DNA Assembly Webinar

Part 1: Primer Design

Dennis Mishler, PhD
Assistant Professor of Practice
The University of Texas at Austin

6/30/2020

DNA Primers

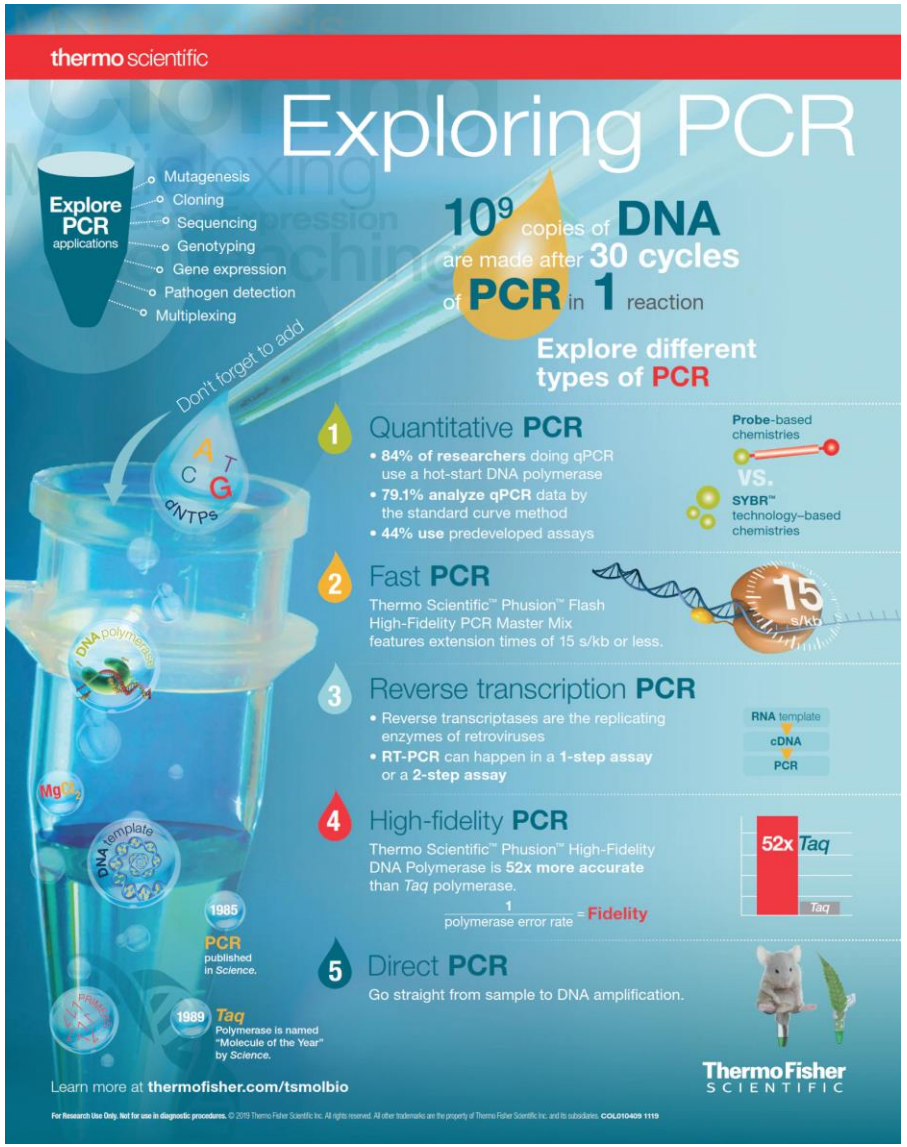
- Why do we use them?
- What are DNA primers?
- Factors to consider when designing primers
- Where and how to design and order DNA primers
- Primer Design example using Benchling and IDTDNA.com

Why do we use DNA Primers?

- **PCR or… Polymerase Chain Reaction**
  - Mentioned in our previous webinar, "DNA parts and basic molecular biology" (Week 2b, Part 3).
  - If you are a bit fuzzy on PCR, I would recommend heading back to that webinar and the linked video.

- There are many different types of PCR with different purposes, but all of them employ DNA primers.

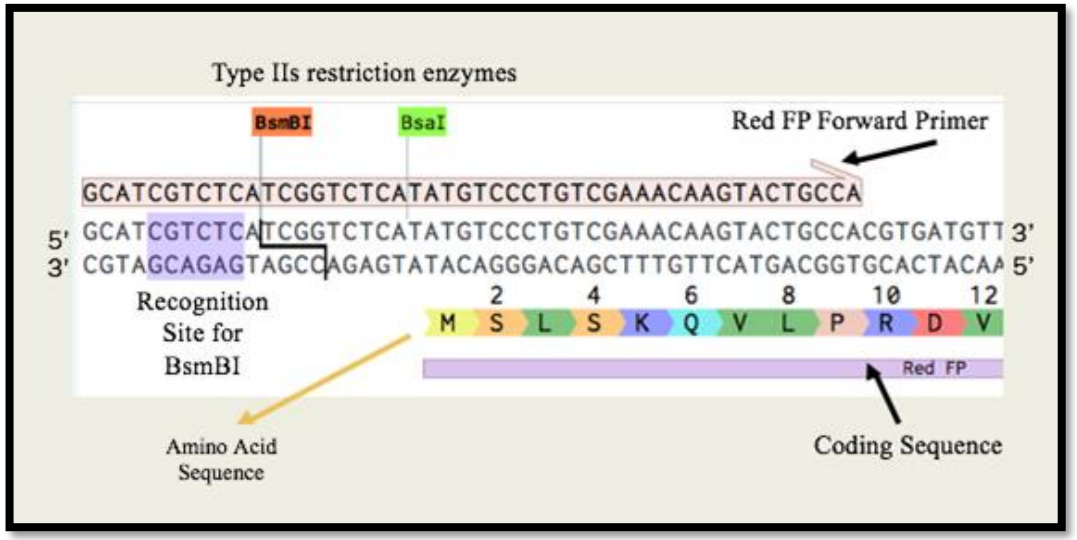
- The primer(s) you design vary greatly, depending on the type of PCR and the template DNA.



Exploring PCR, ThermoFisher.com

What are DNA Primers?

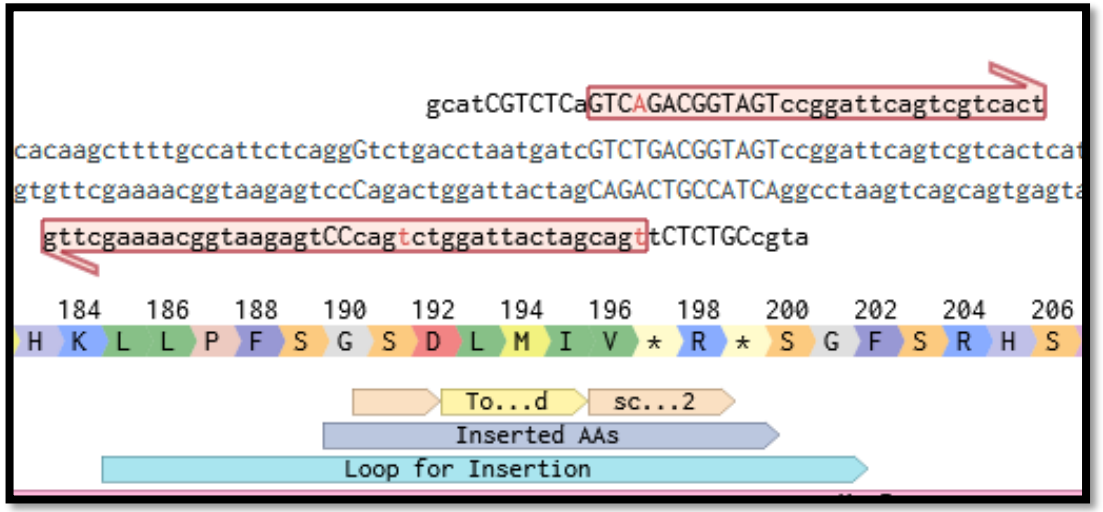
- **DNA primers are short "oligonucleotides"** that can base pair to a strand of DNA.
- **DNA primers are complementary** to the DNA template that they bind to.
- **In the example on the right,** the DNA primer ("Red FP Forward Primer") is bound to the DNA template. Notice that the primer ends with a half-arrow, denoting directionality.
- **Remember that a primer binds to the complementary strand of DNA.** Thus, in this case, the primer is actually binding to the reverse strand, the strand on the bottom.



Primer bound to template. Image generated using Benchling.

Factors to consider when creating DNA Primers

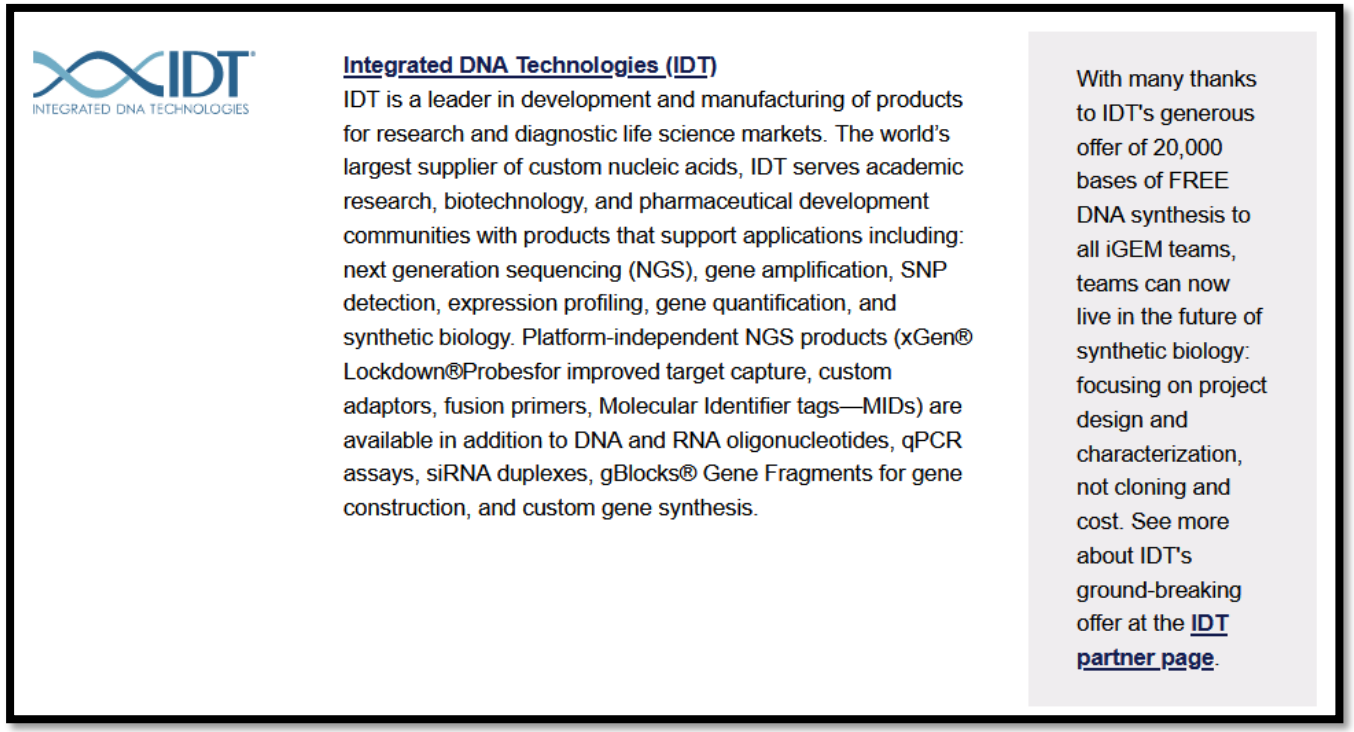
- **Fully complementary primer?**
  - Common for sequencing, amplification from genomes, various diagnostics
- **Partially-complementary primer?**
  - Common for mutagenesis, cloning, various diagnostics
- **When designing the actual sequence of the primer, you need to take into account:**
  - Length and GC-content
  - Melting temp (T_m) and Annealing temp
  - Secondary structure of primer
  - Other factors depending on your purpose



Partially-complementary primers… pointing in the wrong direction? Image made with Benchling

Where to design and order primers

- There are many options.
- Note: iGEM has partnered with several companies to provide YOU with free DNA, which could include primers as well as full genes.
- Go to the iGEM partners page!!!
- We will use Benchling and IDT during this webinar to demonstrate how to make your own primers.
- We will make 1-2 example primers.



iGEM partners page. IDT offer on the right.

Let’s make a primer!

- Benchling.com
- IDTdna.com

Benchling Template sequence:
https://benchling.com/s/seq-e81GjK8N7VnP8asHrxFi

- You won’t be able to edit this file, but you can select all, and then copy and paste the DNA into your own sequence file.
- You can rewatch this webinar later and make your own primers, if interested.
- We made two example primers (assuming time)

Example of PCR Product sequence:
https://benchling.com/s/seq-3Z30OYy0CVIQeYajEjwO

- measurement@igem.org [ask for me!]

Questions?

- From this portion of the webinar, hopefully you learned about some of the important factors affecting primer design.

- Utilize the free resources! They want you to succeed. It's okay to order "gblocks" or whole-gene synthesis.

- **We will get to our next part of the webinar in a few minutes, after questions**. I will introduce Golden Gate Assembly.

- measurement@igem.org [ask for me!]

DNA Assembly Webinar

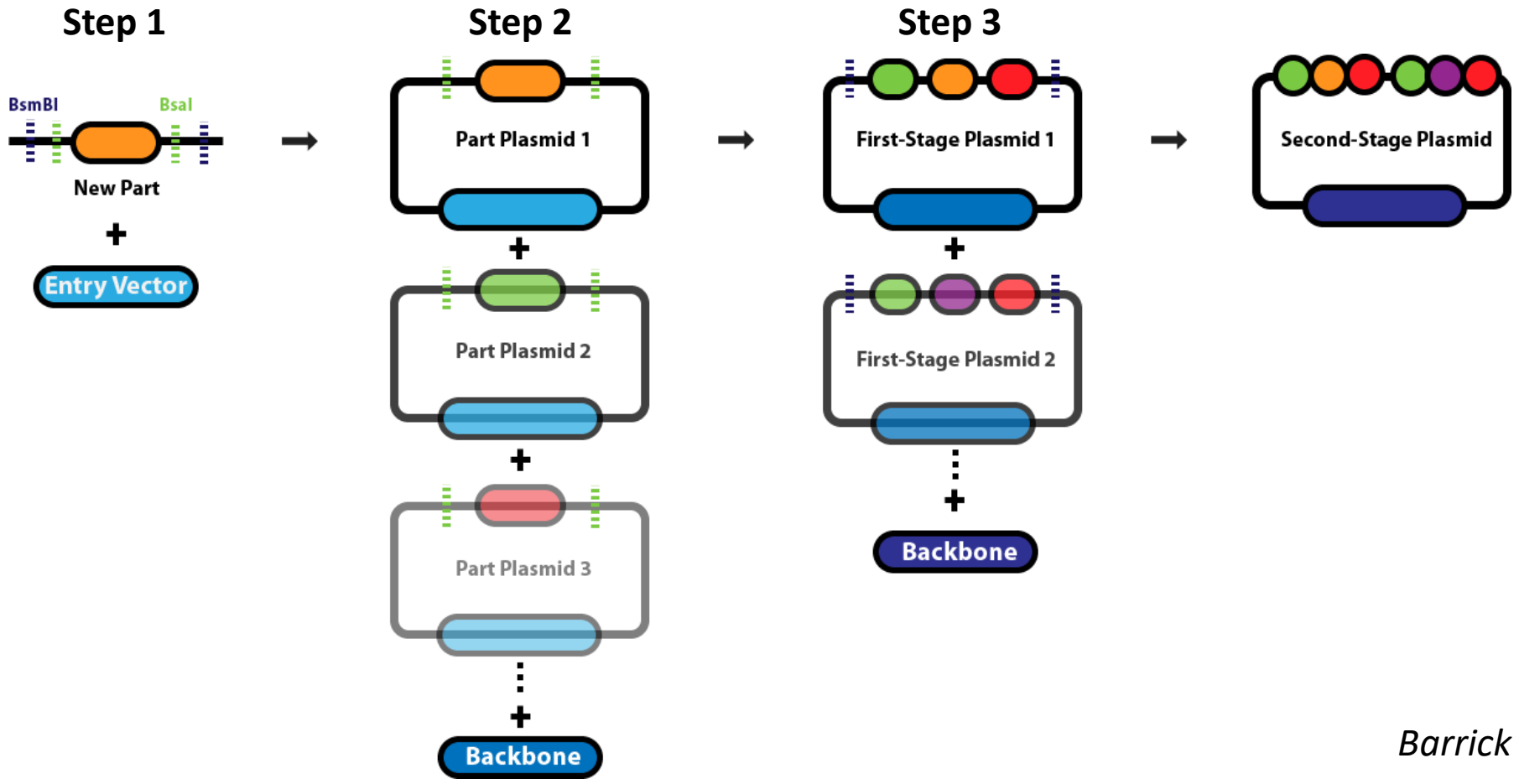
Part 2: Golden Gate Assembly

Dennis Mishler, PhD
Assistant Professor of Practice
The University of Texas at Austin
Some slides by Rene Inckemann
6/30/2020

Golden Gate Assembly

- An assembly method that works efficiently with standardization and parts!
- First introduced in 2008: A one pot, one step, precision cloning method with high throughput capability
  C Engler, R Kandzia, S Marillonnet - PloS one, 2008
- Many pieces of DNA can be assembled in a single “pot”, combining not just multiple pieces of DNA but also the digestion AND ligation steps… all in one tube.
- **Requires “Type IIS” restriction enzymes**

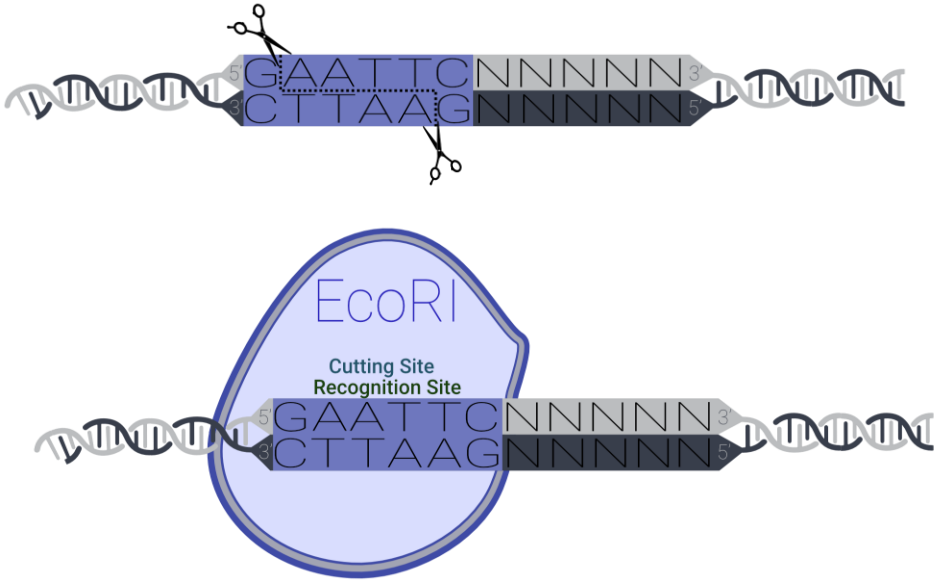
Golden Gate Assembly



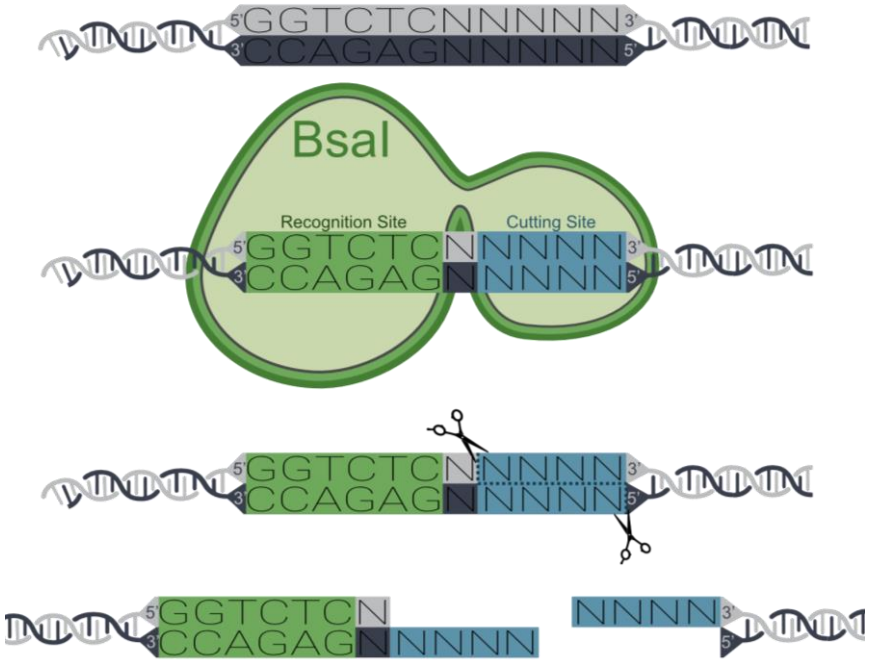
Barrick lab wiki

Step 1: Take PCR product and assemble into a plasmid = Part Plasmid.
Step 2: Take multiple part plasmids and assemble into a functioning plasmid = “First-Stage Plasmid” or “Assembly Plasmid”.
Step 3: Combine “First-Stage Plasmids” to create more complex circuits.

Golden Gate Assembly: The Magic of Type IIs



Type II restriction enzyme:
BioBrick cloning/assembly

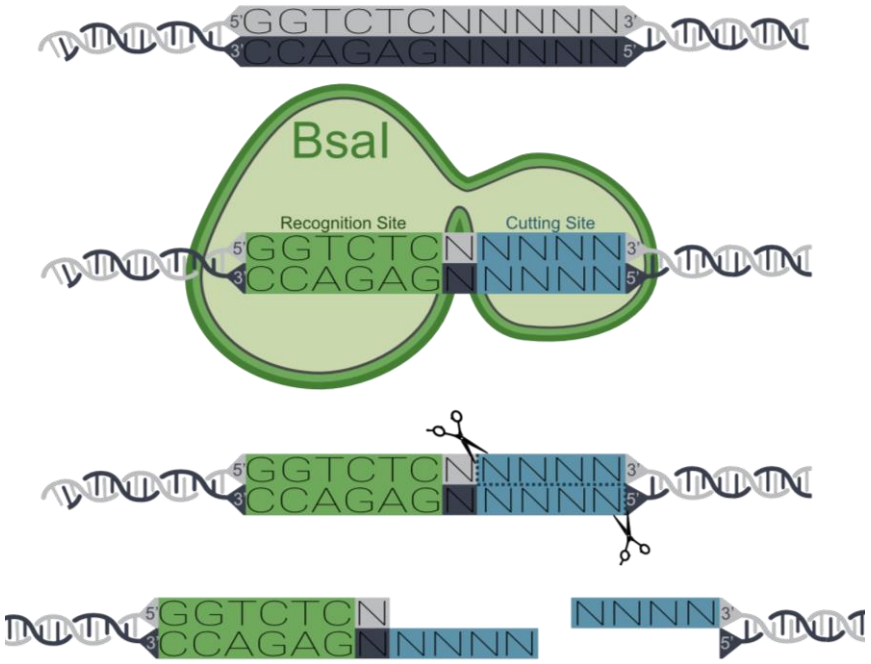


Type IIs restriction enzyme:
Golden Gate cloning/assembly

Type IIs Restriction Enzymes: Lose the recognition site

During the assembly reaction, the DNA containing the recognition site is removed, creating a product that lacks a cut site.

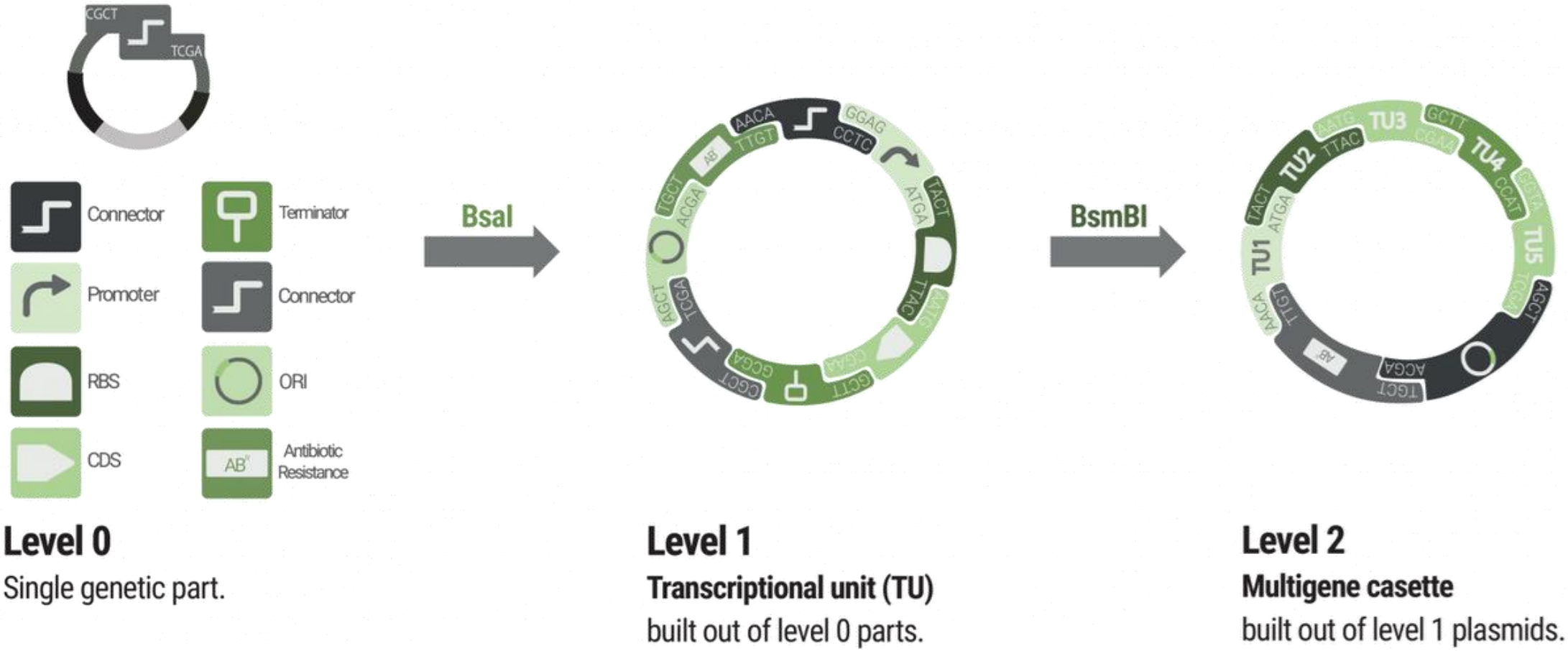
Type IIs Restriction Enzymes



Type IIs restriction enzymes allow for:

- Seamless or scarless cloning
- Varying of the cut site nucleotides, allowing you to generate multiple distinct overhangs in a single reaction.
- This allows you to assemble many pieces of DNA together in a predictable order.

Assembling multiple parts at once



Level 0
Single genetic part.

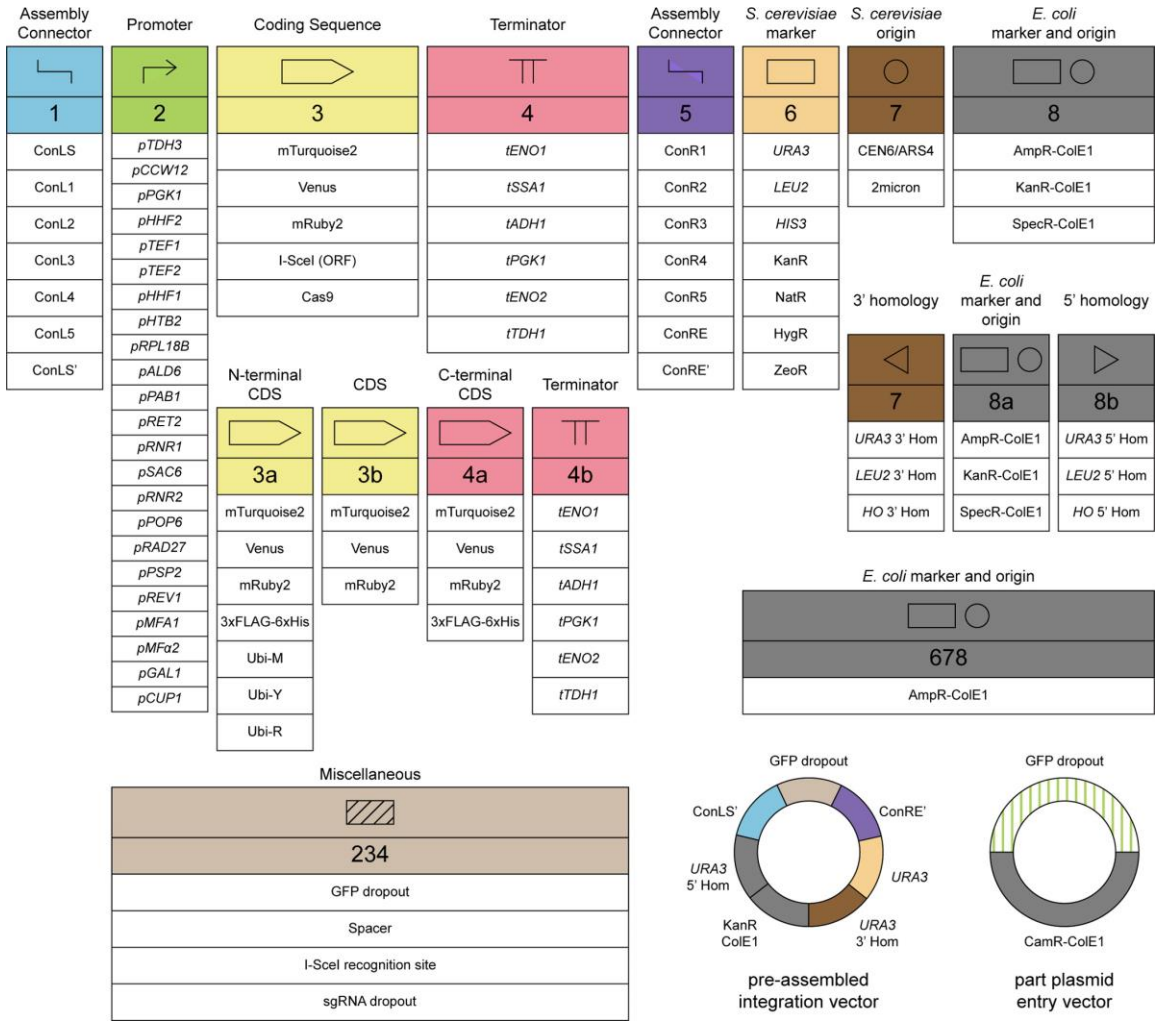
Level 1
Transcriptional unit (TU)
built out of level 0 parts.

Level 2
Multigene casette
built out of level 1 plasmids.

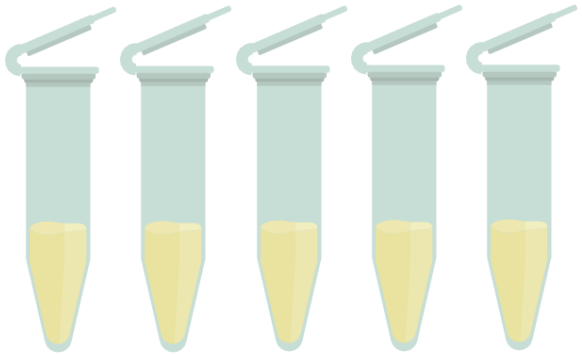
Notice the overhangs between one part and the next match identically

Golden Gate Assembly

- Collection of parts, where each part type has a specific overhang, designed to be complementary to the “adjacent” part type.
- Leads to Modular Cloning, which Rene will speak about in the third part of this webinar.
- Most common Type IIs enzyme include BsmBI (also, Eps3I), BsaI, and BbsI [note: some are replaced with newer, more efficient enzymes].
- Many web Apps have “assembly wizards” that will allow you to design a Golden Gate Assembly reaction.

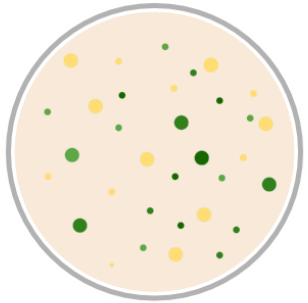


ME Lee, 2015



Assembly reaction

Transformation
(webinar 6b)



Golden Gate in your Lab

Pipette all your parts together

Golden Gate assembly allows for cloning in a one pot reaction. Parts, restriction enzyme, and ligase are pipetted together and put into a thermocycler.

Cyclic/thermocycler reaction

Step 1	37°C	2 min	x 50
Step 2	16°C	5 min	
Cycle steps 1 & 2 x 50			
Step 3	50°C	10 min	
Step 4	80°C	10 min	
Total Time ~6h (370min)			

37°C for the restriction enzyme
16°C for the ligase
50°C as a final digest (to remove original plasmid)
80°C for enzyme inactivation

Golden Gate Assembly: Key Takeaways

- DNA pieces are combined using a Type IIs restriction enzyme (commonly, BsmBI or BsaI).
- Each “part type” has a specific prefix and suffix sequence.
- The prefix/suffix identity dictates the sequential ordering of the DNA being assembled.
- The single reaction includes both digestion and ligation steps, without the need for DNA purification or gel extraction.
- Advantages – Many pieces of DNA can be assembled simultaneously in a single reaction = very fast and powerful.
- Disadvantages – It can be more difficult to initially plan and master the techniques.

Questions?

- From this portion of the webinar, hopefully you learned about the key concepts and purpose of Golden Gate Assembly.

- Mastering Golden Gate Assembly take practice. I recommend using the “wizards” that your favorite Web App has. Rene will hopefully have time to run through an example during his portion of the webinar

- **We will get to our next part of the webinar in a few minutes, after questions**. Rene will talk about “Modular Cloning” (MoClo).

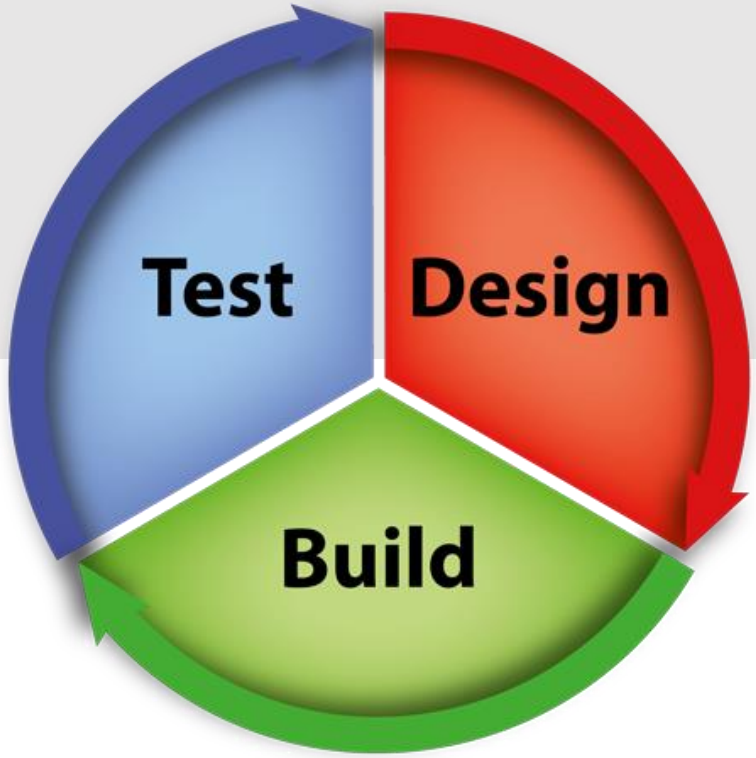
Example of PCR Product sequence that is Golden Gate compatible (if time):
https://benchling.com/s/seq-3Z30OYy0CVIQeYajEjwO [insert]
https://benchling.com/s/seq-wzAHPJOO5esrFFTv9Z3H [backbone]

- measurement@igem.org [ask for me!]

Next up:
Modular Cloning
with Rene Inckemann

MoClo Webinar

Why should you use MoClo?



Applications

- Modular designs with exchangeable parts
- Metabolic engineering
- Genetic circuits
- Multi gene constructs

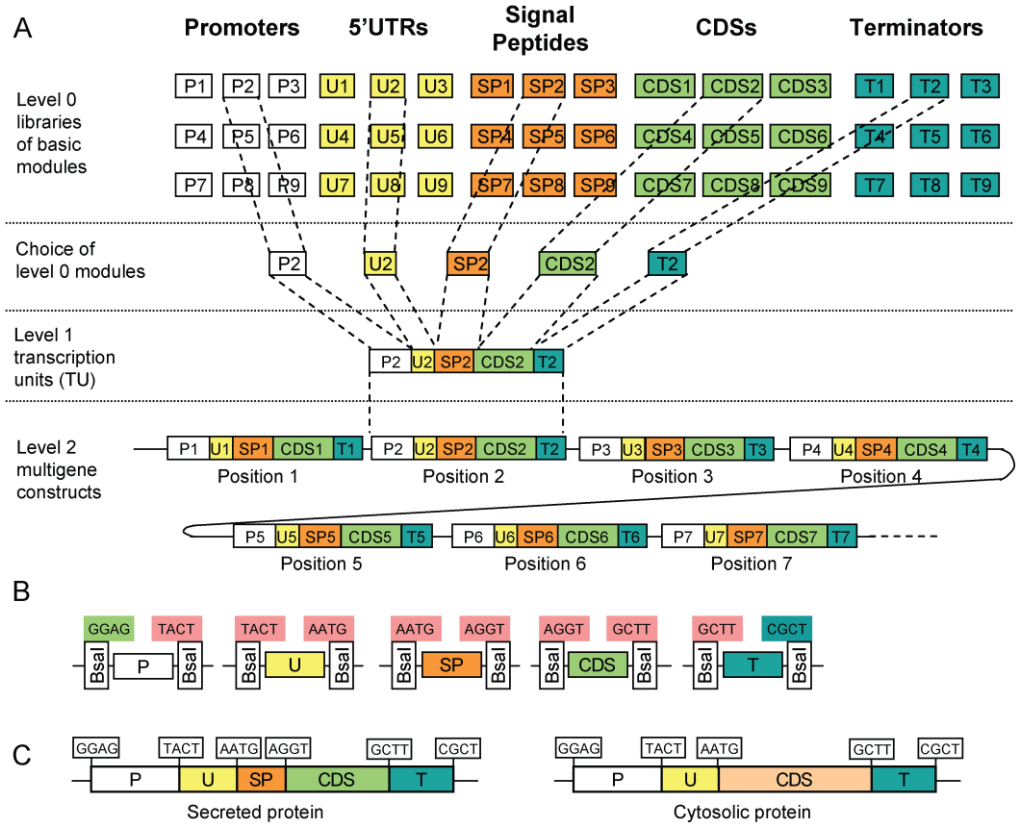
Advantages

- Up to 24 part cloning in one pot reaction
- Sequencing not necessary (if parts are sequenced)
- No time consuming primer ordering for new designs
- High throughput very easy achievable
- Can be automated

What has been done before?

Origin of Modular cloning (Moclo)

- A Modular Cloning System for Standardized assembly of multigene constructs (Weber, Marillonnet *et al* , 2011)
- Based on many vectors as a toolkit



What has been done before?

OpenPlant

sharing tools for a sustainable future

Origin of MoClo

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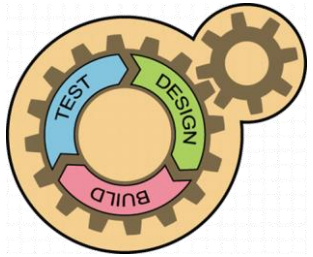
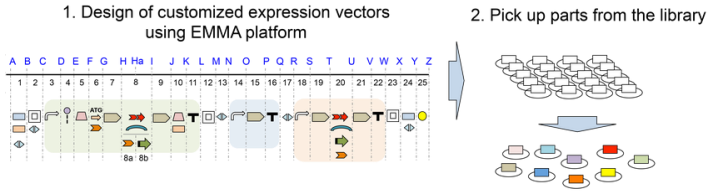
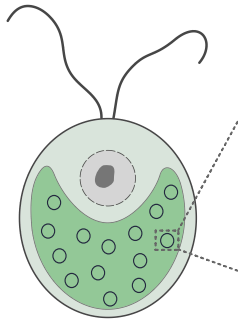
- Based on many vectors as a toolkit

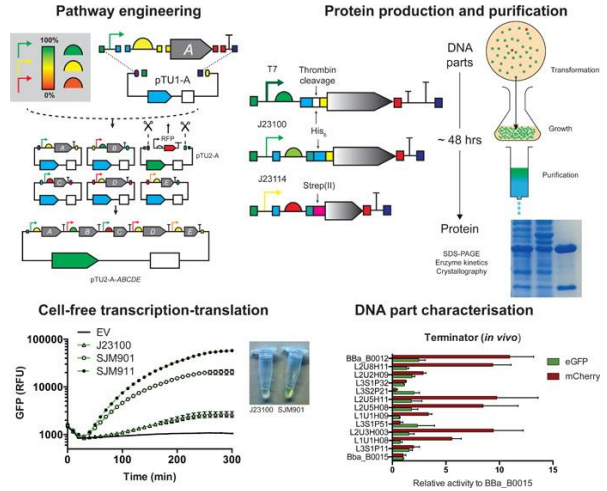




Different Variants/ Chassis Organisms

- common standard for plant/phototrophic chassis
- Bacterial (*E.Coli*/*Vibrio*), bakers yeast, *pichia*, *yarrowia*, mammalien cells, cyanobacteria
- Iterative cloning / hierarchical cloning





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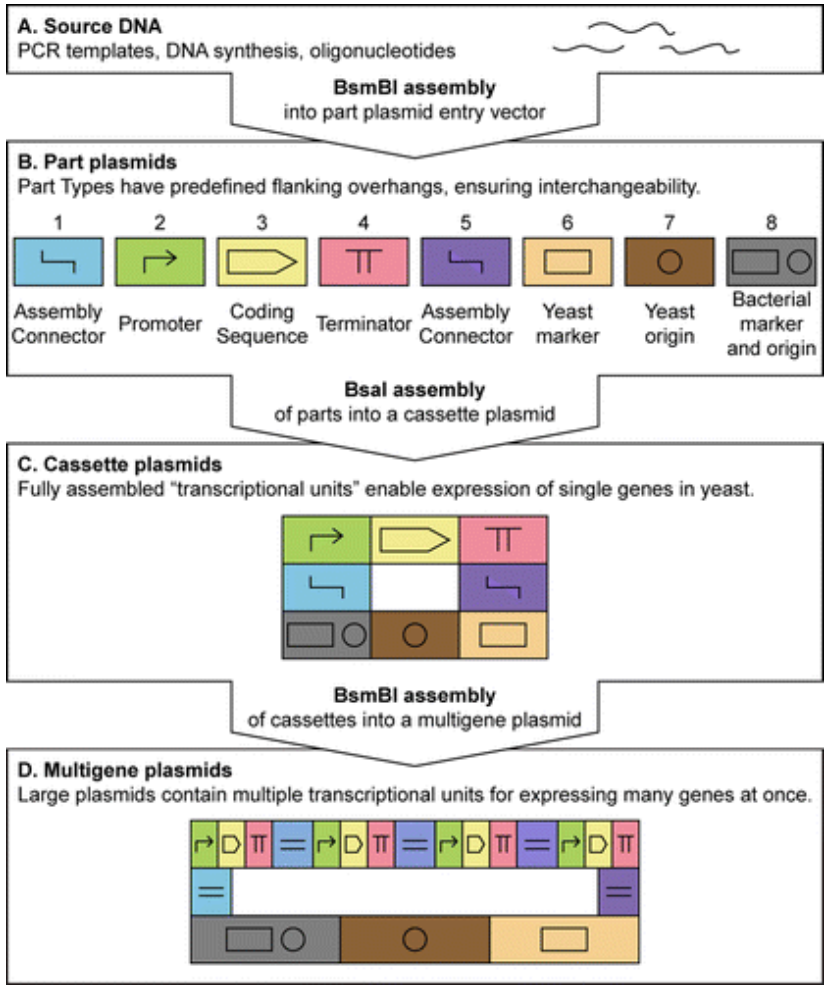
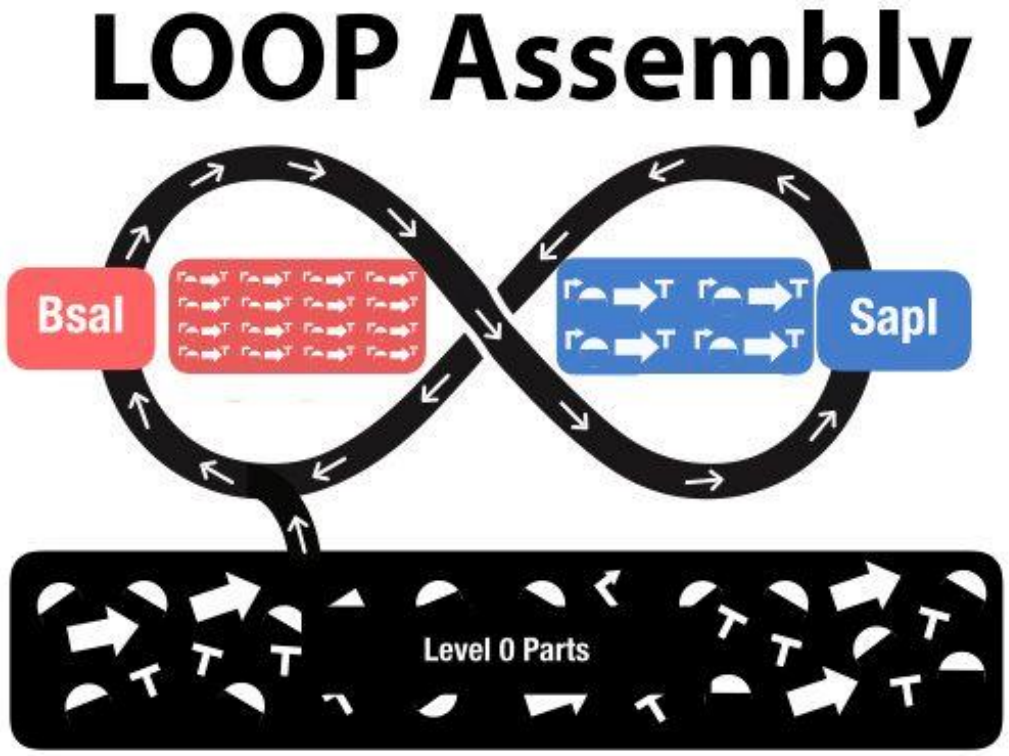
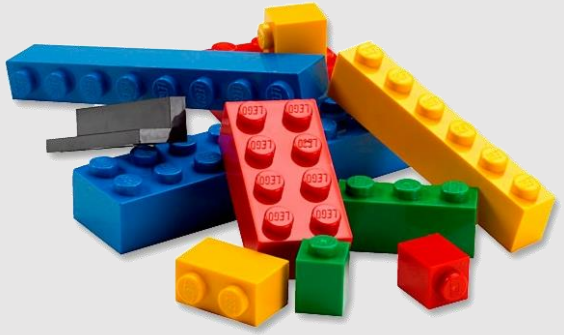


VS.



The Modular cloning principle



BsaI

BsmBI

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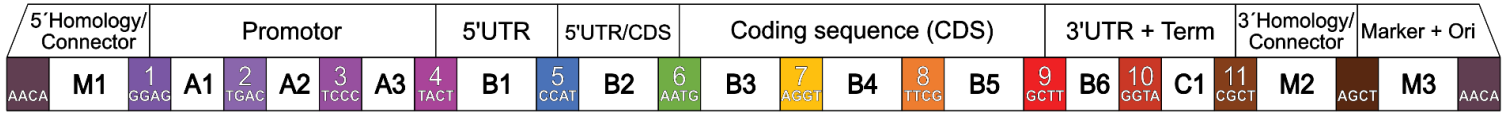
101 Golden Gate

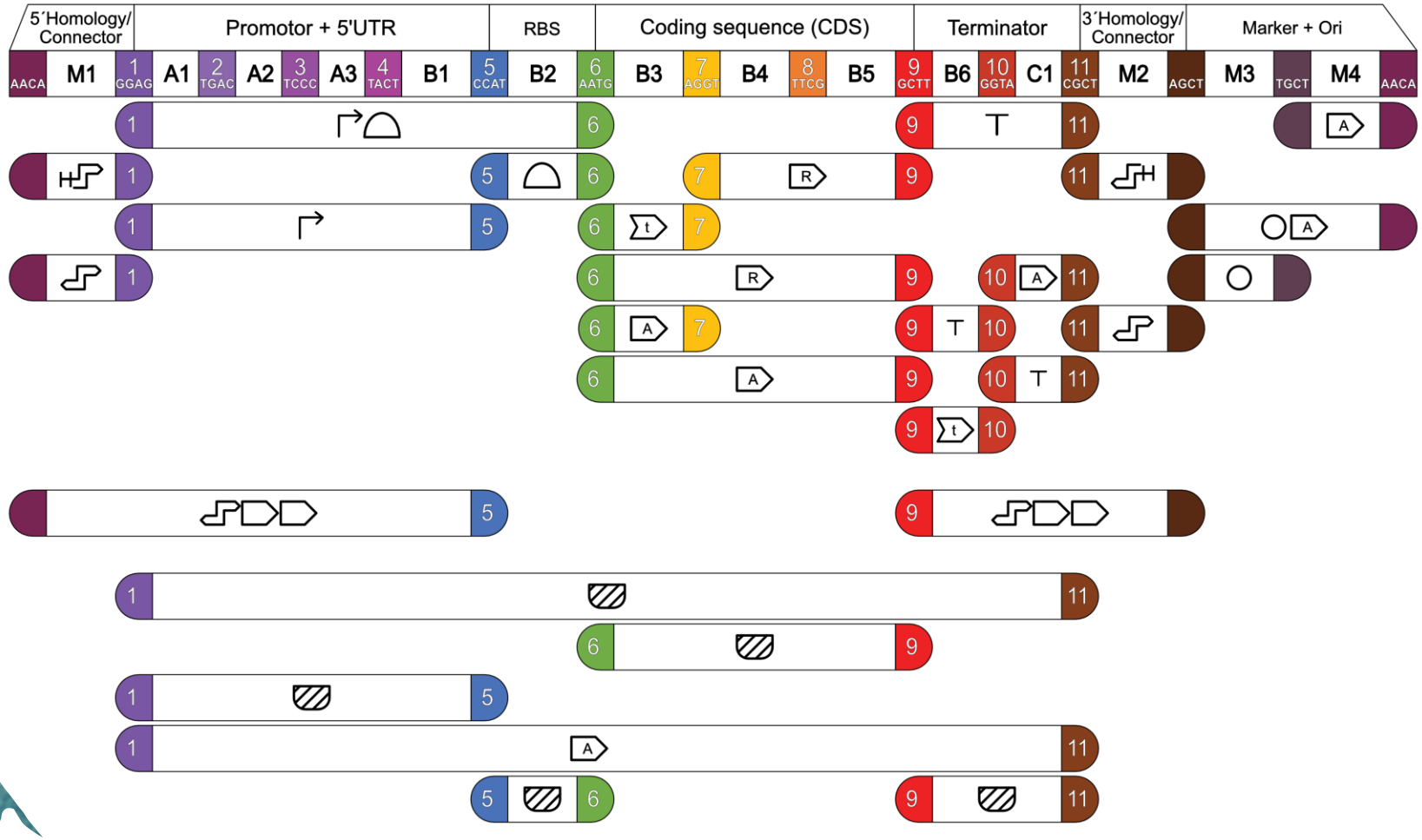


Modular Cloning

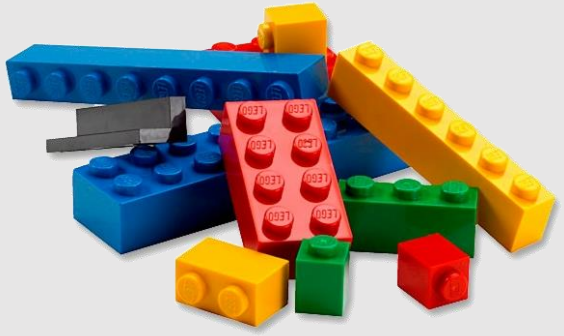
Fusion sites are standardized

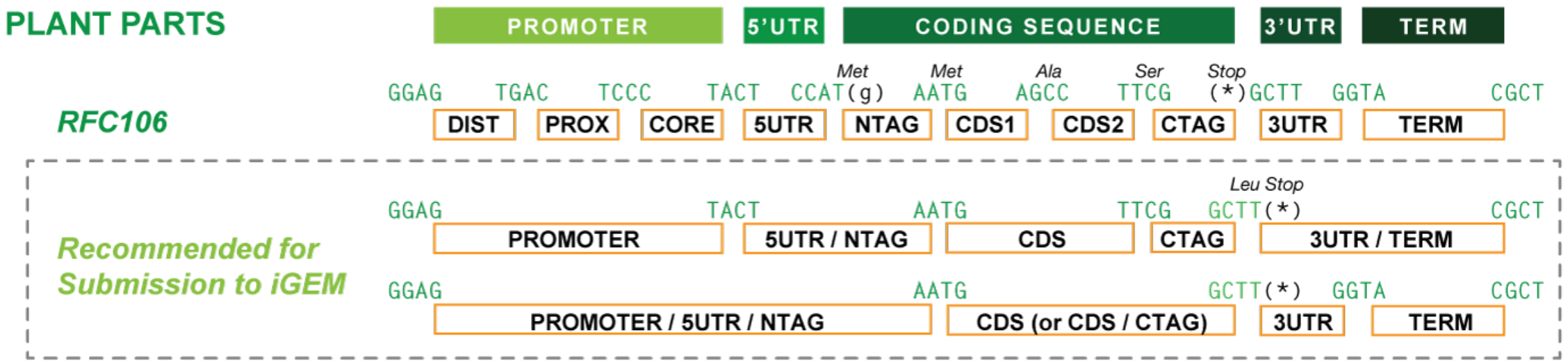
Allows exchangeability of parts (level 0, e.g promoter) with other labs/groups

101 Golden Gate



The Modular cloning
The iGEM standard(s)





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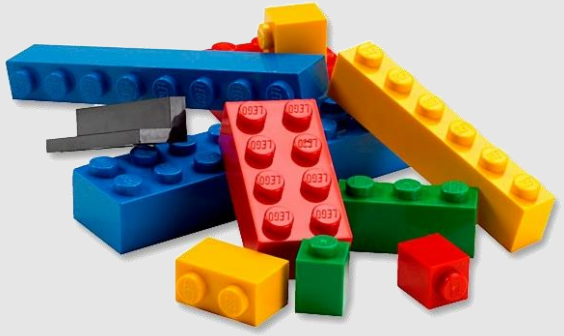

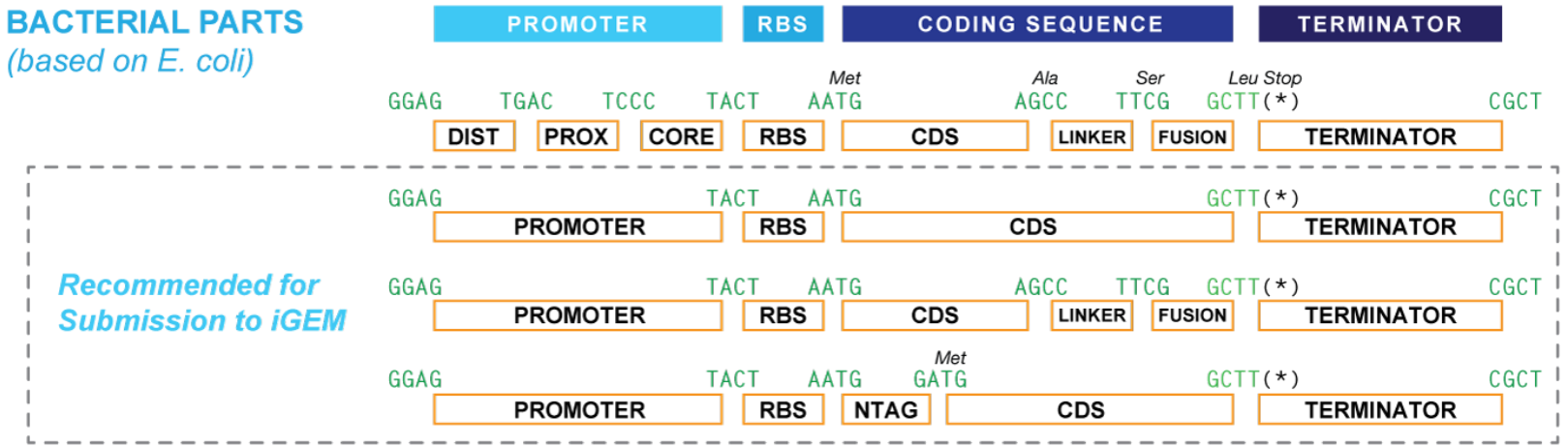

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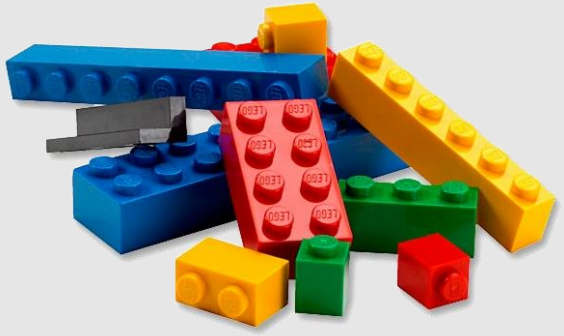
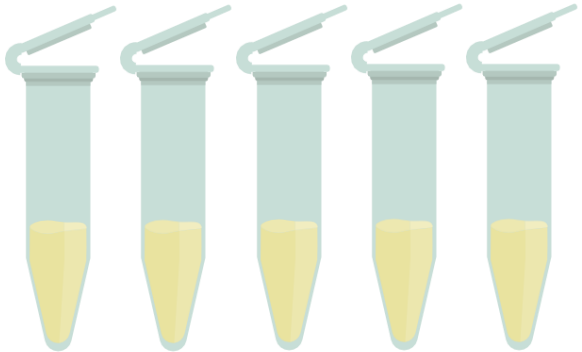


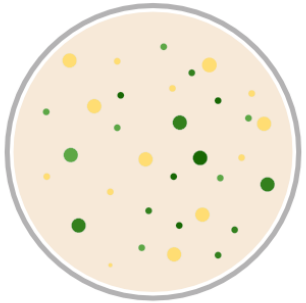
			Assembled into	
Level	Abstraction	Types	Loop Vector	Enzyme
Level 0	Basic	Promoter, RBS, CDS, Termintaor, etc.	pOdd (Level 1)	Bsa1
Level 1	Composite	Transcriptional units (devices, reporters, etc.)	pEven (Level 2)	Sap1
Level 2	Composite	Multi-transcriptional units (up to 4)	pOdd (Level 3)	Bsa1
Level 3	Composite	Multi-transcriptional units (up to 16)	pEven (Level 4)	Sap1

Level 1 Assembly								
GGAG	Promoter	TACT	5'UTR	AATG	CDS	GCTT	Terminator	CGCT
GGAG	Transcriptional Unit							CGCT

Fusion Site 5'	Transcriptional Unit (TU)	Fusion Site 3'
ATG	TU 1	GCA
GCA	TU 2	TAC
TAC	TU 3	CAG
CAG	TU 4	GGT

- **Loop assembly standard**
  Introduced (planned) just last year.





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37°C for the restriction enzyme
16°C for the ligase
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Practical part

From level 0 parts to multigene constructs