Recent Advances in Ligation-Free Cloning

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August 6, 2025

Traditional approach to cloning DNA fragments

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The traditional approach to cloning DNA fragments in a plasmid involves the following steps:

- Adding restriction endonuclease (RE) recognition sites on either ends of the fragment
- RE digestion of the fragment
- RE digestion of the plasmid
- Ligation of compatible ends using DNA ligase
- Transformation of the ligated product in competent *Escherichia coli* cells

Drawbacks associated with this approach

This strategy is constrained by availability of suitable RE sites in

- the DNA fragment to be cloned
- the vector backbone

Cloning more than one gene in a single vector or cloning large DNA fragments can be challenging due to the increased probability of finding RE sites within the DNA to be cloned.

Overcoming this challenge

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One way to circumvent this issue is to modify the DNA sequence to remove RE sites found within DNA fragments to be cloned by

1. site-directed mutagenesis, or
2. chemical synthesis of the DNA fragment.

Both these strategies have their associated disadvantages, necessitating alternate cloning strategies.

DNA fragments as 'parts'

In the synthetic biology approach to molecular cloning, DNA fragments are treated as parts that can be joined to each other in a standard manner.

Parts are flanked by DNA sequences that are compatible with ends of other DNA sequences, making it possible to join parts in a defined and precise manner.

Standardization of DNA parts

Many standards have been described for DNA parts:

- NOMAD
- BioBricks
- BglBricks
- ...

Standard parts can be easily swapped and replaced, simplifying the process of vector construction.

These standards allow facile cloning and assembly of DNA parts.

The BioBrick RFC10 standard

A BioBrick consists of a prefix and a suffix.

BioBricks conforming to the RFC10 standard have the following RE sites:

- Prefix: EcoRI and XbaI
- Suffix: SpeI and PstI

As XbaI and SpeI generate complementary sticky ends, parts can be joined together unidirectionally into a vector that has been digested by EcoRI and PstI.

The composite part can be released from the vector by digestion with EcoRI and SpeI (or XbaI and PstI), and joined upstream of another part that has been digested with XbaI and PstI (or downstream of a part digested by EcoRI and SpeI).

BioBrick RFC10 standard part

https://parts.igem.org/Help:Assembly/Scars

Other ligation-dependent cloning strategies

- Golden Gate
- Golden Braid
- Modular Cloning (MoClo)

These strategies depend on Type IIS restriction endonucleases, which cut DNA outside of their recognition sites.

Disadvantages still remain

The problem associated with RE sites present within DNA fragments to be cloned remains unresolved. Moreover, reagents for these methods are expensive.

Need for ligation-independent cloning methods

Restriction-digestion and ligation reactions create scar sequences between DNA parts that are assembled together.

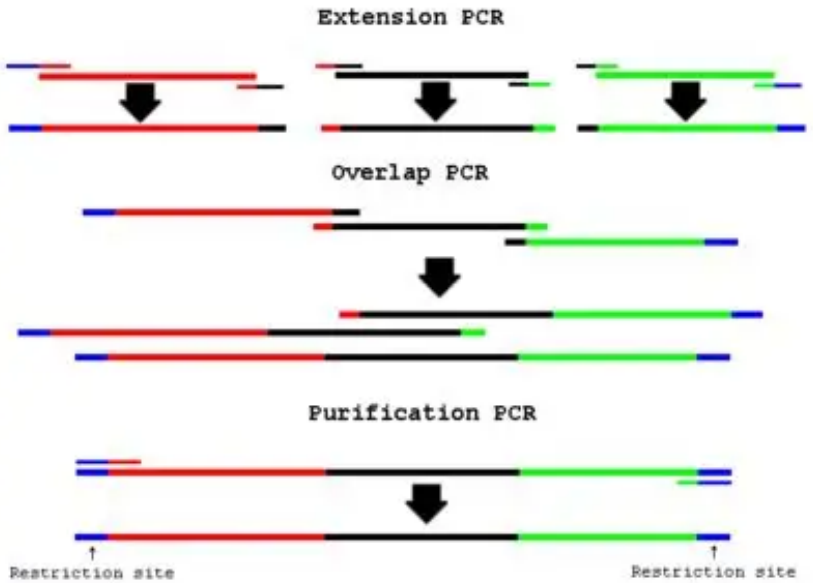
Recombination-based assembly of DNA parts allows the generation of constructs without intervening scar sequences.

Recombination-based assembly requires homologous sequences to be present on ends to be joined together.

DNA parts need not be engineered to remove restriction sites, as the only requirement for joining parts together is the presence of complementary overlap sequences.

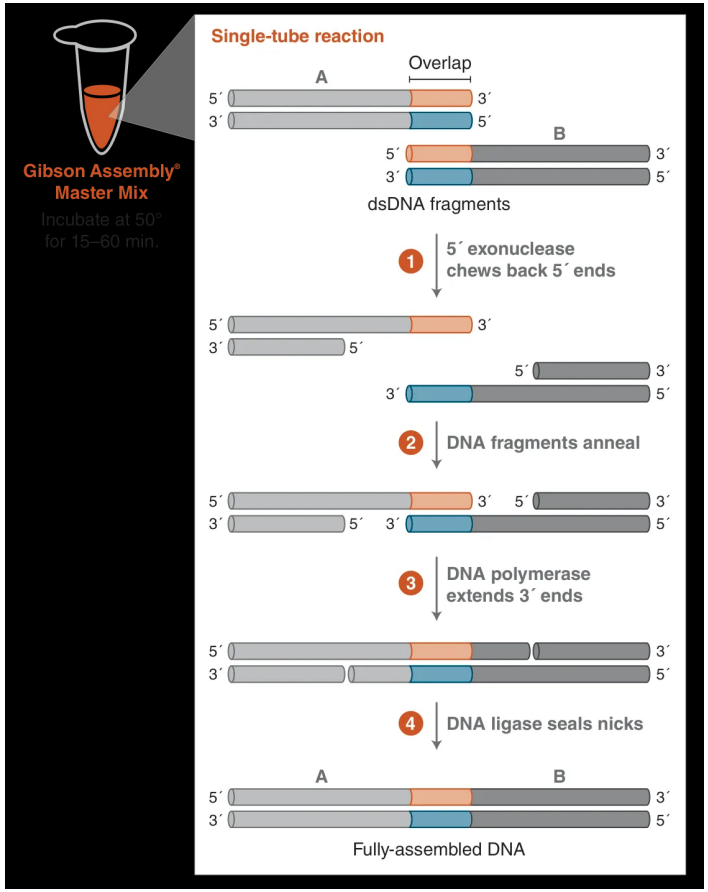
Ligation-independent cloning strategies

- Overlap extension PCR (OE-PCR)
- Sequence- and ligation-independent cloning (SLIC)
- Circular polymerase extension cloning (CPEC)
- Gibson Assembly (sequence-independent)
- In-Fusion Cloning (sequence-independent)



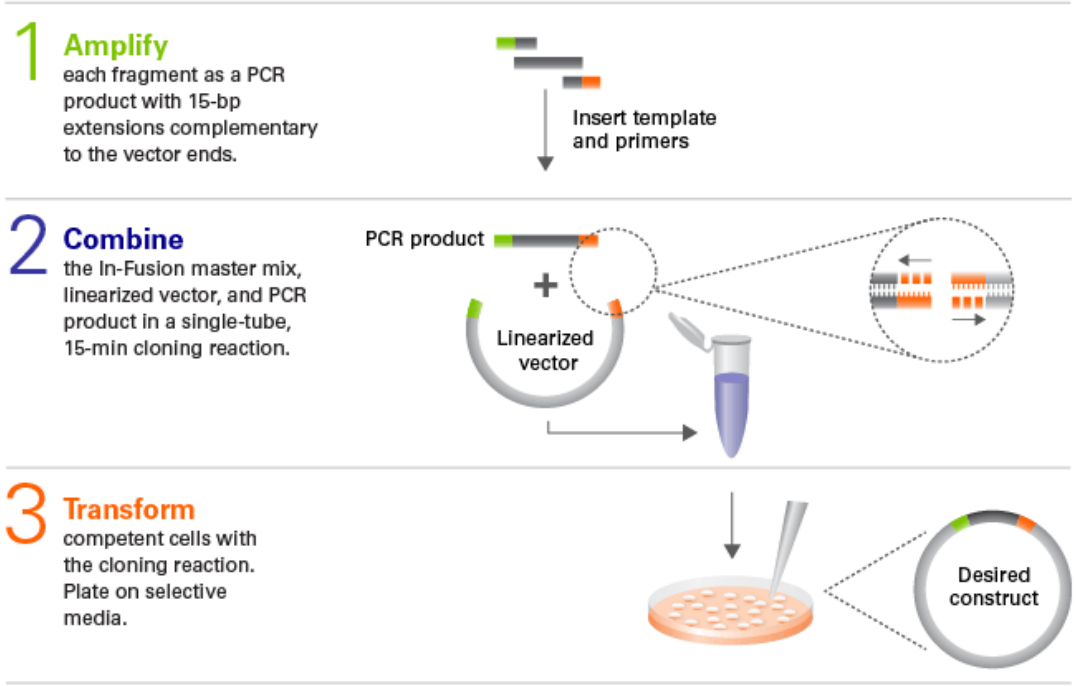
Overlap extension PCR

https://openwetware.org/wiki/PCR_Overlap_Extension



Gibson Assembly

https://www.neb.com/en/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/gibson-assembly



In-Fusion Cloning

https://www.takarabio.com/learning-centers/cloning/in-fusion-cloning-general-information/in-fusion-cloning-overview

Gibson Assembly and In-Fusion Cloning are widely used techniques, but are expensive.

In vivo cloning methods

In vivo cloning leverages the natural recombination processes of living cells to join DNA fragments that share short homologous regions.

This approach enables the direct and seamless assembly of plasmids within the host, eliminating the need for traditional restriction digestion and ligation steps.

Choosing a host organism for in vivo cloning

Saccharomyces cerevisiae is a preferred host organism due to its strong recombination efficiency.

E. coli presents several notable advantages for in vivo cloning:

- faster growth
- higher transformation efficiency
- greater plasmid yield

Additionally, recovering recombinant plasmids from yeast is more labour-intensive compared to *E. coli*.

Open Cloning

We have developed a methodology that can be used to clone DNA fragments in plasmids in a truly sequence- and ligation-independent manner.

The only requirement is that DNA fragments to be cloned should share 40 bp of homology at both ends.

Linear DNA fragments are transformed into *E. coli* expressing the λ phage Red recombinase.

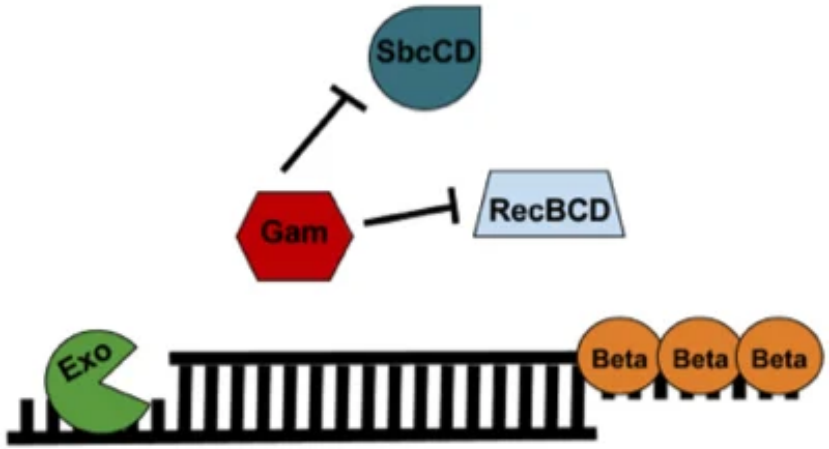
The λ Red system

The λ Red system comprises three genes: *exo*, *bet* and *gam*.

The *exo* gene encodes λ exonuclease. It processes DNA ends to produce 3' single-stranded overhangs, which are essential intermediates in most recombination mechanisms.

The Bet protein binds to single-stranded DNA and facilitates the annealing of complementary sequences. It can also mediate limited strand exchange.

The Gam protein binds to the RecBCD complex, blocking its exonuclease and helicase activities.



λ Red system

https://blog.addgene.org/
lambda-red-a-homologous-recombination-based-technique-for-genetic-engineering

Open Cloning workflow

1. Amplify vector backbone.
2. Amplify insert with primers carrying 40 nt homology to vector backbone.
3. Transform both DNA fragments into *E. coli*.
4. Screen transformants for correct insertion.

Advantages of Open Cloning

- Sequence-independent – no RE recognition sites required
- Ligation-free – inefficient ligation avoided
- Works with chemical competent *E. coli* cells – no need for electroporation
- Single step cloning – saves time
- Cost-effective – no expensive reagents or enzymes used

Thank you!