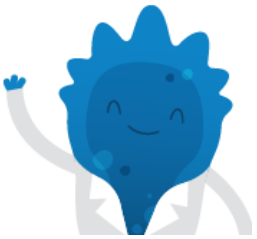
Dec 31, 2019 Version 2

Plasmid Cloning by Restriction Enzyme Digest (aka Subcloning) V.2



In 1 collection

DOI
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Addgene The Nonprofit Plasmid Repository[1]

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Joshua Tran Protocols

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We use this protocol and it's working

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Abstract

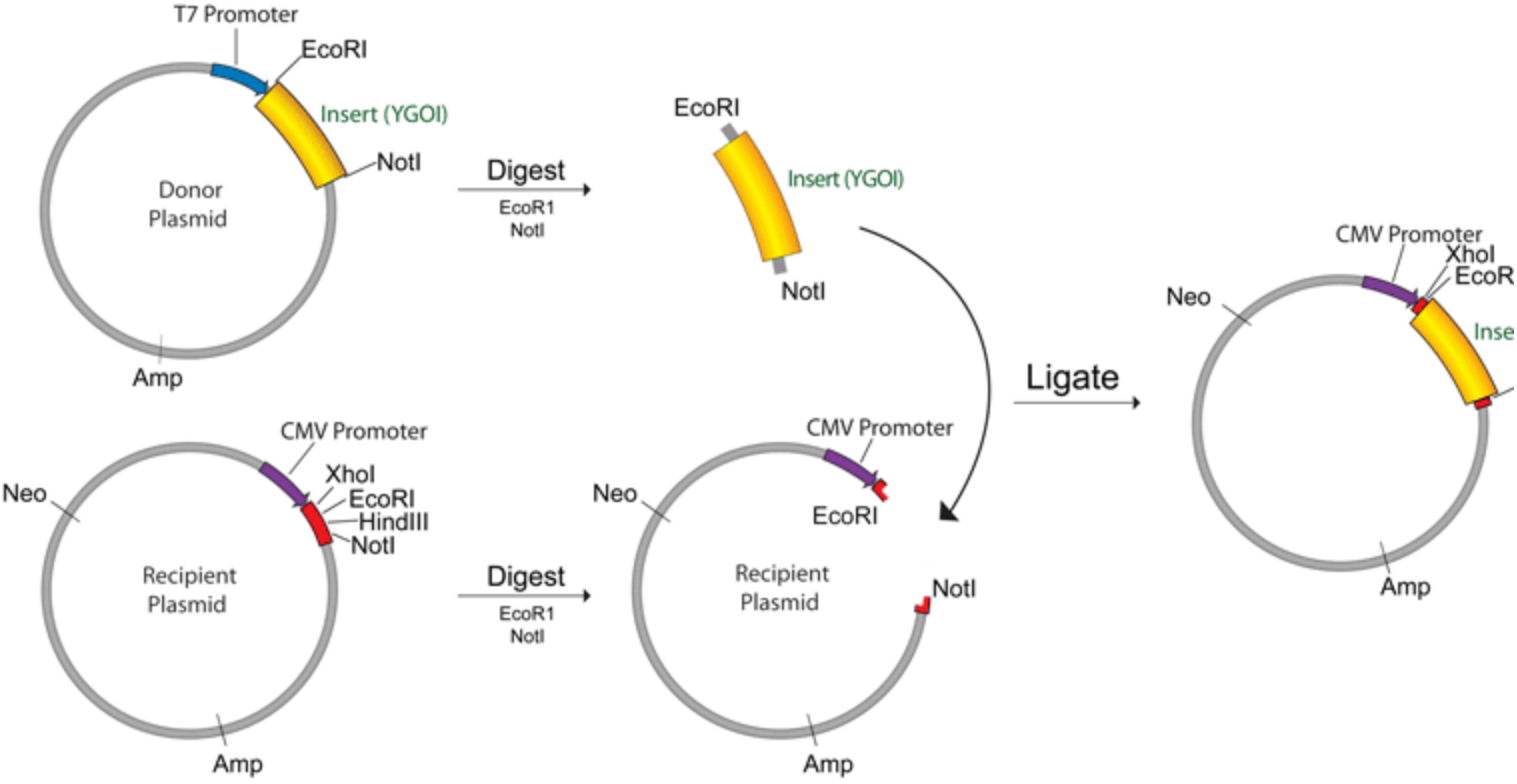
This protocol describes plasmid cloning by restriction enzyme digest (aka subcloning). To see the full abstract and additional resources, please visit the **Addgene protocol page**.

Guidelines

Cloning by Restriction Enzyme Digest Example and Diagram

Subcloning by restriction digest is a commonly used lab technique. For the purposes of this tutorial we will discuss how to move a cDNA from one plasmid to another. However, the same technique can be used to move promoters, selectable markers, or any other DNA element between plasmids.

Let's assume that you are beginning a new project on your gene of interest (YGOI for short). You might need to express YGOI in cultured mammalian cells. The problem is that the only version of full-length cDNA you can find for YGOI is in a bacterial expression vector. Using subcloning, you can easily move YGOI into a mammalian expression vector.



Troubleshooting

Design (Choosing enzymes)

1 Many DNA analysis tools, including **Addgene's Sequence Analyzer**, allow you to identify which restriction sites are present in a given sequence.

When selecting restriction enzymes, you want to choose enzymes that:

- Flank your insert, but do not cut within your insert
- Are in the desired location in your recipient plasmid (usually in the Multiple Cloning Site (MCS)), but do not cut elsewhere on the plasmid
- Will result in your insert being in the correct orientation in the recipient plasmid. (You don't want to express the antisense version of your gene!)
- Are in frame with tags or fusion proteins in the recipient plasmid (if you are creating a fusion protein)

Ideally, you will find two different restriction enzymes for your subcloning. It is also possible to use a single enzyme, but this will require phosphatase treatment of your recipient plasmid as well as a specifically designed test digest later to verify that the insert was cloned in the correct orientation.

If you cannot find enzymes that meet these criteria, do not fear. You have other options, such as:

- Adding desired restriction sites to flank your insert : You can use **PCR Based Cloning** and add restriction sites to the ends of your oligos. This will allow you to produce a version of your insert flanked by restriction sites compatible with the recipient plasmid's MCS. However, you still need to avoid restriction enzymes that cut within your insert.

- Adding desired restriction sites to your recipient plasmid : You can modify the MCS of your recipient plasmid using **Annealed-oligo Cloning**.

If you are lucky enough to have multiple options for enzymes that flank your insert and will result in correct orientation in the recipient plasmid, it is useful to see if one set of enzymes will work in the same restriction enzyme buffer (see **New England Biolabs** for more information about restriction enzyme buffers). If you select enzymes that can function in the same buffer, it will save you time in future steps.

Experimental Procedure

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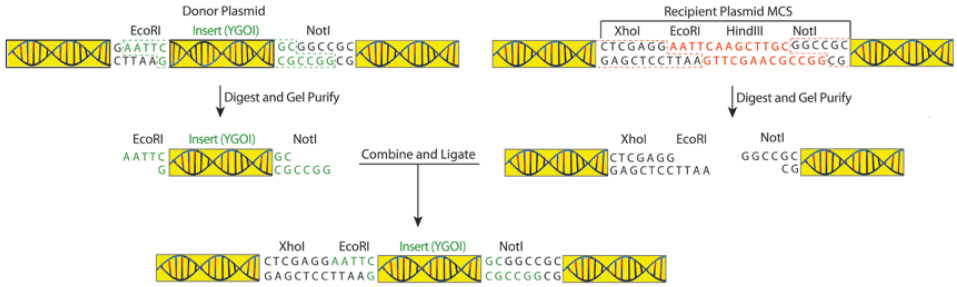

Digest your DNA

Set up **restriction digests** for your donor and recipient plasmids. Because you lose some DNA during the **gel purification** step, it is important to digest plenty of starting material. We recommend 1.5 µL - 2 µg of donor plasmid and 1 µg of recipient plasmid. It is also critical that as much of the recipient plasmid as possible be cut with both enzymes, and therefore it is important that the digest go at least 04:00:00 and as long as overnight.

If you are going to use only one restriction enzyme, or enzymes that have compatible overhangs or no overhangs after digestion, you will need to use a phosphatase to prevent re-circularization of the recipient plasmid. You should treat your digested recipient plasmid with a phosphatase prior to the ligation step or prior to the gel purification step, depending on the phosphatase you choose. CIP (calf alkaline phosphatase) or SAP (shrimp alkaline phosphatase) are commonly used. Follow the manufacturer's instructions.

3 **Isolate your insert and vector by gel purification**

Run your digested DNA on an **agarose gel** and conduct a **gel purification** to isolate the DNA. When running a gel for purification purposes it is important to have nice crisp bands and to have space to cut out the bands. Because of this we recommend that you use a wide gel comb, run the gel on the slower side, and skip lanes between samples. In addition to a DNA ladder standard, it is also a good idea to run an uncut sample of each plasmid to help with troubleshooting if your digests don't look as you expected.

Once you have cut out and purified your insert and recipient plasmid backbone bands away from the gel via your favorite **gel purification** method, it is important to**determine the concentration of recovered DNA**.

4 **Ligate your insert into your vector**

Conduct a **DNA Ligation** to fuse your insert to your recipient plasmid.

We recommend around 100 ng of total DNA in a standard ligation reaction. You ideally want a recipient plasmid to insert ratio of approximately 1:3. Since the number of base pairs for each varies, it is difficult to calculate this based on DNA concentration alone. One method is to conduct 2 ligations for each plasmid you are trying to create, with varying ratios of recipient plasmid to insert.

It is also important to set up negative controls in parallel. For instance, a ligation of the recipient plasmid DNA without any insert will tell you how much background you have of uncut or self-ligating recipient plasmid backbone.

5 **Transformation**

Transform your ligation reaction into your bacterial strain of choice. Follow the manufacturer's instructions for your competent cells.

For most standard cloning, you can transform 1 µL - 2 µL of your ligation reaction into competent cells such as DH5alpha or TOP10. If using much less total DNA (< 1 ng) or if you are having trouble getting colonies, you might want to use higher competency cells. Additionally, if your final product is going to be very large (>10kb) you might want to use electro-competent cells instead of the more common chemically-competent cells.

The number of bacterial colonies resulting from your transformation will give you the first indication as to whether your transformation worked. our recipient plasmid + insert plate should have significantly more colonies than the recipient plasmid alone plate. The recipient plasmid alone control will tell you your "background" level or more specifically it will tell you how many colonies you can expect on your recipient plasmid + insert plate that are not correct.

If you have a high number of colonies on your recipient plasmid alone plate, you can try ligating the recipient plasmid alone in the presence and absence of ligase. If the colonies are a result of uncut empty plasmid, you will still have colonies when you do not add ligase. If the colonies are a result of recipient plasmid self-ligation, you will see significantly more colonies when you add ligase.

If you do not see any colonies, you should conduct a positive control to ensure that your transformation worked. You should also verify that you are plating on the appropriate antibiotic and try varying the amount of recipient plasmid to insert in the ligation reaction.

6 **Isolate the finished plasmid**

Finally, you will need to pick individual bacterial colonies and check them for successful ligations. Pick 3-10 colonies depending on the number of background colonies on your

control plate (the more background, the more colonies you will need to pick) and grow overnight cultures for DNA purification.

After purifying the DNA, conduct a diagnostic restriction digest of 100 ng - 300 ng of your purified DNA with the enzymes you used for the cloning. Run your digest on anagarose gel. You should see two bands, one the size of your vector and one the size of your new insert. If you used only one enzyme or used enzymes with compatible overhangs you will need to verify the orientation of your insert, so you may want to design a diagnostic digest for this purpose.

Note

Congratulations, you now have YGOI in a mammalian expression vector and can begin your studies. (See guidelines section for background on example)