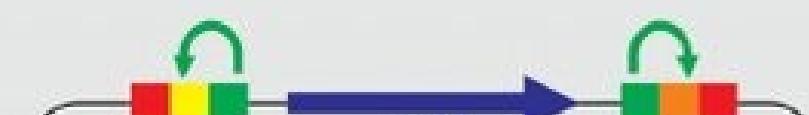
Re-ligated vector  
= blue colony

Level 0 part release

## Type-IIIS-based cloning



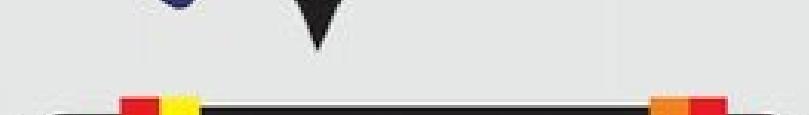
+ Type IIIS enzyme DNA ligase



One pot reaction:  
Type IIIS enzyme DNA ligase



Bpil



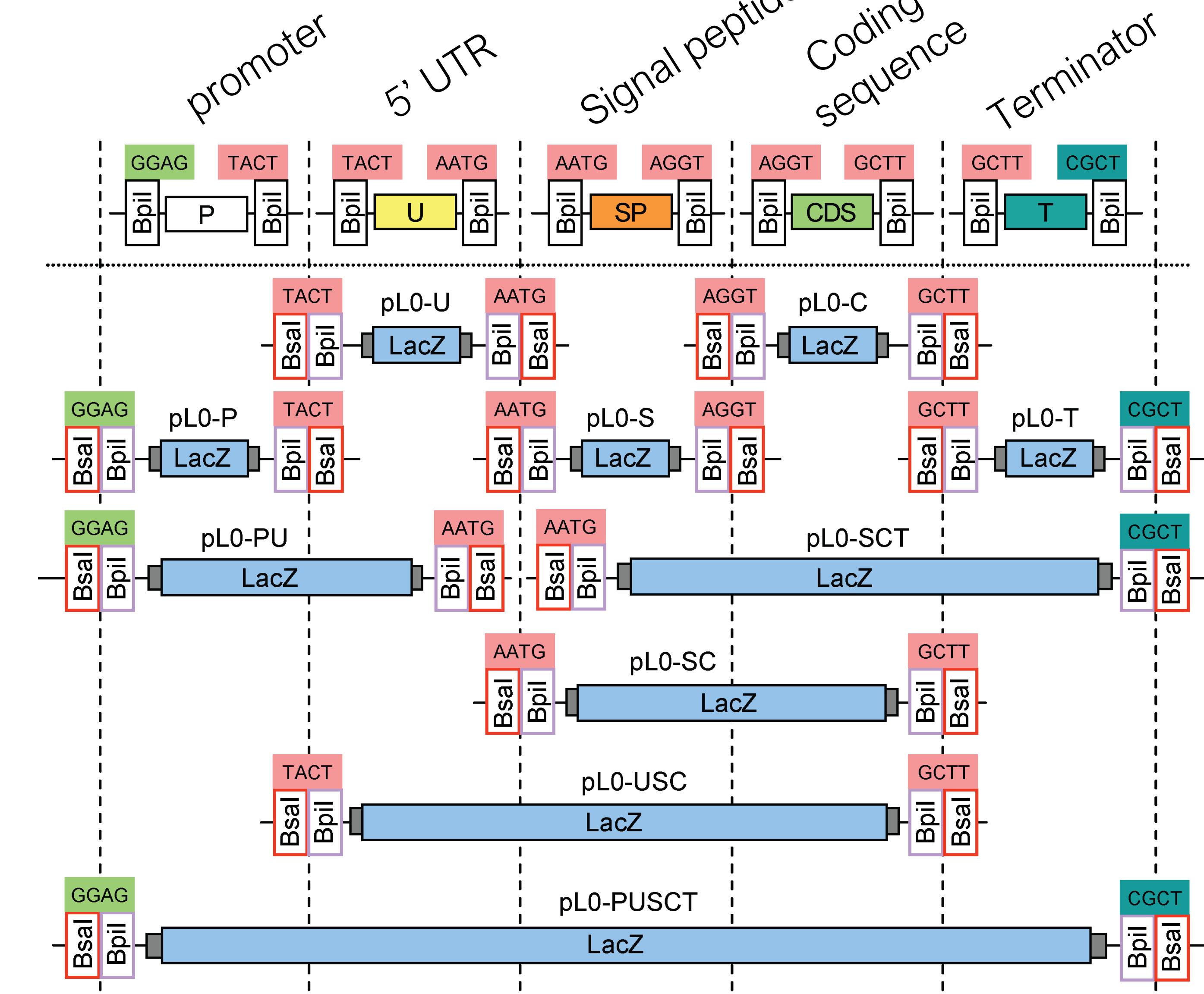
Transformation & Selection marker A



Bsal



Type IIIS enzyme



Level 0 entry vectors (SPEC)

# Building level 1 transcription units (TUs)

Level 0 modules

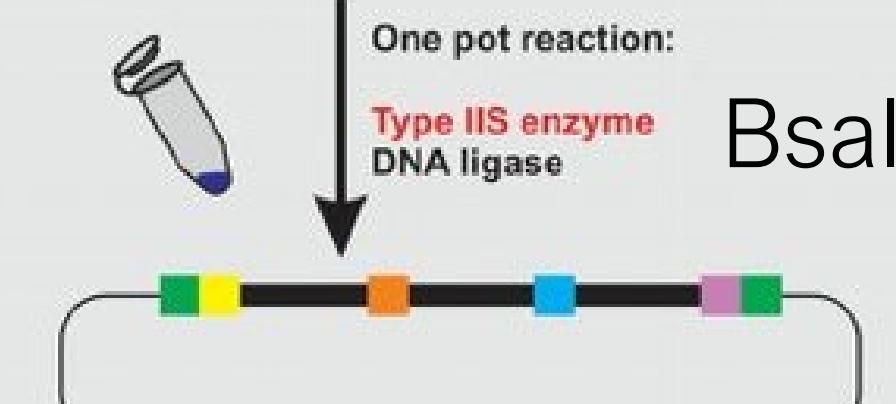
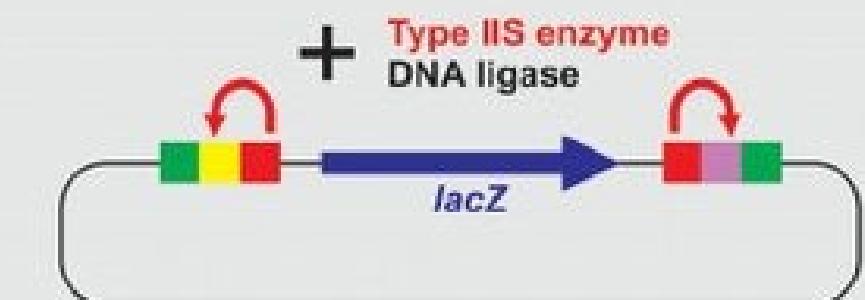
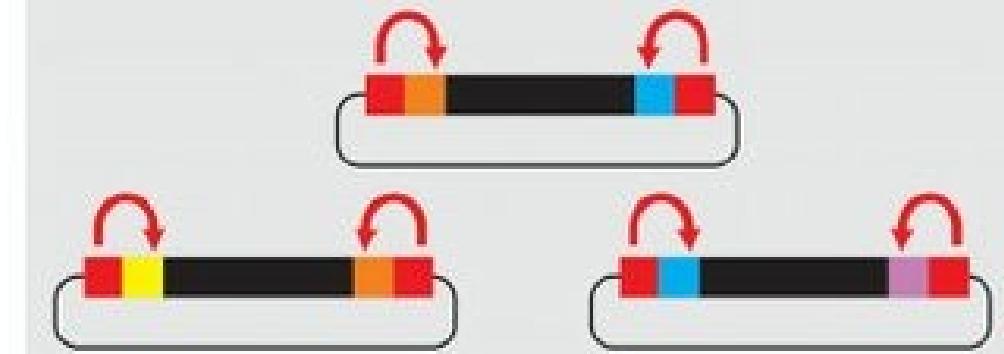
Level 1  
destination vector

Level 1  
transcription unit

Re-ligated vector  
= blue colony

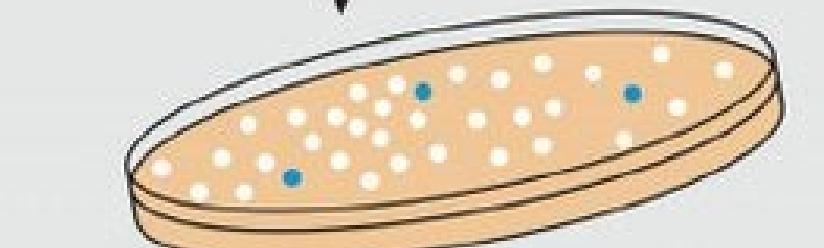
Level 1 part release

## Type-IIS-based assembly



One pot reaction:  
Type IIS enzyme  
DNA ligase

Bsal

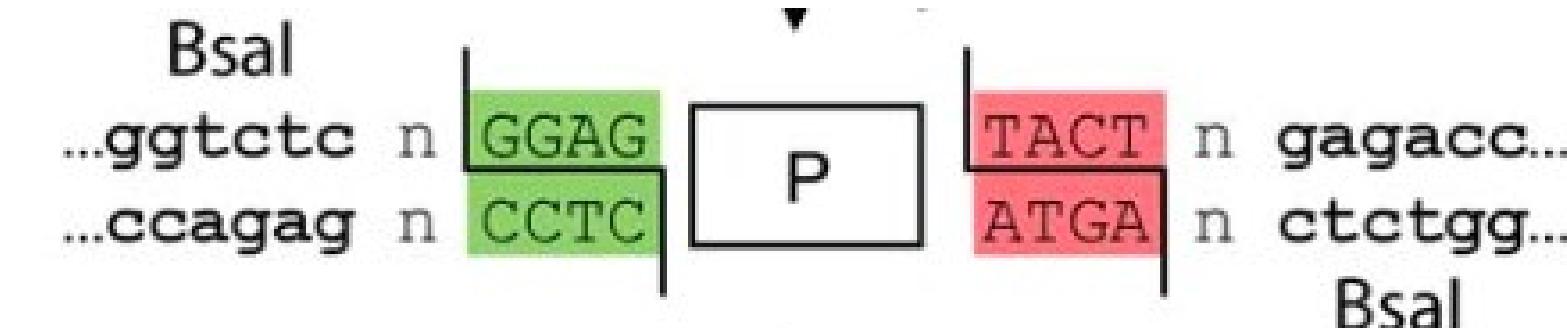


Transformation & Selection marker B

Bpil



Bpil

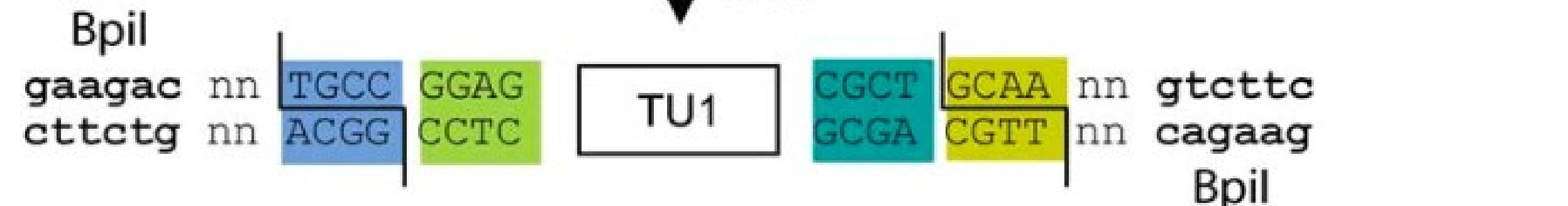


+



Bsal

Bpil



Bsal

Bpil

TU1

Bpil



Level 0 modules

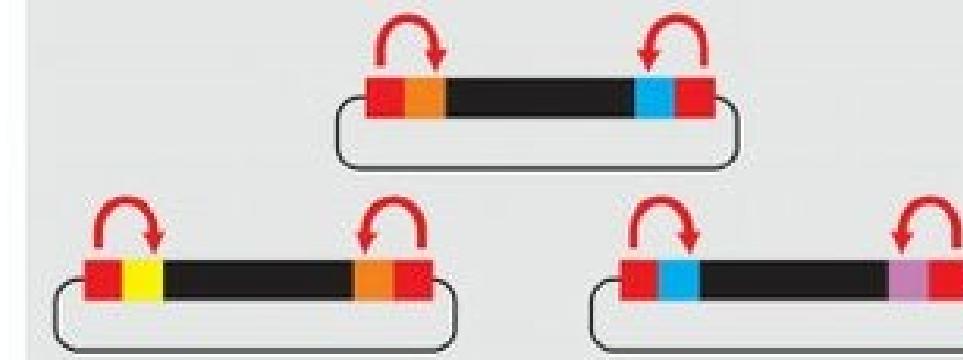
Level 1  
destination vector

Level 1  
transcription unit

Re-ligated vector  
= blue colony

Level 1 part release

### Type-IIS-based assembly



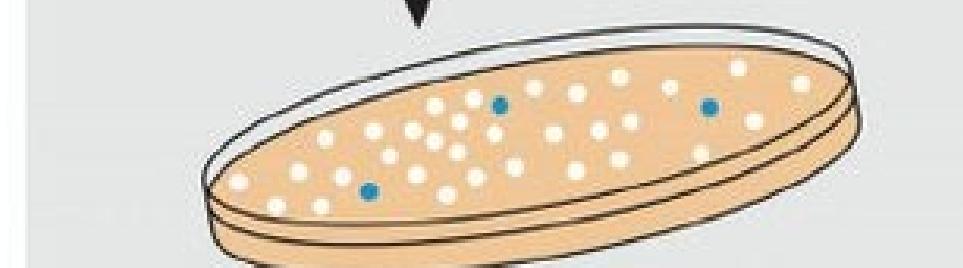
+ Type IIS enzyme  
DNA ligase

One pot reaction:  
Type IIS enzyme  
DNA ligase

Bsal

Bsal

Transformation  
&  
Selection marker B

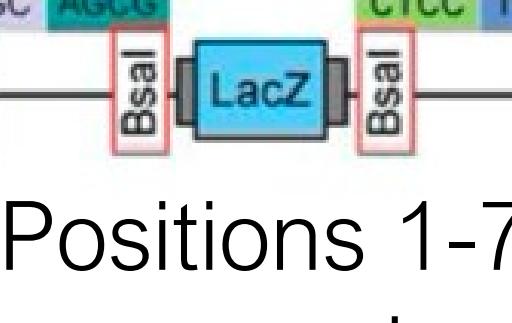
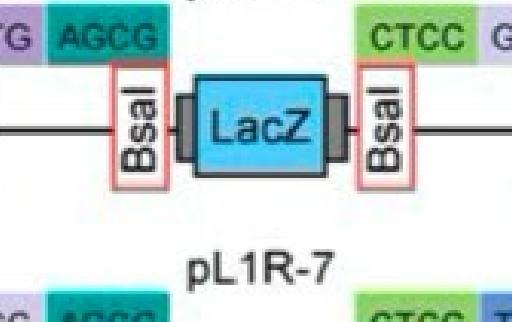
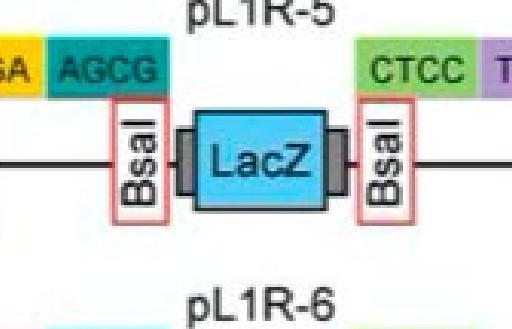
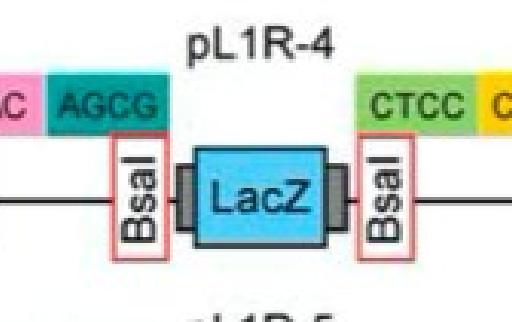
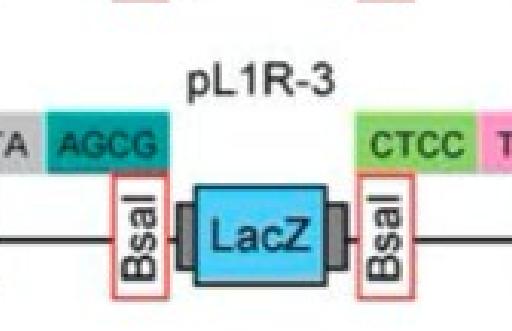
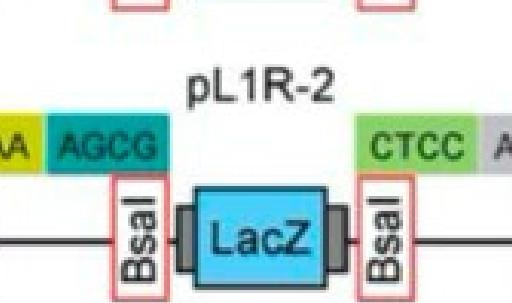
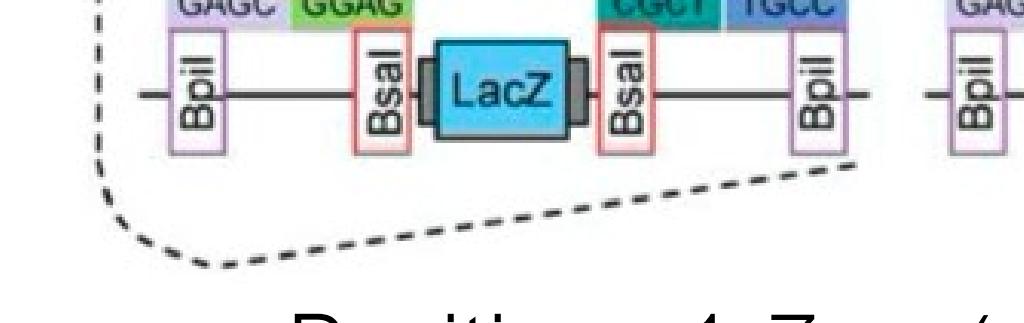
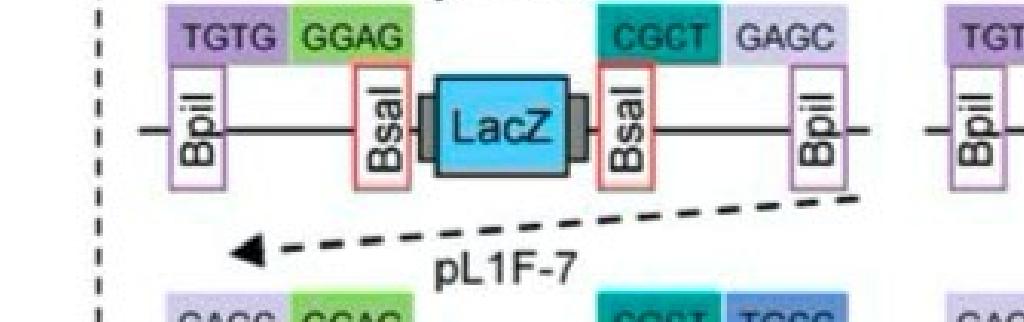
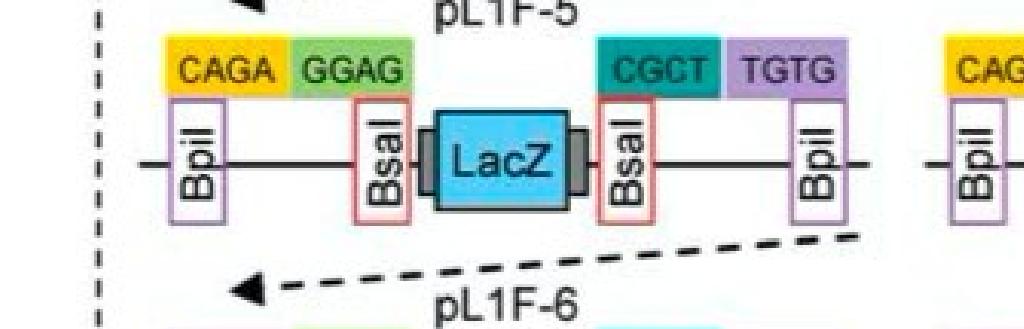
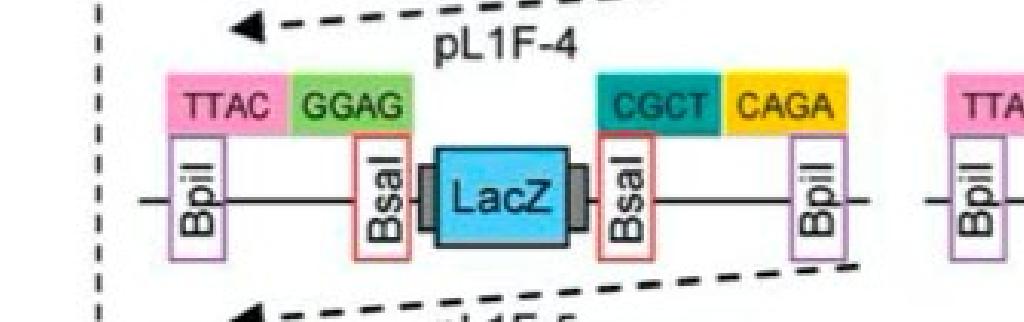
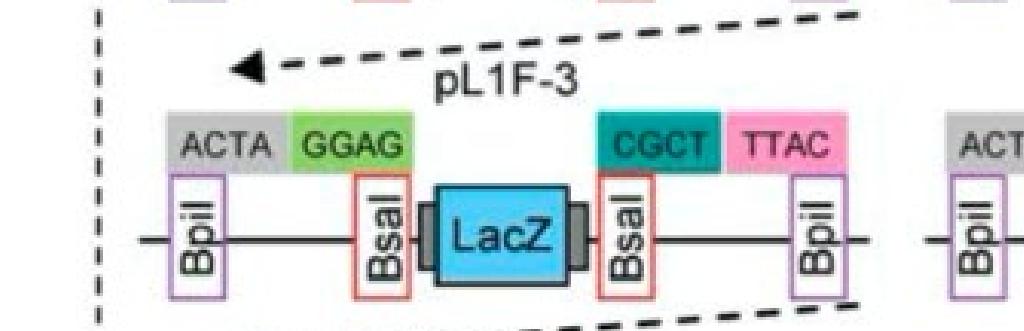
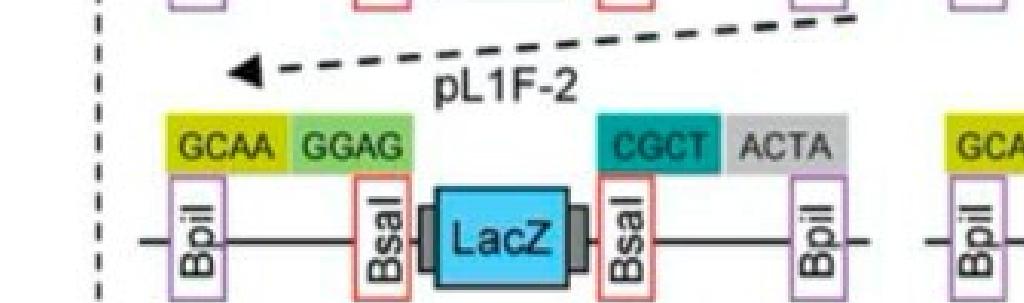


Type IIS enzyme

BpiI

BpiI

### Level 1 destination vectors ( $\text{Ap}^R$ )



Positions 1-7  
(reverse orient.)

# Building level M circuits

Level 1  
transcription units

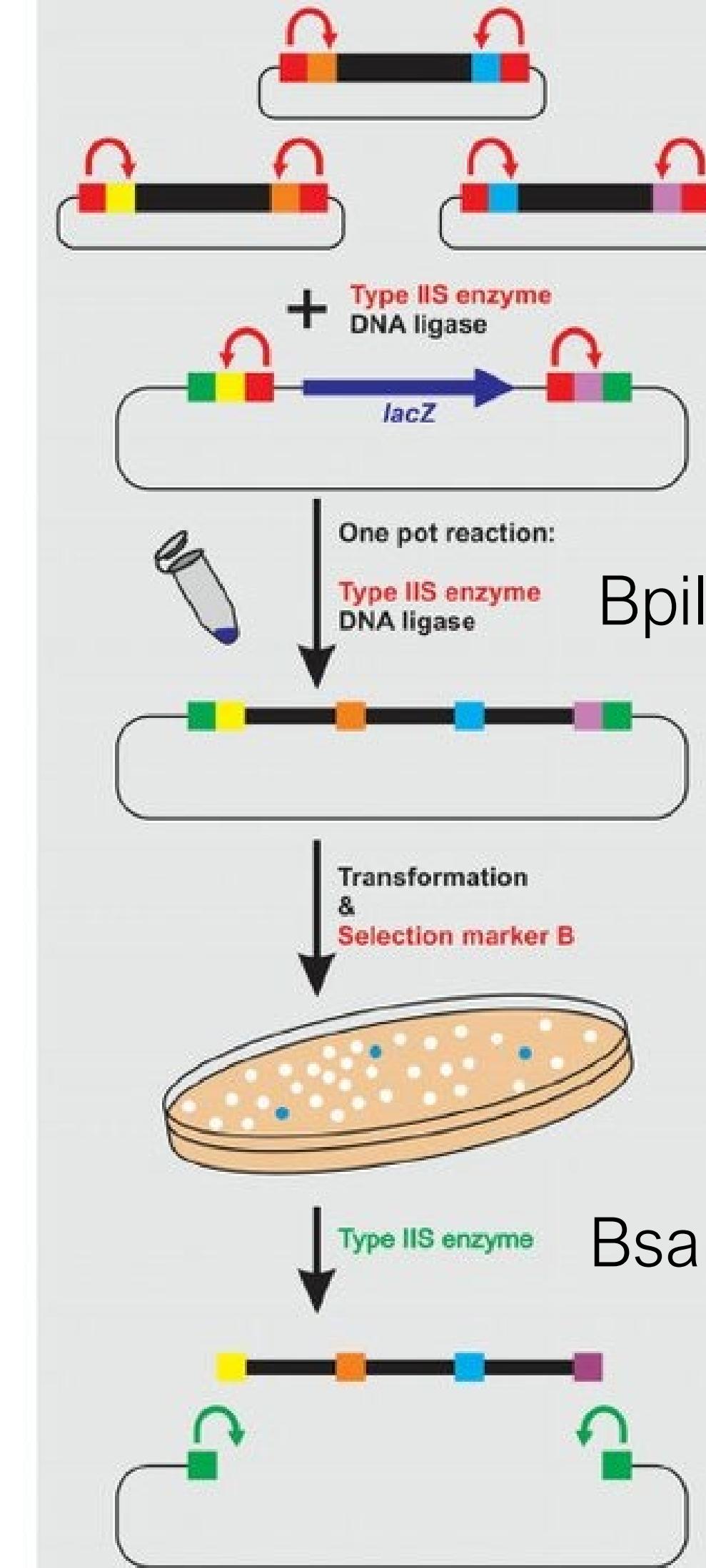
Level 2  
destination vector

Level 2 circuit

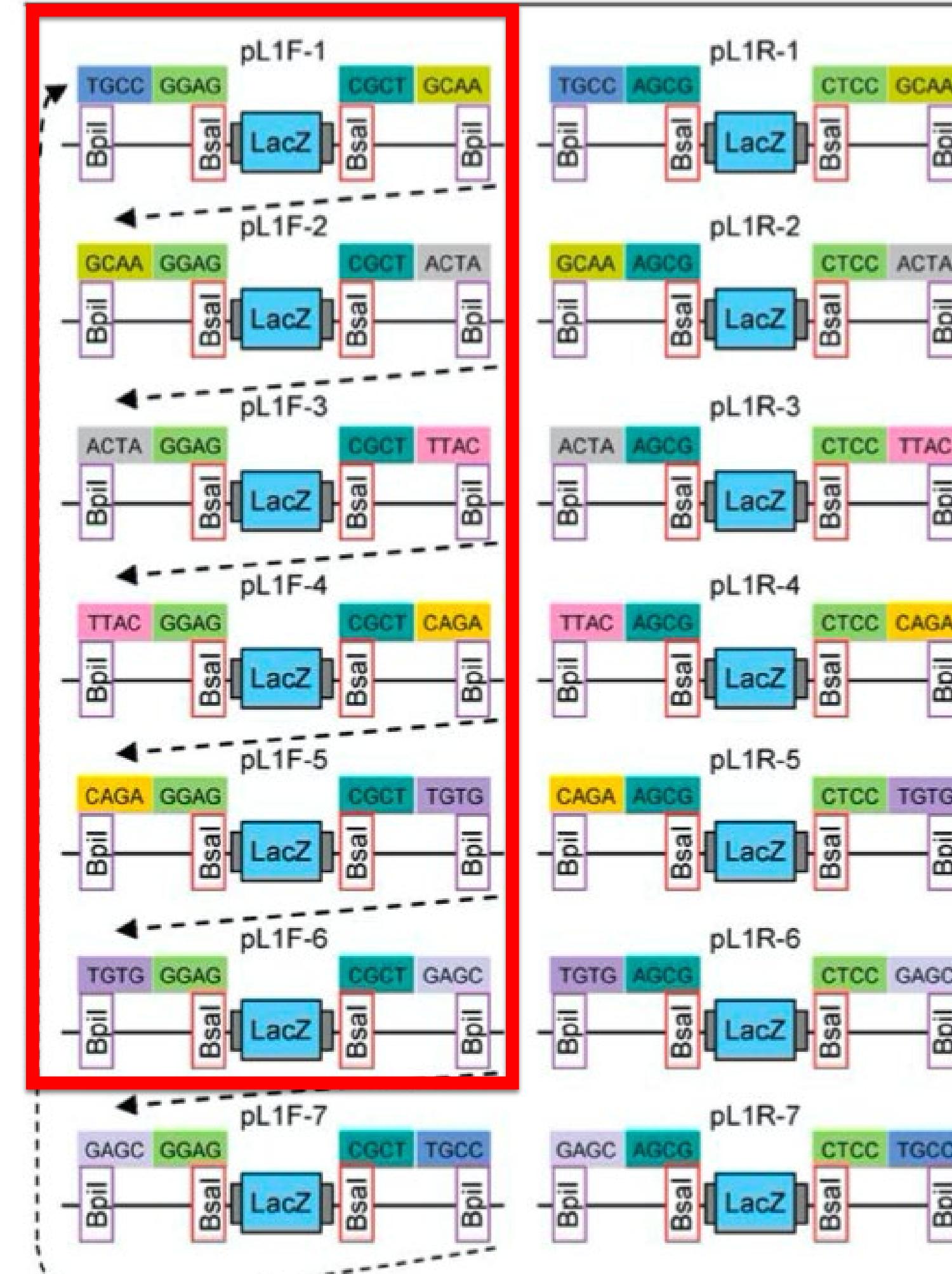
Re-ligated vector  
= blue colony

Level 2 part release

## Type-IIS-based assembly

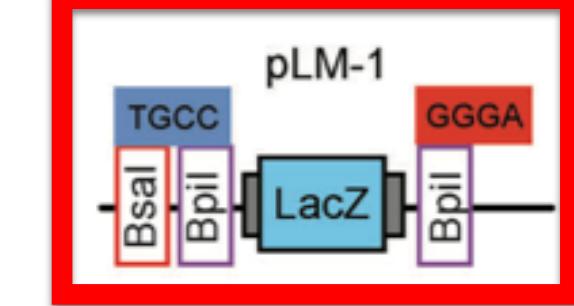


## Level 1 destination vectors ( $Ap^R$ )

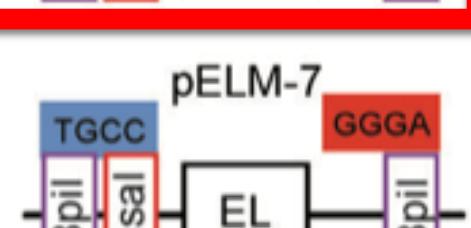
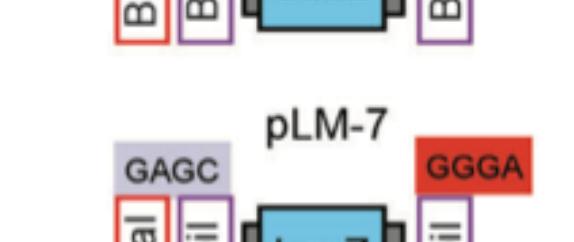
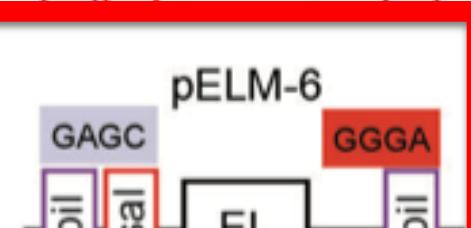
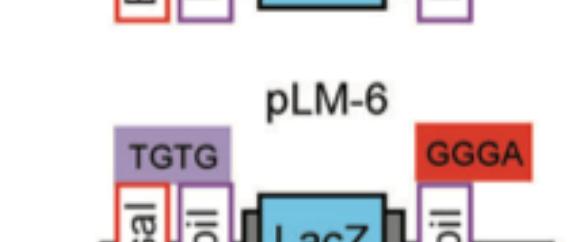
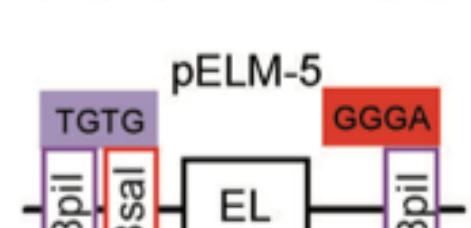
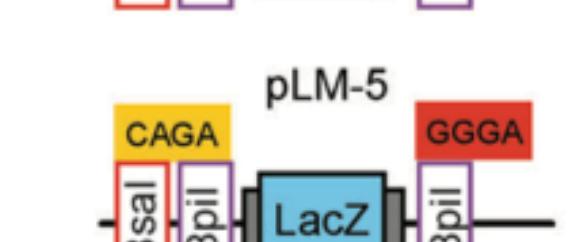
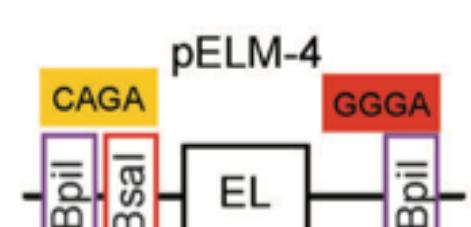
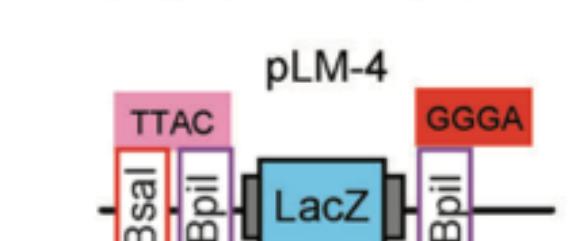
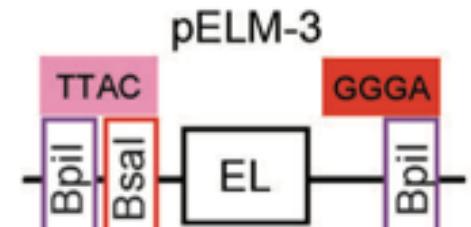
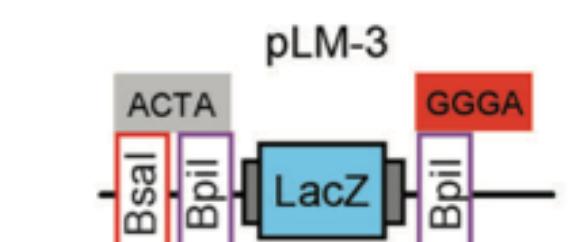
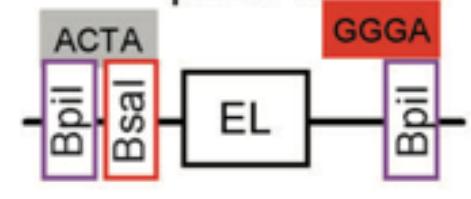
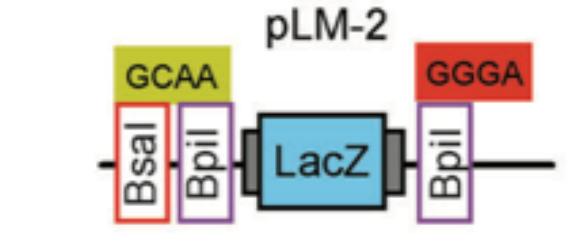
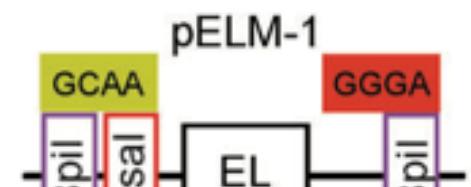


## Level M

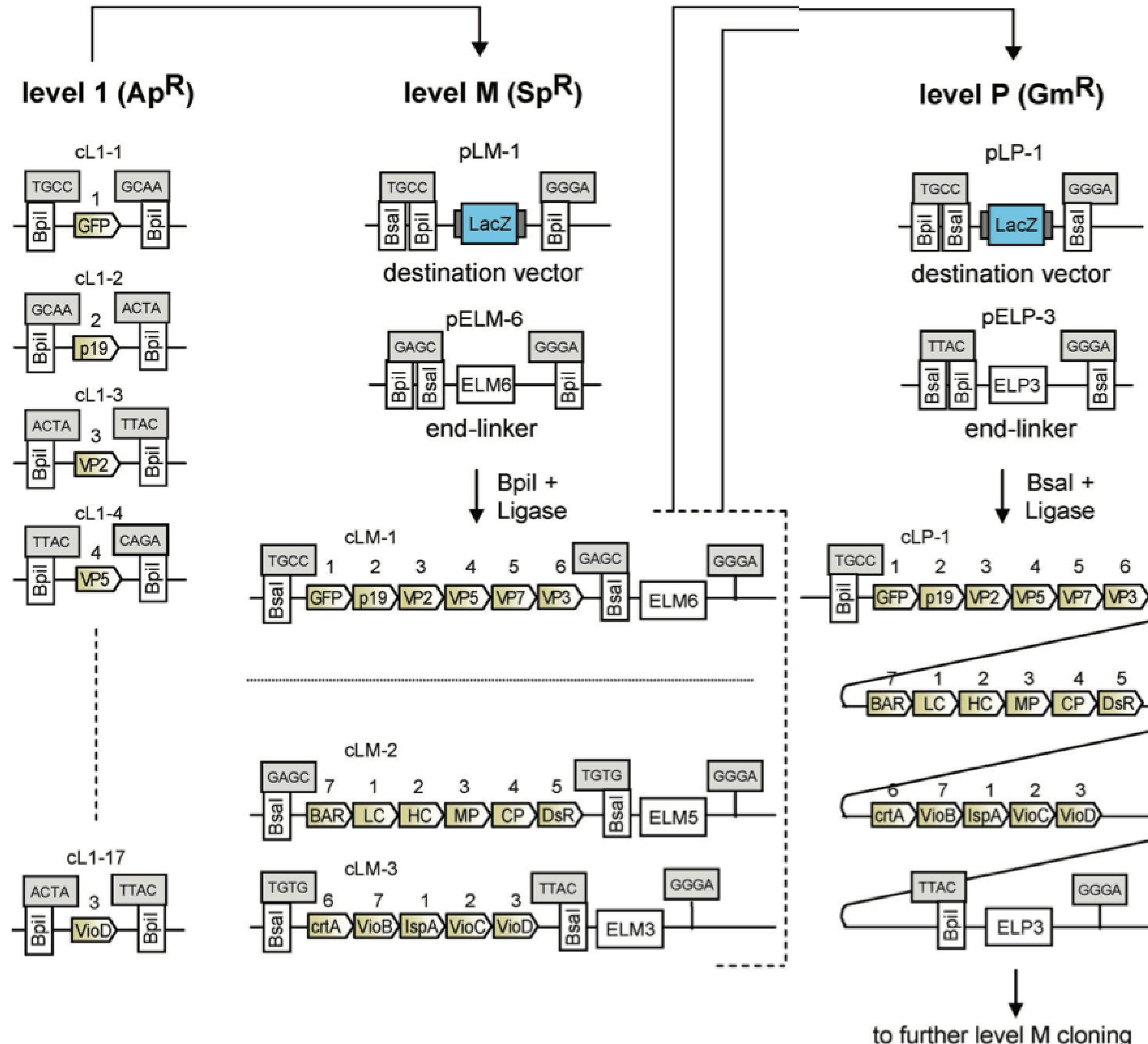
Destination vectors  
( $Sp^R$ )



End-linkers  
( $Ap^R$ )



# From level 1 TUs to level P circuits

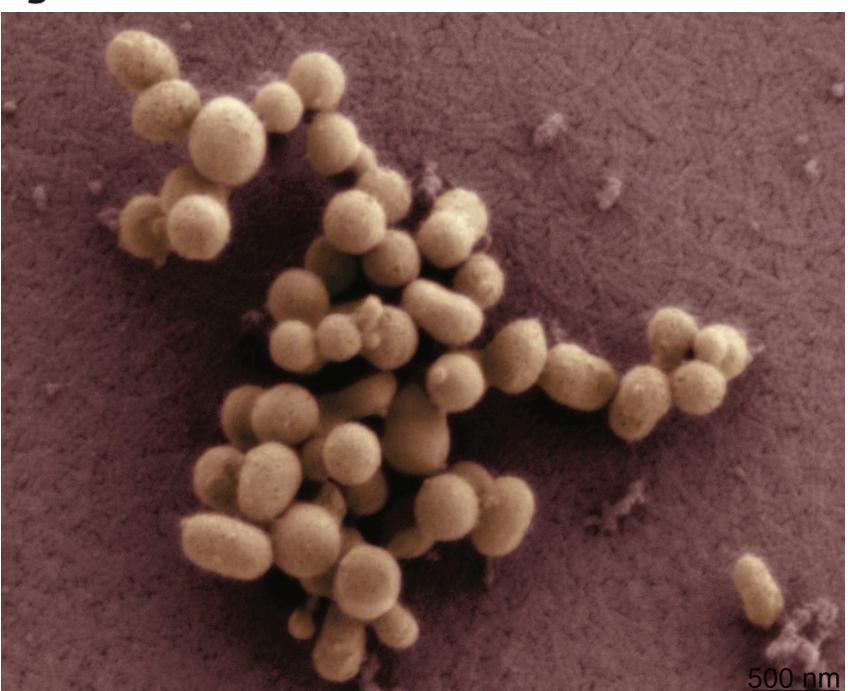


Stefan Werner, Carola Engler, Ernst Weber,  
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 (2012) Fast track assembly of multigene  
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 MoClo system, *Bioengineered*, 3:1, 38-43, DOI:  
[10.4161/bbug.3.1.18223](https://doi.org/10.4161/bbug.3.1.18223)

2 JULY 2010 VOL 329 SCIENCE

## Creation of a Bacterial Cell Controlled by a Chemically Synthesized Genome

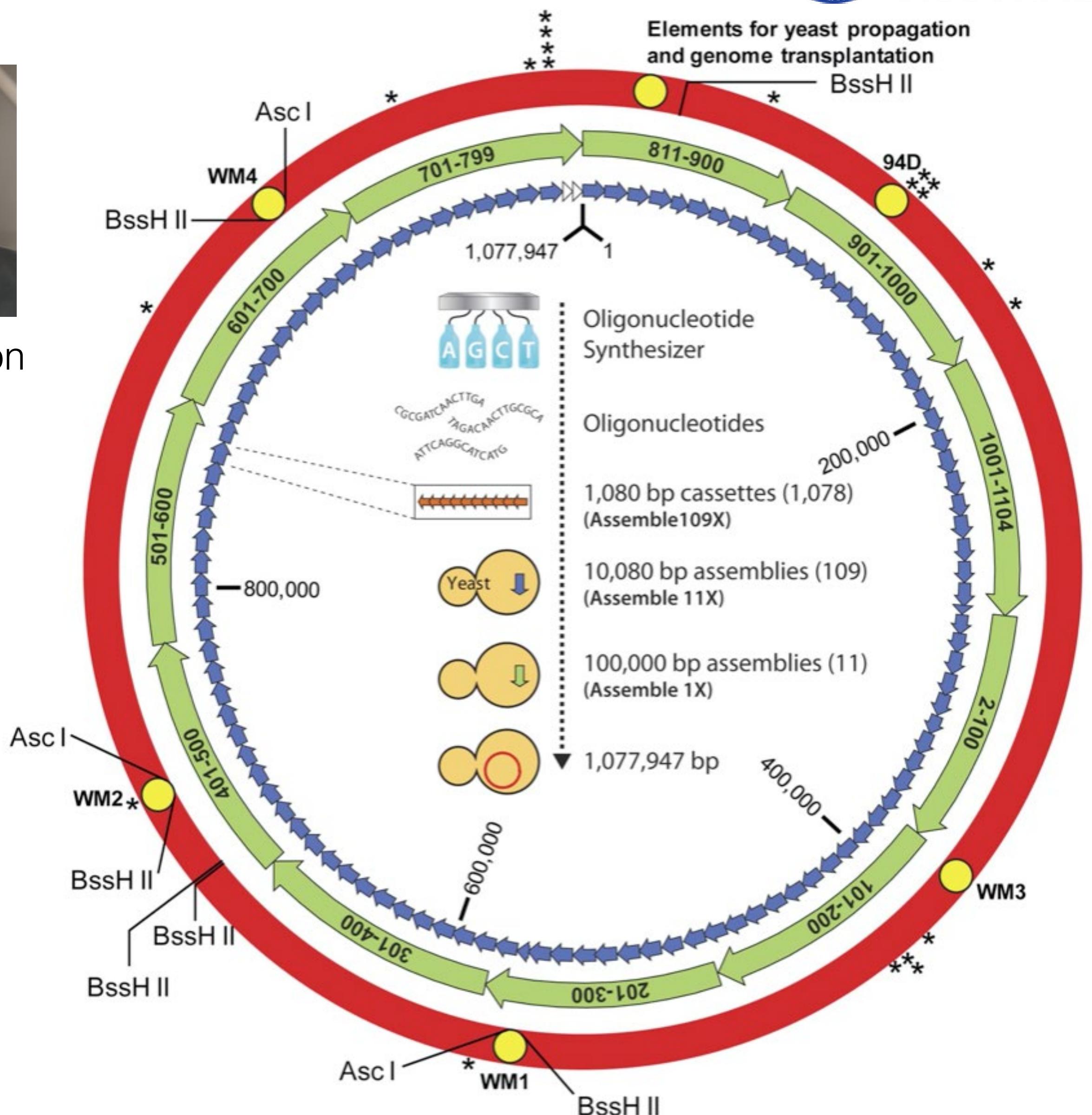
Daniel G. Gibson,<sup>1</sup> John I. Glass,<sup>1</sup> Carole Lartigue,<sup>1</sup> Vladimir N. Noskov,<sup>1</sup> Ray-Yuan Chuang,<sup>1</sup> Mikkel A. Algire,<sup>1</sup> Gwynedd A. Benders,<sup>2</sup> Michael G. Montague,<sup>1</sup> Li Ma,<sup>1</sup> Monzia M. Moodie,<sup>1</sup> Chuck Merryman,<sup>1</sup> Sanjay Vashee,<sup>1</sup> Radha Krishnakumar,<sup>1</sup> Nacyra Assad-Garcia,<sup>1</sup> Cynthia Andrews-Pfannkoch,<sup>1</sup> Evgeniya A. Denisova,<sup>1</sup> Lei Young,<sup>1</sup> Zhi-Qing Qi,<sup>1</sup> Thomas H. Segall-Shapiro,<sup>1</sup> Christopher H. Calvey,<sup>1</sup> Prashanth P. Parmar,<sup>1</sup> Clyde A. Hutchison III,<sup>2</sup> Hamilton O. Smith,<sup>2</sup> J. Craig Venter<sup>1,2\*</sup>



- Synthesis of 1.08-mega-base pair *Mycoplasma mycoides* JCVI-syn1.0 genome
- Genome transplantation in *M. capricolum* cells
- Lead to viable cells with all physiological properties of *Mycoplasma mycoides*

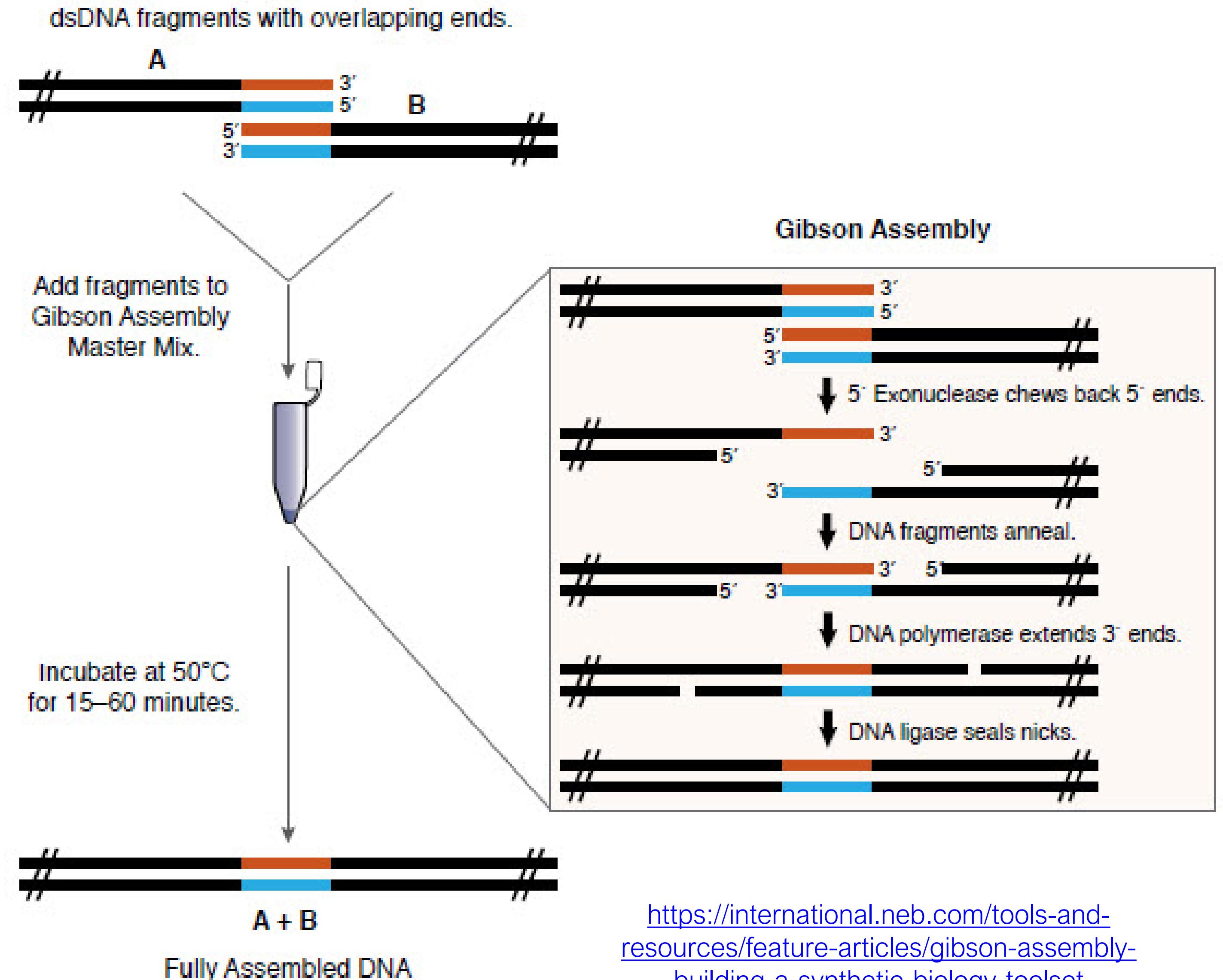


Daniel Gibson

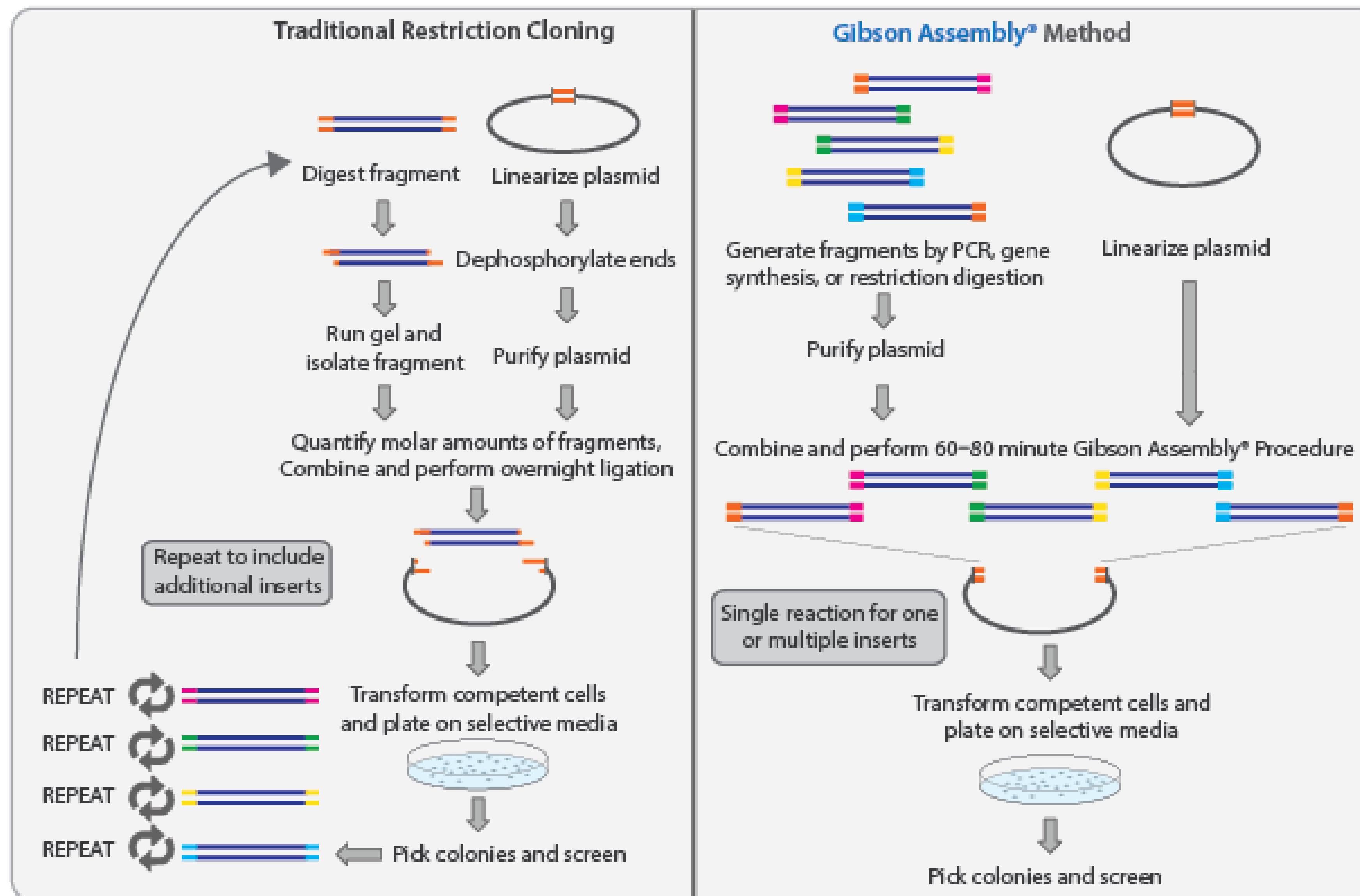


# Gibson assembly (*in vitro*)

- Neighbouring dsDNA fragments to contain a 20-40 bp overlapping sequence
- 5' exonuclease activity creates sticky ends -> DNA fragments anneal
- 3' extension by DNA polymerase
- Seal nicks by DNA ligase
- Assembly of up to 6-8 DNA fragments
- Assembly of large (~5kb) DNA fragments possible
- Problems with small fragments!



# Faster & more efficient than traditional cloning

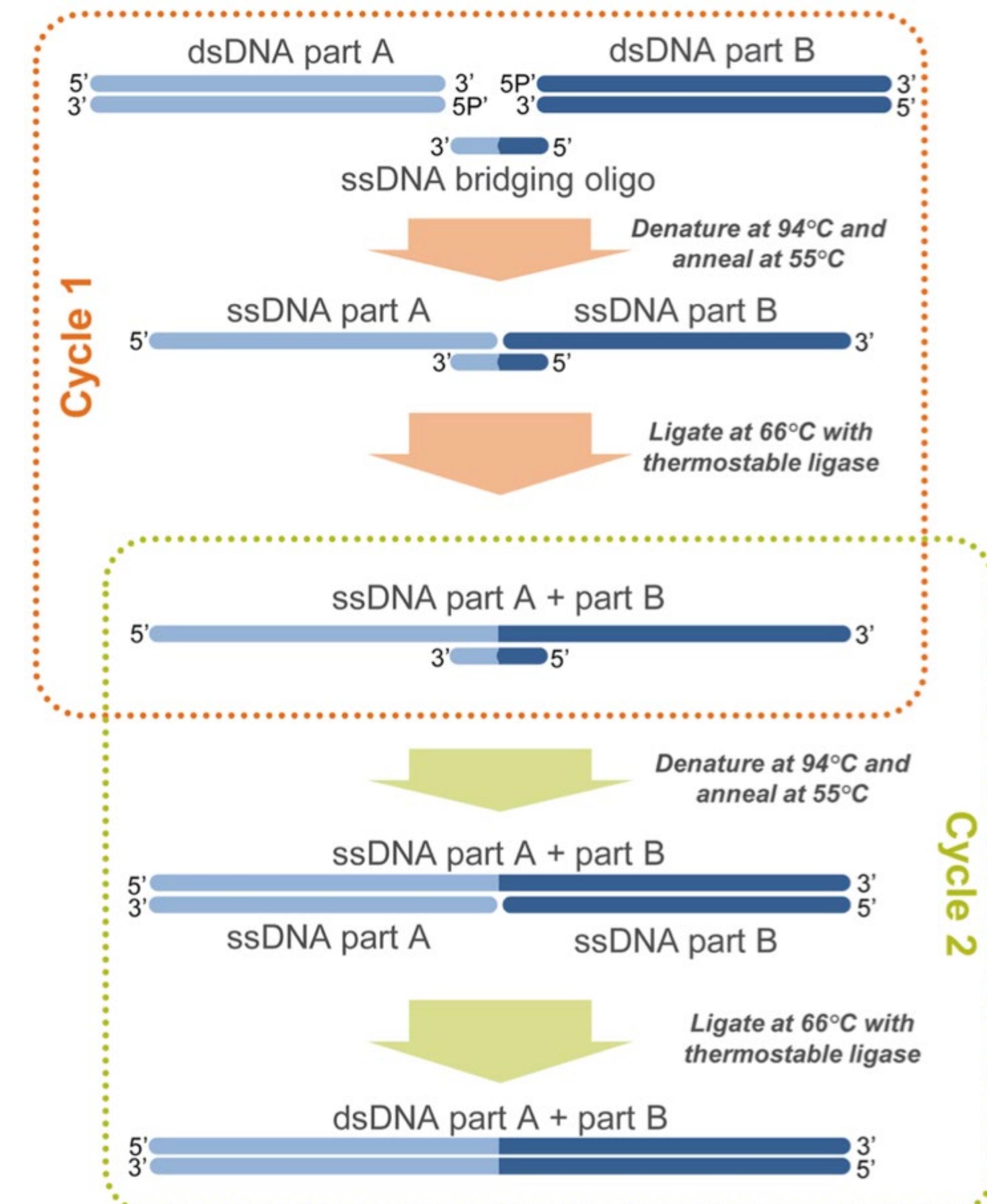


# The Gibson assembly song (2010)



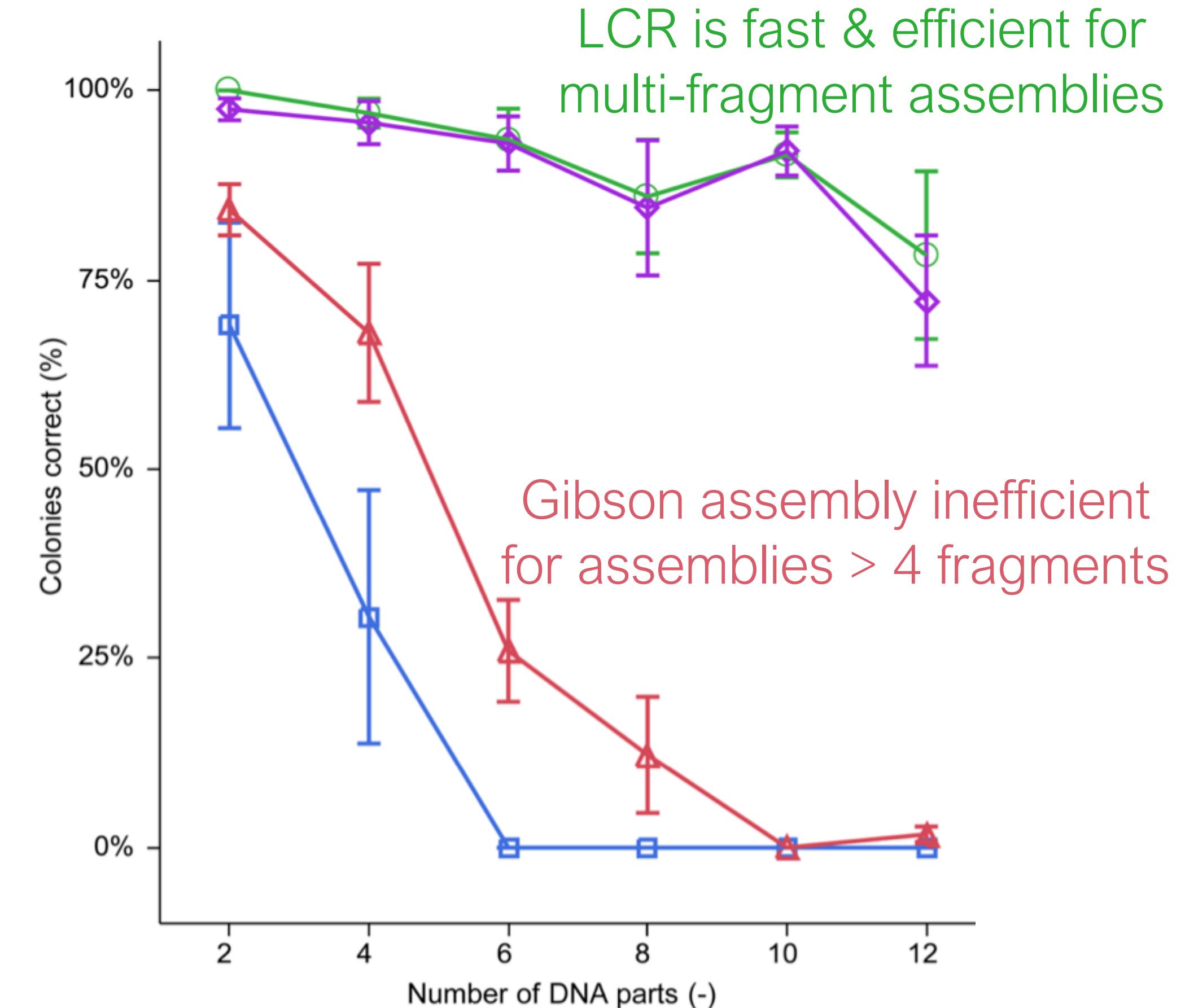
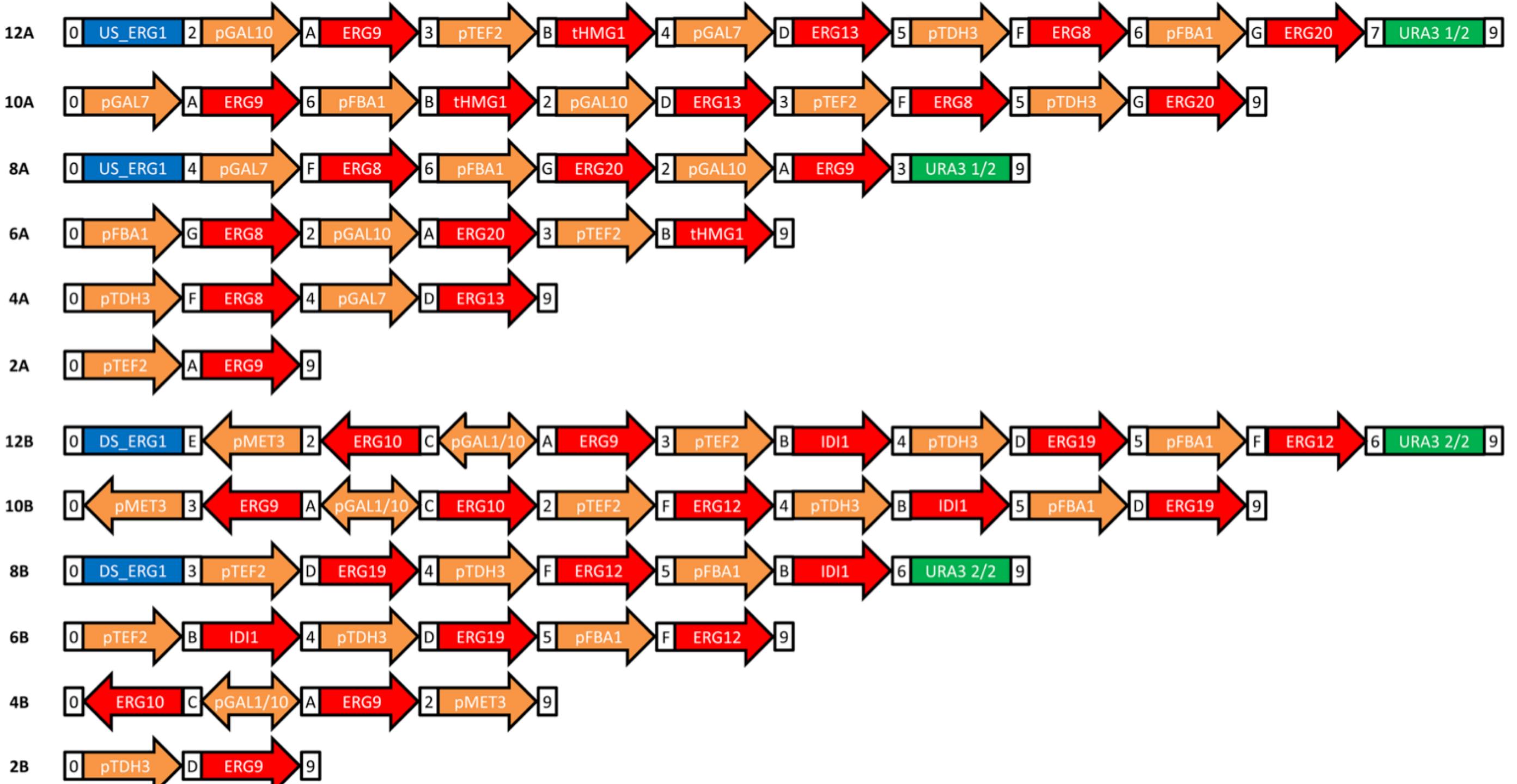
# Ligase cycling reaction (LCR)

- Custom single-stranded bridging oligos complementary to the ends of neighbouring DNA parts serve as a template to bring the upper strands of denatured (5' phosphorylated) DNA parts together
- Target melting temperature of 70 °C for both halves of the bridging oligo -> typical bridging oligo lengths of 60–90 bp
- A thermostable ligase joins the DNA backbones
- In the second and subsequent temperature cycles, the assembled upper strand serves as a template for ligation of the lower strand
- Typically, 50 denaturation– annealing–ligation temperature cycles are used for assembly of many DNA parts into complex DNA constructs.



# Performance of LCR vs. Gibson assembly

Design of DNA constructs to compare DNA assembly methods

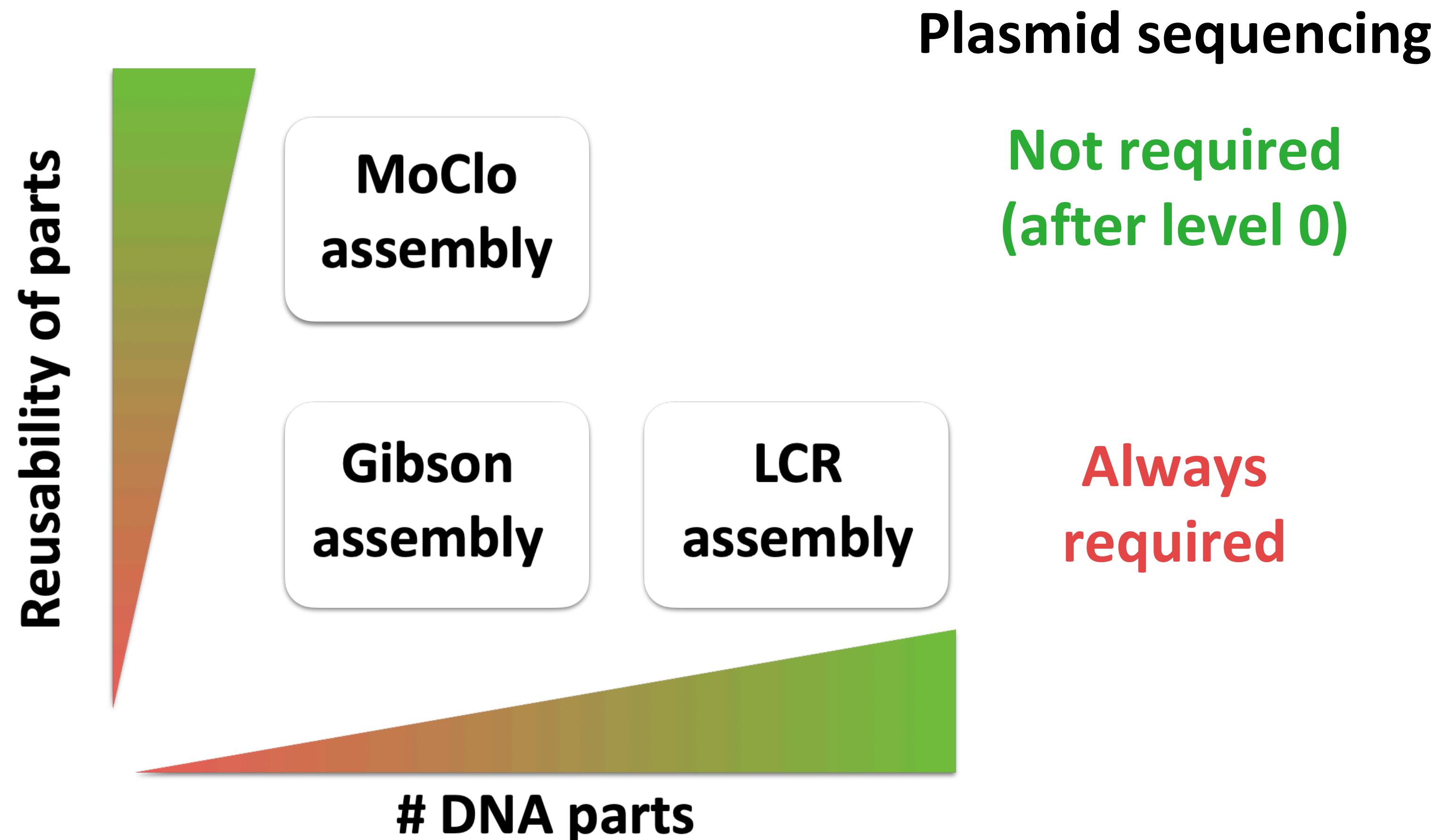


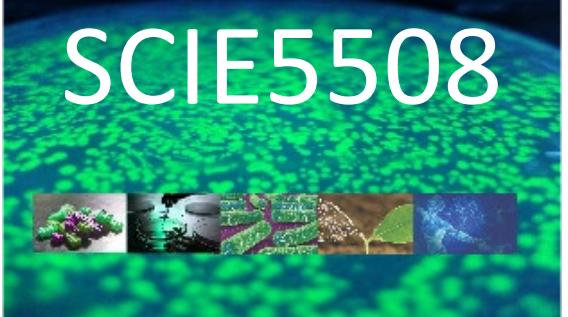
LCR is fast & efficient for multi-fragment assemblies

Gibson assembly inefficient for assemblies > 4 fragments

DNA assembly method	Assembly time (h)
Circular Polymerase Extension Cloning (CPEC)	1.5-3
Gibson isothermal assembly	1
Yeast homologous recombination	36-72
Ligase Cycling Reaction (LCR)	1.5-2

# Comparison of the discussed DNA assembly methods





# (Partial) overview of DNA assembly standards

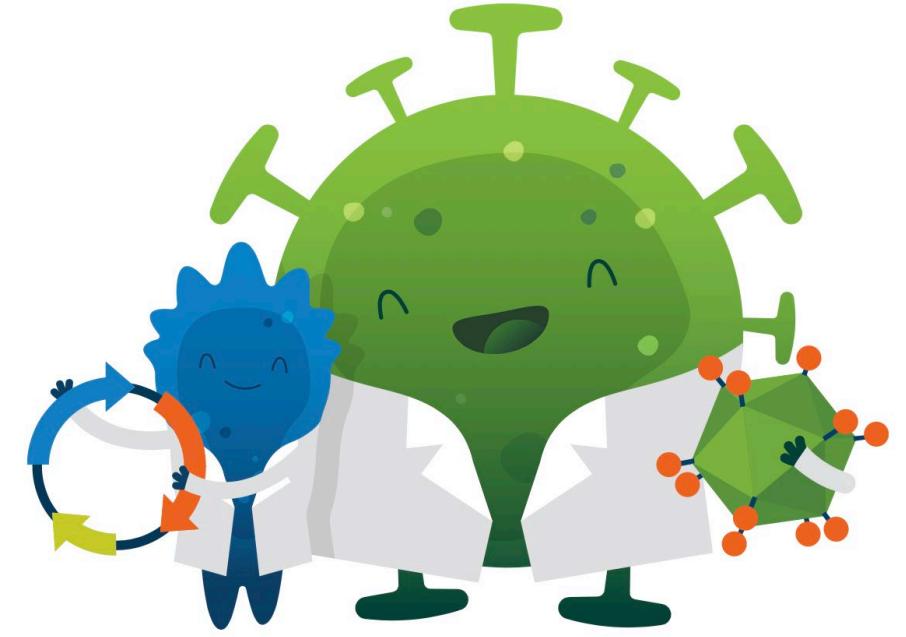


Physical standards	Underlying methodology					Limitations		Workflow		
	Restriction and ligation	HE	Type IIS RE	SSR	Long overlap	PCR required*	Forbidden restriction sites‡	Number of assembly tiers	Multipart assembly§	Hierarchical assembly
BioBrick <sup>13</sup> and BglBrick <sup>15</sup>	✓					No	4	1	No	Yes
iBrick <sup>21</sup>		✓				No	0	1	No	Yes
HVAS <sup>22</sup>		✓	✓			No	0	2	Yes; no	No <sup>¶</sup>
MoClo <sup>29</sup>		✓				No	3	2	Yes; yes	Yes
GoldenBraid 2.0 (REF. 30)		✓				No	3	≥2	Yes; no	Yes
GreenGate <sup>32</sup>		✓				No	1	2	Yes; no	Yes
Binder <i>et al.</i> <sup>31</sup>		✓				No	3	2	Yes; yes	Yes
PSA <sup>37</sup>		✓				No	0	1	No	Yes
DNA assembler <sup>53</sup>			✓			Yes	0	2	Yes; yes	No
MODAL <sup>9</sup>			✓			Yes	0	1	Yes	No
BASIC <sup>58</sup>		✓	✓			No	1	1	Yes	Yes
Torella <i>et al.</i> <sup>55¶</sup>	✓		✓	✓		No	≥4 <sup>#</sup>	2	No; yes	No
Guye <i>et al.</i> <sup>59</sup>		✓	✓	✓		No	0	2	Yes; yes	Yes
PaperClip <sup>56</sup>			✓			No	0	1	Yes	No

Casini, A., Storch, M., Baldwin, G. *et al.* *Nat Rev Mol Cell Biol* 16, 568–576 (2015).

<https://doi.org/10.1038/nrm4014>

# Modular Cloning systems to become the standard of the standards: Toolkits for many model systems



<https://www.addgene.org>

Sharing 89,020  
plasmids on behalf  
of 4,307 labs

🔥 MoClo Tool Kit

🔥 EcoFlex MoClo Kit

🔥 CIDAR MoClo Parts Kit

🔥 MoClo Yeast Toolkit

🔥 MoClo Pichia toolkit

🔥 MoClo Plant Parts Kit

🔥 MoClo Plant Parts II and Infrastructure Kit

MoClo CRISPR/Cas Toolkit for Plants

# Summary

- Re-usability of biological parts demands standardisation in DNA assembly
- The registry of standard biological parts is a repository of ~20,000 parts that adhere to the BioBrick assembly standard; The standard uses Type IIP restriction enzymes for binary assembly of BioBricks; Compared to contemporary techniques BioBrick assembly is slow + cumbersome
- The Modular Cloning (MoClo) standard relies on Golden Gate assembly via Type IIS restriction enzymes, enabling quick, hierarchical assembly of complex DNA constructs; many toolboxes with characterized parts available for model organisms
- Gibson Assembly enables fast & efficient assembly of DNA parts; Cons: Parts need to be larger than 100bp; reduced fidelity for >4-5 parts
- Ligase Cycling Reaction (LCR) works via bridging oligos and efficiently assembles many (~10-12) fragments



# Thanks for watching!

Please post your questions on the LMS  
Discussion board & tune in the Q&A session



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