الكلية	العلوم
القسم	علوم الحياة
المادة باللغة الانجليزية	Biotechnology and genetic engineering
المادة باللغة العربية	التقنيات الاحيائية والهندسة الوراثية
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عنوان المحاضرة باللغة الانجليزية	Molecular cloning methods
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	Gao, J. (2012). Tissue culture and rapid multiplication techniques of Apocynum L. Agricultural Science & Technology, 13(11), 2269.

Molecular cloning methods

There are several different approaches to clone and you will need to find the right approach for your research. Below are some examples of popular cloning methods to generate a recombinant DNA construct:

Restriction Enzyme Cloning

Restriction enzyme cloning was the first cloning method developed and is still widely used today. This method uses restriction enzymes to cut the insert DNA and the vector at specific sites, leaving either overhangs or “sticky ends,” where one strand of DNA is longer than the other, or blunt ends, where both strands of DNA are the same length. If the sequences of the sticky ends are compatible with one another, then the vector and insert can be pieced together. Regardless whether the fragments have sticky ends or blunt ends, fragments are joined together using an enzyme called DNA ligase to create the recombinant piece of DNA (Figure 1).

This method is ideal when cloning single fragments, or a small number of fragments, into the vector.

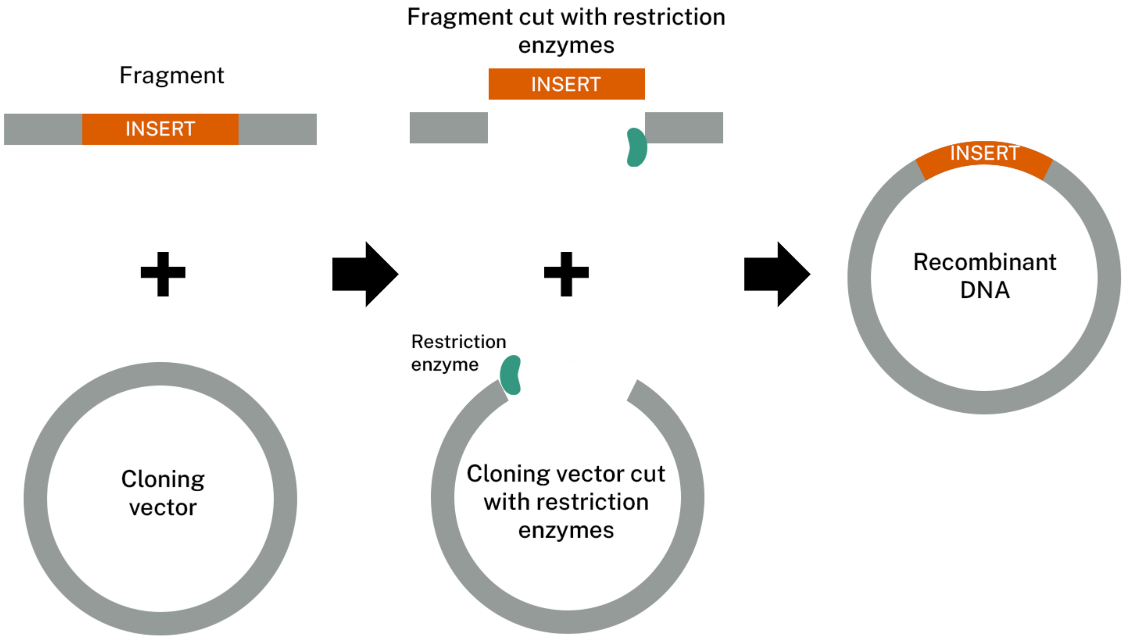

Figure 1. Restriction enzyme cloning.

Pros and cons of restriction enzyme cloning

Pros

- A widely used and established method with many options for cloning vectors and restriction sites
- Many tools available to design restriction enzyme cloning projects

Cons

- Can be time consuming as it is a multi-step process (digest first, then ligate)
- Need to ensure enzyme does not cut within insert sequence
- Cut vector may re-circularize on itself if it is not ligated to the insert
- If you're creating many different plasmids for your experiment, you might need multiple restriction enzymes as one enzyme may not be suitable for all inserts

Resources for restriction cloning in Geneious Prime

➤ **Restriction Cloning**: Video series covering how to find and analyse restriction sites and how to simulate restriction cloning

➤ **Restriction Cloning Tutorial**: Written tutorial on using the restriction cloning tool in Geneious Prime

TOPO Cloning

Unlike restriction enzyme cloning, which uses a restriction enzyme and a DNA ligase, TOPO cloning accomplishes DNA cleavage and ligation with one enzyme: topoisomerase I. Topoisomerase I has two activities which make it ideal for cloning:

- It cuts DNA at the sequence 5´-(C/T)CCTT-3'
- It ligates DNA fragments when bound to the 3' phosphate group in thymidine

Topoisomerase I enables three types of TOPO cloning:

TA cloning

TA cloning, or sticky-end TOPO cloning, uses an A overhang on the 3' end of a PCR product to place it into a TOPO vector containing a 3' T overhang with a covalently attached topoisomerase I enzyme (Figure 2). As many high fidelity enzymes do not introduce a 3' A overhang, use *Taq* polymerase to generate the PCR product. Otherwise, a short incubation with *Taq* after a PCR reaction with Hifi polymerase will add the necessary 3' A overhang.

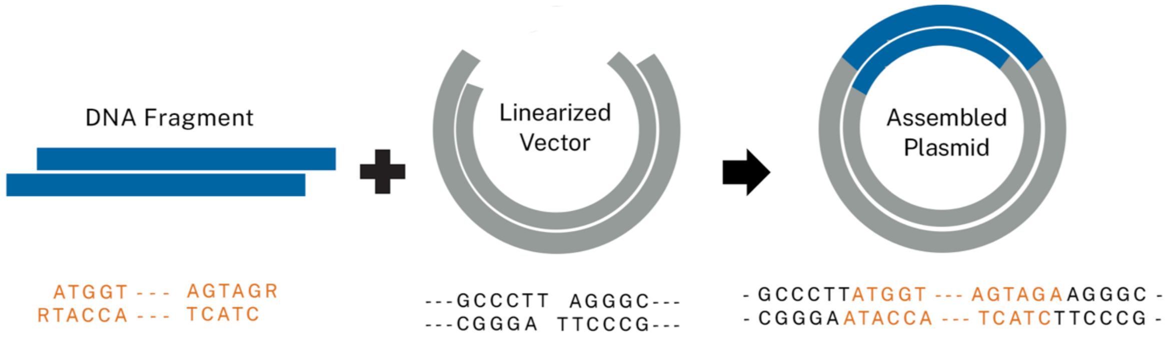

Figure 2. TA TOPO cloning.

Blunt end cloning

Inserts are created either with high-fidelity polymerases that leave blunt ends or with restriction enzymes that form blunt ends (Figure 3). When mixed with a blunt TOPO vector and topoisomerase I, ligation occurs.

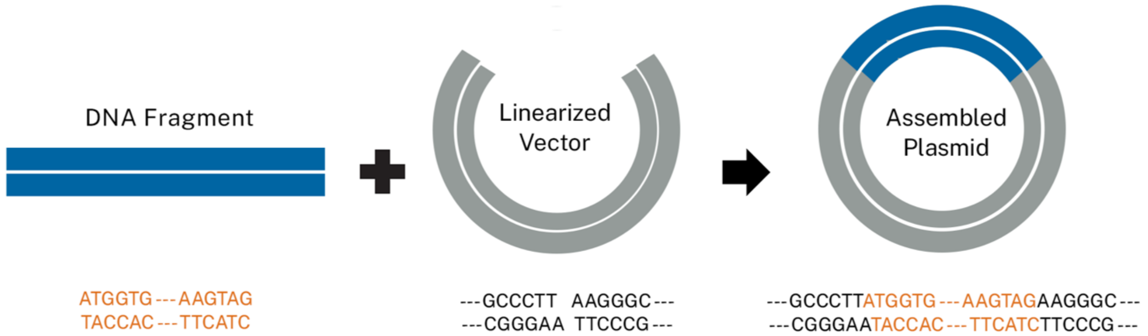

Figure 3. Blunt end TOPO cloning.

Directional cloning

Insert contains a 5’ CACC overhang and a 3’ blunt end to give directionality (Figure 4). Topoisomerase I ligates the insert with a vector that contains a 5’ GTGG and a 3’ blunt end

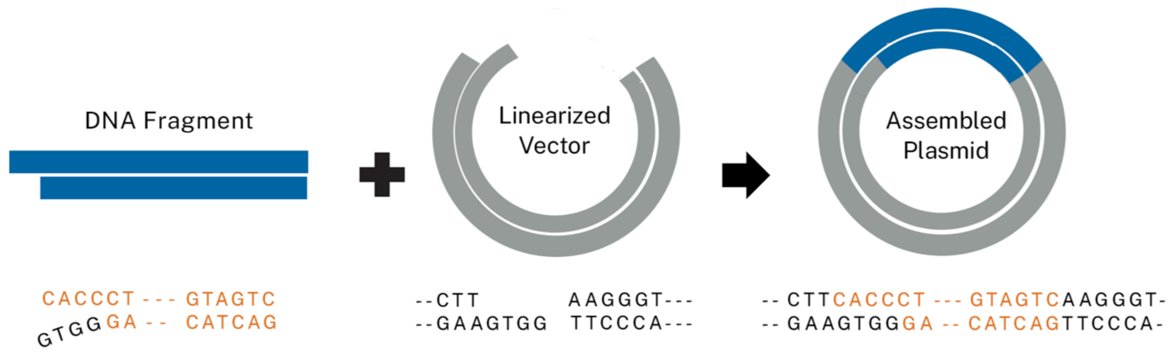

Figure 4. Directional TOPO Cloning

Pros and cons of TOPO Cloning

Pros

- Routine process without the need to design restriction digest protocol and purchase restriction enzymes specific for the exact cloning experiment
- Can offer directionality depending on method

Cons

- Insert size limited to 2-3kb
- Limited vector choices and may not be suitable for custom vectors

Resources for TOPO cloning in Geneious Prime

➤ **TOPO Cloning**: Video series on simulating blunt, directional, and TA TOPO cloning in Geneious Prime

➤ **TOPO Cloning Tutorial**: Written tutorial with practice exercises on simulating TOPO cloning methods in Geneious Prime

Gateway Cloning

Gateway cloning takes advantage of site-specific recombination enzymes that recognize specific sequences on DNA to swap out the fragments between two strands (i.e. vector and insert). These sequences are known as recombination sites and the sites used in Gateway cloning are called *attB*, *attP*, *attL*, and *attR*.

Gateway cloning proceeds in two steps (Figure 5):

1. **BP reaction:** The BP reaction generates the entry clone based on recombination of *attB* and *attP* sites on fragment and donor vector. The enzyme BP clonase catalyzes this reaction and creates the entry clone containing the DNA fragment with *attL* recombination sites.
2. **LR reaction**: The LR reaction generates the expression clone via recombination of the *attL* site of the entry clone and the *attR* site of the destination vector. The enzyme LR clonase catalyzes this reaction to move the insert DNA into the destination vector.

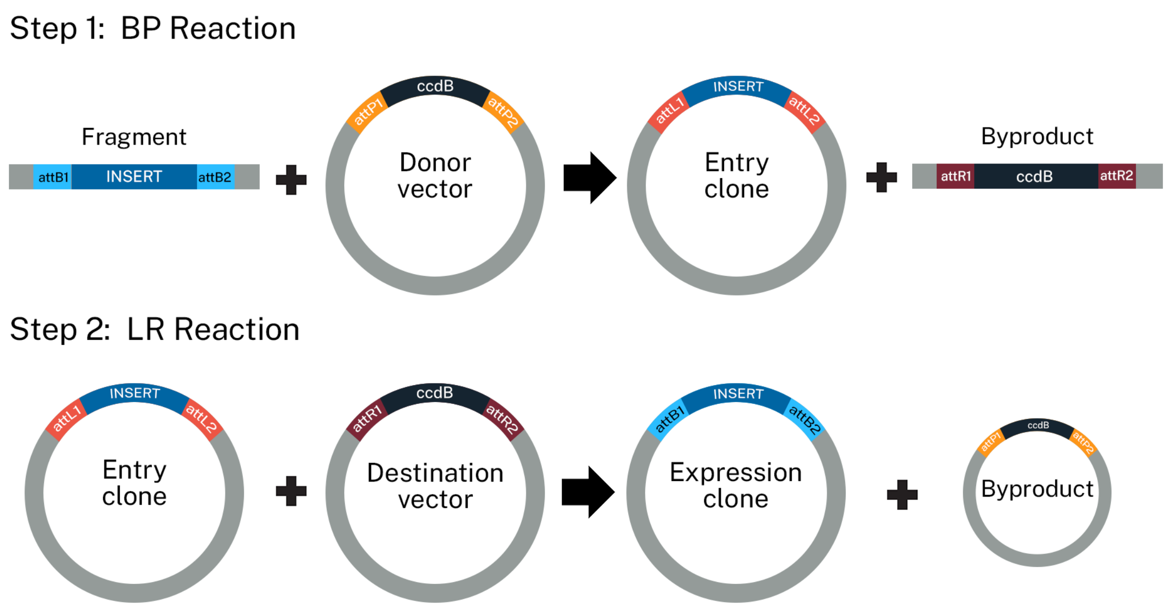

Figure 5. Gateway cloning BP and LR reactions.

Gateway cloning is particularly useful if you need to move your insert between multiple destination vectors. To do this, you'd create one entry clone using the BP reaction and then use that clone to create multiple expression clones using the LR reaction with different destination vectors. Gateway cloning can also assemble multiple fragments in one tube by taking advantage of the specificity of the recombination sites. For example, *attB1* only recombines with *attP1*, so designing inserts with the recombination sites in a specific order can give your construct directionality.

Pros and cons of Gateway Cloning

Pros

- Faster than restriction cloning (BP and LR reactions can be done in one step)
- Libraries of cloned and sequenced gene fragments available for purchase

Cons

- Assembles only up to four fragments
- Scars are left in plasmids
- Can be more difficult to execute

Resources for Gateway cloning in Geneious Prime

➤ **Gateway Cloning**: Video series describing how to clone one or more fragment with Gateway cloning.

➤ **Gateway Cloning Tutorial**: Written guide with practice exercises on simulating Gateway cloning in Geneious Prime.

Gibson Cloning

Gibson assembly uses T5 exonuclease, Phusion polymerase, and Taq ligase to assemble up to 15 overlapping DNA fragments (Figure 6). These fragments are created using PCR with primers that contain both template specific sequences and sequences that overlap with the adjacent fragment (either another insert or the vector) in the final construct. Before assembly begins, the vector is linearized to produce overhangs using either restriction digest or inverse PCR.

Gibson assembly begins with T5 exonuclease chewing back the 5' ends of the DNA to create overhangs. When compatible, overhangs from two fragments bind to one another and DNA polymerase extends exposed DNA. Finally, DNA ligase repairs the nicks in between the fragments.

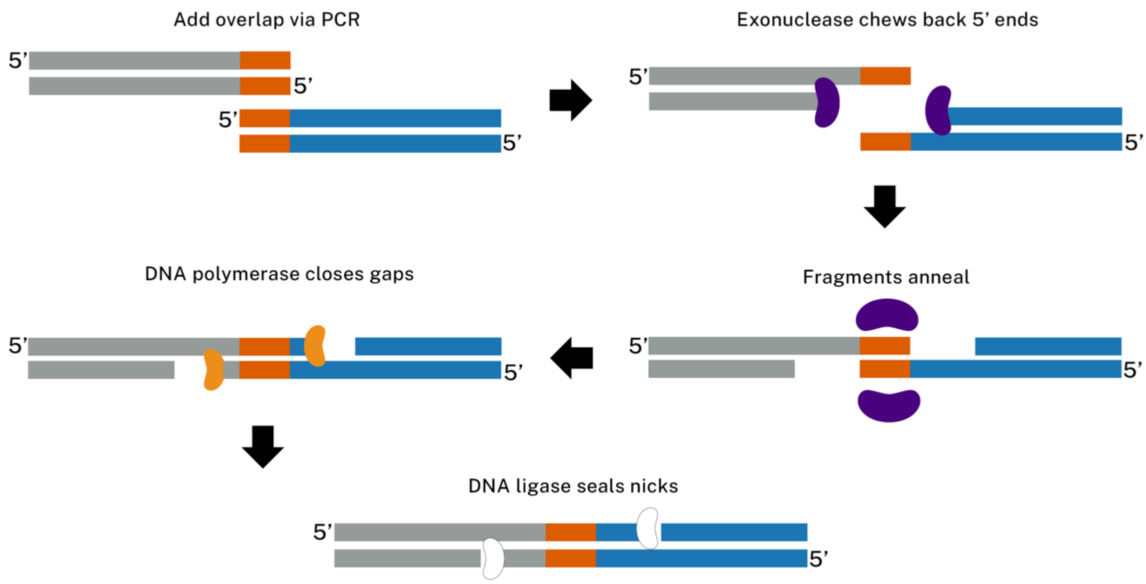

Figure 6. Gibson assembly of two DNA fragments with overlapping regions.

Pros and cons of Gibson assembly

Pros

- A "one pot" assembly
- Fast
- Can be used to join up to 15 fragments (but success rate decreases over 5 fragments)

Cons

- Can be more expensive than other methods
- Not suitable for small fragments (<200 bp)

Resources for Gibson assembly in Geneious Prime

➤ **Gibson Assembly**: Video on how to perform gibson assembly in Geneious Prime.

➤ **Gibson Assembly Tutorial**: Practice exercises on simulating single fragment or multiple fragment Gibson assembly in Geneious Prime.

In-Fusion Cloning

In-Fusion cloning is similar to Gibson assembly methods in that a linearized vector is mixed with overlapping PCR product(s). The difference: In-Fusion cloning uses one enzyme instead of three. This cloning method takes advantage of the DNA polymerase from the vaccinia virus, which contains a 3' -> 5' exonuclease activity. During cloning, the In-Fusion enzyme first chews back the 3' ends of the fragments to create overhangs. This complex is transformed into *E. coli* where it is subsequently ligated.

In-Fusion cloning has many advantages as it's suitable when you need scarless assembly, directional cloning, and the capacity to insert multiple fragments at once.

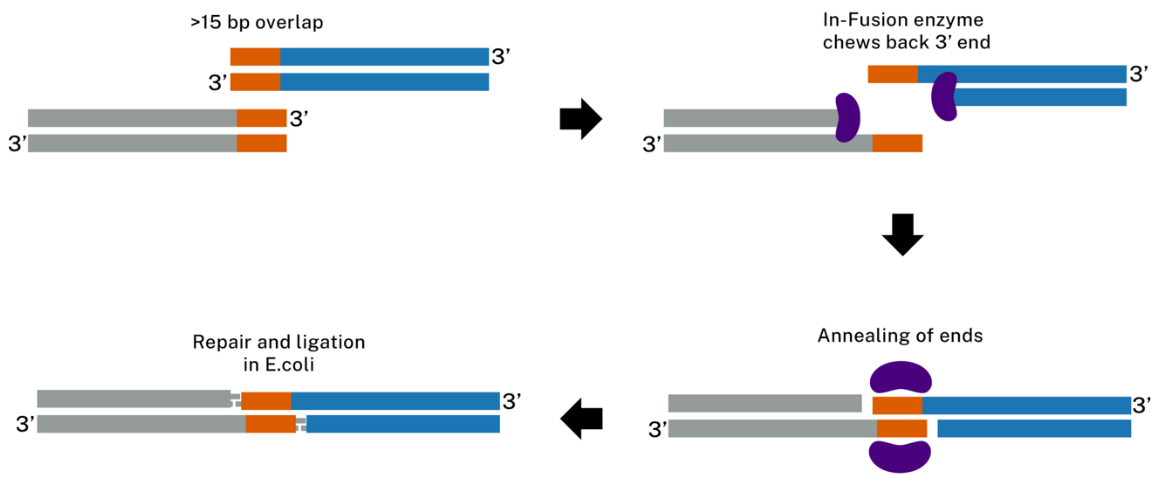

Figure 7. In-Fusion cloning uses In-Fusion enzymes to chew back DNA ends before they are repaired and ligated in E. coli.

Pros and cons of In-Fusion Cloning

Pros

- No restriction enzymes or ligase required
- Directional cloning
- Seamless cloning
- Fast

Cons

- Requires careful primer design
- Any secondary structure in the overlapping regions can be difficult to deal with

Resources for In-Fusion cloning in Geneious Prime

➤ **In-Fusion Cloning**: Video on how In-Fusion cloning works and how to perform In-Fusion cloning in Geneious Prime.

Golden Gate Cloning

Golden Gate cloning uses Type IIS restriction enzymes that cleave DNA outside of the recognition site. Different Type IIS restriction enzymes cut the DNA at different distances from the recognition site and thus create overhangs with different lengths. As the cut site is not sequence dependent, scientists can create overhangs with any possible sequence. This has two advantages: (1) scientists can design constructs with multiple overlapping inserts and (2) destination vectors won't circularize on itself because their sticky ends are incompatible. These features allow Golden Gate cloning to proceed in just one step where the digestion and ligation occurs within one mixture.

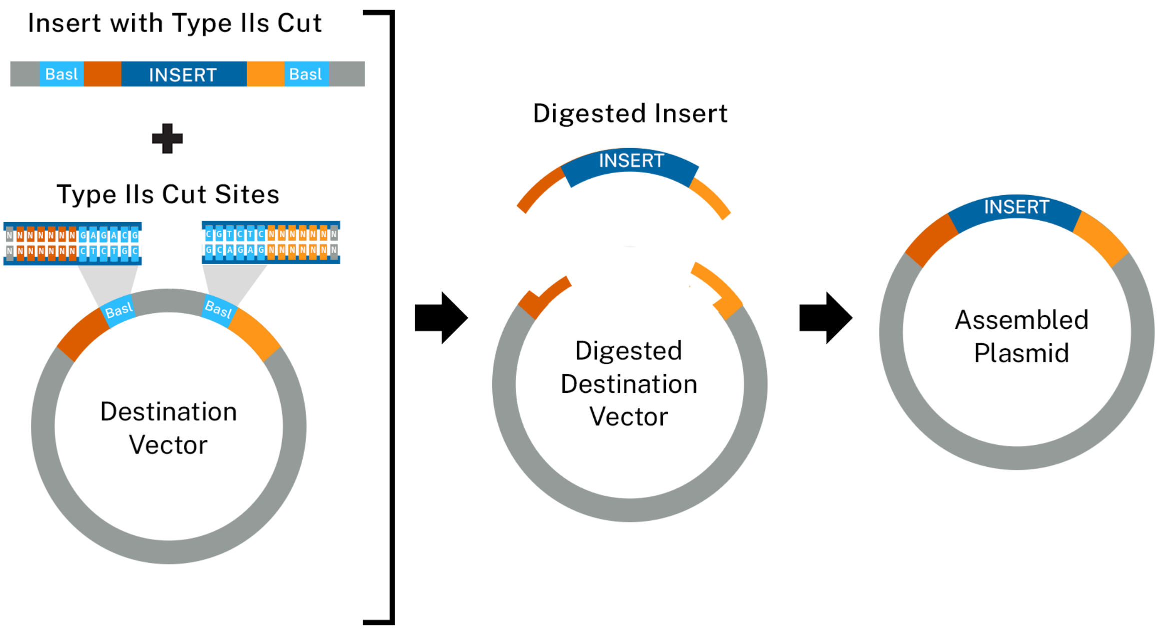

Figure 8. Golden Gate cloning eliminates the Type IIS restriction sites from the final product.

Pros and cons of Golden Gate cloning

Pros

- One step, one pot process in less than 30 min
- Assemble multiple fragments
- Destination vector cannot recircularize after digestion

Cons

- Type IIS recognition sites cannot be present in the insert. If the insert has the recognition site, eliminate it by introducing a point mutation before cloning.

Resources for Golden Gate cloning in Geneious Prime

➤ **Golden Gate Cloning**: Video series on simulating Golden Gate cloning in Geneious Prime.

➤ **Golden Gate Cloning Tutorial**: Written exercises on simulating Golden Gate cloning in Geneious Prime.

Parts Cloning (Combinatorial Cloning)

Parts cloning, or combinatorial cloning, uses libraries of genetic elements (ex: promoters, coding regions, terminators, etc.) and various cloning techniques (ex: Golden Gate, Gateway, etc.) to create multi-part constructs. Synthetic biology and engineering applications use parts cloning to create biological systems from standardized, modular components. Thus, parts cloning can only occur when using well characterized or standardized components that are found within a modular toolkit of parts.

Pros and cons of parts cloning

Pros

- Easy to mix and match modules
- Parts are well characterized so how they will behave in the construct is predictable

Cons

- Only available for standardized parts
- May be difficult to integrate non-modular parts
- Cost associated with obtaining the modular parts toolkit

Resources for parts cloning in Geneious Prime

➤ **Parts Cloning**: Video on simulating parts cloning and concatenate libraries of genetic elements outputting all possible combinations.

Uses of DNA cloning

DNA molecules built through cloning techniques are used for many purposes in molecular biology. A short list of examples includes:

- **Biopharmaceuticals.** DNA cloning can be used to make human proteins with biomedical applications, such as the insulin mentioned above. Other examples of recombinant proteins include human growth hormone, which is given to patients who are unable to synthesize the hormone, and tissue plasminogen activator (tPA), which is used to treat strokes and prevent blood clots. Recombinant proteins like these are often made in bacteria.

- **Gene therapy.** In some genetic disorders, patients lack the functional form of a particular gene. Gene therapy attempts to provide a normal copy of the gene to the cells of a patient's body. For example, DNA cloning was used to build plasmids containing a normal version of the gene that's nonfunctional in cystic fibrosis. When the plasmids were delivered to the lungs of cystic fibrosis patients, lung function deteriorated less quickly\[^2\].
- **Gene analysis.** In basic research labs, biologists often use DNA cloning to build artificial, recombinant versions of genes that help them understand how normal genes in an organism function.

Hide explanation

For instance, researchers studying neurons in fruit flies might use DNA cloning to assemble a **reporter construct** for a neural gene. In this construct, the regulatory region (promoter) of the gene might be pasted in front of a gene encoding a fluorescent protein. When transferred into a fly, the "reporter gene" would be expressed in the same neurons as the neural gene itself, causing those neurons to glow (fluoresce) under UV light.

These are just a few examples of how DNA cloning is used in biology today. DNA cloning is a very common technique that is used in a huge variety of molecular biology applications.