

Ilsa's Guide to Cloning

Introduction

Hello reader. This manual contains step-by-step instructions for cloning. These are the steps I have found to produce the best results in the quickest time. Although initially I go through my "quick" version of cloning, don't worry, there are notes along the way instructing you of other things I've had to try (or have heard of) for troubleshooting.

I. Polymerase Chain Reaction (PCR)

Purpose: exponentially amplifying DNA

Components: DNA template
Two primers
DNA-polymerase
Nucleotides
Buffer
Water

A. General Questions To Ask Yourself Prior to Starting

1. **What am I trying to clone?** (i.e. What gene, UTR, etc.? From now on, I will refer to what you're cloning as a "gene.") Get this base pair sequence and put it in a text-editing file.

****NOTE are there introns spanning the area you want to clone – if so you must use cDNA??****

Some places to get your sequence:

PlasmODB (<http://www.plasmodb.org/plasmo/home.jsp>)

Search Genes by PlasmODB Gene ID; Keyword (product name, notes, etc.); Gene Type;

Search Genomic Sequences by Genomic Sequence ID;

Sanger Center (<http://www.sanger.ac.uk/Projects/Protozoa/>)

GeneDB (<http://www.genedb.org/>)

Search for gene by ID/description in selected organism's database

NCBI (<http://www.ncbi.nlm.nih.gov/>)

Search by Accession Number, GenBank ID, etc.

2. **What is the purpose of the clone I am trying to make?** In other words, what vector am I trying to insert my "gene" into? [pET24a (-His₆); pGEX4T-1 (-GST); pLN-GFP, etc.]
3. **What enzymes are available on the vector to insert my gene?** On commercial plasmids like pET24a and pGEX4T-1 there are cloning cassettes with many enzymes to choose from. On plasmids that labs engineer, you are usually taking their existing insert out and replacing it with your own. There may or may not be a multiple number of enzymes you can use.
4. **Does this restriction enzyme cut my insert?** If so, you need to choose another restriction enzyme.

Ways to answer this question:

MacVector: I use this program (for Macintosh computers) to analyze my gene's sequence. I ask the program to list all the "Noncutters" from a New England Biolabs restriction enzyme list. I copy this Noncutter list into the same text-editing file I created with my "gene" sequence. This list comes in handy later for a diagnostic digest of your ligation reaction (explained later).

VectorNTI: PC program

NEBcutter V2.0: (<http://tools.neb.com/NEBcutter2/index.php>)

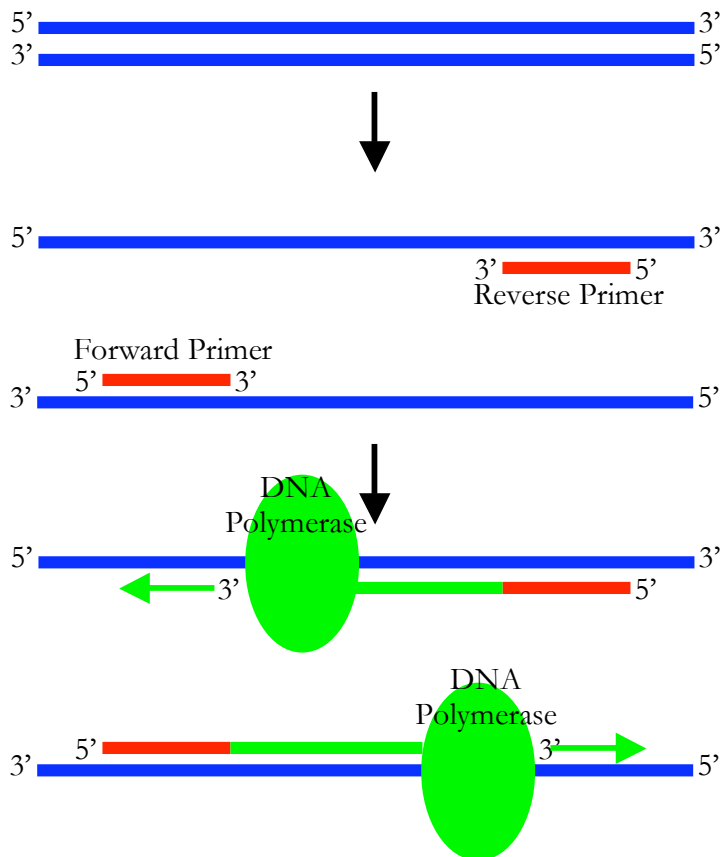
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Find: on the New England BioLabs website or in their *Catalog and Technical Reference* look up the target sequence of the enzyme you want to use and do a search in the text file you created.

5. **Does the enzyme(s) create a frameshift?** Check your codons!! Are they changed in anyway because of the enzyme(s) you are using? Is your tag and stop codon still in frame?
6. **Are you going to have to add a start and/or stop codon with this vector/enzyme(s) combo when designing your primers for PCR?** *i.e.* For pET24a I try to use Nde I [CATATG/GTATAC] and Xho I, because Nde I has a start codon, and Xho I is the closest I can get to the C-terminal His₆-tag. Additionally, I would NOT include a stop codon when using this vector because then my His₆-tag would not get translated.

B. Primer Design

Primers are short, artificially generated DNA strands, usually between 20-40 bp with a melting temperature between 60°C and 75°C, where the DNA-Polymerase binds and begins the synthesis of the new DNA strand. Below is a cartoon showing the first three steps in a PCR. The forward and reverse primers are shown in red. The DNA polymerase and the amplified DNA is shown in green:



Here you see how your forward and reverse primers need to anneal to your DNA. Forward primer anneals to the strand that runs 3'→5'; Reverse primer anneals to the strand that runs 5'→3'.

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The following are the steps I take to design primers:

1. Use the general equation that As and Ts = 2°C, Gs and Cs = 4°C to get to a primer T_m between 60-75°C. I always try to get 60°C or just above. I also try to end with a G or C (because they have 3 hydrogen bonds, in turn, making the affinity stronger). I only count the bases that will anneal during the first round of PCR. I do not count the restriction target sequence or the extra bases used as an aid for the restriction enzyme to bind the DNA.¹
2. For the forward primer I count from the beginning of the 5'→3' sequence enough bases to reach 60°C.
3. For the reverse primer I count from the end of the 3' end in, enough bases to reach 60°C. Then you must take the complement of this sequence for your reverse primer. To order primers from IDT your primer must be in the 5'→3' order, so this means you need the reverse of this sequence. (I like this program: http://bioinformatics.org/sms/rev_comp.html)
4. Both primers are going to need an additional sequence on the end of your primer in order for the restriction enzyme to bind to your DNA (The back of the New England BioLabs *Catalog and Technical Reference* give you a guideline of how many bases is needed depending on the enzyme, but if you use 5-6 base pairs, you can't go wrong).

Here is an example of a gene (PF11_0163) being cloned into pET24a using Nde I and Xho I (No start codon needed, no stop needed, no adjustments needed with the addition of the restriction enzyme target sequences):

>PF11_0163²

```
ATGAATTTATTTTATAACCTAATTAAC TCAATAAAAAGAAAAATGAAAACAAGAAATTTTACA
TCCAATTTTGTA AAAACGATTTTCATATAAGCACAATATATCAACATAGGAAAAAATCAATATG
TTTAAATTC AATACAAAATAATTC AAGTGTAATAATAATATATGTATTGTA AAAAGTGAAAA
CAAGAAATAGATA AAAACAATTCTGATTTTATTCAATATGTTTTTAAATGGGGAATAGGAAATA
AATTTTCGTTCCGATCCAGAAAACAGATATCATCCAGTACATTTTAAACGCTCCAAGAAGTAAC
TATAAGAAAAGACTACTTTGATAATGTTAATGAAAACATAAGGTATGAAGAATTAATGAACAA
TGGGAAGTATATTGGTTTGAAAATAATAAACTAAATGCAAAACCCTTTCCTGTTAAAAAGTTTG
GAATAAATGATGCAAAAAGGGAAGCAATCAATTTTATGAATCCTTAAAGGTACAACAACGTAT
AATGCCAAAACCTAAATACGAATCTGGCGTTGAAGGTGTTCACTATGATATTATTACCAACTGT
TGGGTTGCCTTTTATCGTTCAAACAACCTCCCTGTTTGCAGATCTTCTCAGCTGAATATCATG
GATTTGAAACTGCTAAACAGATGGCCATAGAAA GAGTTGAAAAACACAAGAAG TAA
```

>PF11_0163 Forward (Nde I; start included):

```
GCTACTCATATGAATTTATTTTATAACCTAATTAAC
```

Reverse designing:

```
GAGTTGAAAAACACAAGAAGCTCGAGTGC
```

>PF11_063 Reverse (Xho I; No stop) needed to be ordered:

```
GCACCTCGAGCTTCTTGTGTTTTTCAACTC
```

¹ There are a "million" programs available to calculate T_m for you (IDT's SciTools, and so on). I do NOT recommend this. I have always done it the way I describe and it works. I never tried the fancy tools out there. Just in case you were wondering:

$T_m = 81.5 + 0.41(\%GC) - (675/N) - \%mismatch$; (N = total number of bases). Trust me, use the formula As and Ts = 2°C, Gs and Cs = 4°C. Here you're starting to see I do things a certain way and it works. Please try it my way first. It will work!

² Courier is a great font to use because it lines up your letters on top of each other. I always use it.

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C. Polymerase Chain Reaction

****Note: If you have an intron spanning your gene,** you will need to do a PCR off cDNA. To generate cDNA you will either get some as a gift, or make it yourself by Reverse Transcriptase Reaction (RT) from RNA input. Once you have cDNA proceed.

A. Setup (please see Llinás Lab protocol)

B. Verify fragment size by electrophoresis and ethidium bromide staining (please see Llinás Lab protocol)

Troubleshooting

1. If this was your first PCR, go through and make sure you added everything you needed. A common newbie mistake is to forget a component.
2. Check the DNA loading buffer you are using. Is the running dye about where you expect your band to be? Is it covering it up? Use the Orange G loading buffer instead!
3. If you don't see a band: try adding double the DNA input.
4. Then, try doing a temperature gradient spanning 5°C above and below your T_m . (If you have a smear, your annealing is non-specific. Try a temperature gradient 10°C above your initial T_m).
5. Others have also tried a $MgCl_2$ gradient or a matrix of temperature and $MgCl_2$.

C. Cleanup PCR

I use the Qiagen PCR Cleanup Kit. Make sure the thing you're trying to cleanup is selected for in your cleanup. Read the kit's instructions. Use 30 μ l Milli Q water to elute your PCR product, let stand for 5 min and spin at max speed into clean microcentrifuge tube.

****If you're fragment is too small to do a Qiagen PCR cleanup, people have tried phenol/chloroform extractions, and extractions using β agarase.**

II. Restriction Digest

You're going to need **both** cut vector and cut insert. To generate enough vector to use, if not done so already, transform into a competent cell line, make a -80°C stock, and do a Miniprep on the *E. coli* to isolate the plasmid DNA (a Qiagen Miniprep Kit is sufficient for what you need to do in the remainder of this protocol). Directions for these techniques are found later in this protocol.

A. Digestion Conditions

1. Look up the restriction enzymes' digest conditions (I've always used NEB's enzymes so I use their catalog. If possible I try to do a double digestion (time saver)). Ask yourself: What buffers do the enzymes prefer? What temperatures is best? Does one or both of them need BSA? (You can add BSA in any digest, it will not "hurt" the reaction).
2. **Setup a 20 μ l reaction:**
 - x μ l Milli Q
 - 2 μ l 10X NEB Buffer
 - 2 μ l 10X BSA (if needed)-make yourself a 10X BSA stock from the 100X they supply
 - 5 μ l Miniprep plasmid DNA or 10 μ l cleaned up PCR product
 - 0.5 μ l Enzyme A³ + 0.5 μ l Enzyme B⁴

³ The amount of enzyme used may be adjusted depending on the final double digestion setup. For example: If enzyme A only can be used in Buffer #1, but enzyme B has 50% activity in Buffer #1, I would use double the amount of enzyme B.

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3. Incubate Reaction

Usually enzymes require a two-hour incubation at 37°C. **(Double check this is correct for your enzymes)!!** I like to use a water bath. (When rushed I have had success with 1 hour incubations, however, I like the 2 hour incubation best).

4. Enzyme Inactivation

Usually enzymes are inactivated at 65°C. **(Double check this is correct for your enzymes)!!** Those that cannot be heat inactivated I clean up with a Zymo Clean and Concentrator Column, eluting in 20 µl of Milli Q after a 5 min stand and max spin.

Vector Digests: (Do this prior to doing a Zymo Cleanup if needed). Run 1-2 µl of your reaction on a gel besides an equal concentration of uncut vector to verify you have linearized plasmid. If you have a lot of uncut vector, you may want to do a gel purification of your linearized plasmid, however, I would rather do a diagnostic digest of my plasmid after my ligation rxn (explained later), and would proceed anyway.

III. Ligation Reaction

- 7.5 µl Milli Q
- 5 µl Digested PCR product
- 1 µl Digested Vector
- 2 µl T4 DNA ligase Buffer
- 0.5 µl T4 DNA ligase

1. Incubate O/N on your bench (When rushed I have successfully incubated for an hour with “new” enzyme and proceeded).
2. Heat Inactivate 65°C 10 min. (If you're going directly to the next step MAKE SURE YOU COOL THIS REACTION DOWN before proceeding!!).

IV. Diagnostic Digest of Ligation Reaction

Here is where the list of noncutters you made when you first started cloning also comes handy. Like I said earlier in the *Restriction Digest Section*, I like to do this instead of gel purifications of vectors (I will discuss this more in my *Cloning Troubleshooting Section*).

1. Pick a restriction enzyme that will cut you vector only if it **DOES NOT** have your insert.
2. Setup a reaction:

- x µl Milli Q
- 2 µl 10X NEB Buffer
- 2 µl 10X BSA (if needed)
- 10 µl Ligation Reaction

⁴ Doing a step-wise digestion is completely fine, although, I avoid doing this unless it's a drastic temperature difference. I try and adjust enzyme amounts when possible. Step-wise digestions can be used when the salt concentrations needed are drastically different, or the incubation temperatures are drastically different.

When drastically different salt concentrations are needed: setup your first digest, with the buffer and enzyme requiring the lower salt concentration, incubate for 2 hours, then bring your salt concentration up for the second enzyme, add your second enzyme, then incubate for another 2 hours.

When drastically different salt concentrations are needed: add your first enzyme, incubate at the appropriate temperature for 2 hours, then add your second enzyme (watch the temperature of your reaction will not denature your next enzyme), then incubate at the appropriate temperature for 2 hours.

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0.5 μ l Enzyme

3. Incubate two hours at appropriate temperature. (I don't worry about heat inactivating my enzyme at this step). If you are proceeding to do a heat shock transformation make sure your reaction is cooled on ice prior to proceeding!!

V. 42°C Heat Shock Transformation of Diagnostic Digest of Ligation Reaction

1. Gently thaw rubidium competent cells⁵ in your hands, put immediately on ice.
2. Incubate on ice at least 15 mins.
3. Gently, add 10 μ l⁶ of diagnostic digest of ligation rxn to 50 μ l of competent cells on ice.
4. Incubate at 42°C for 2 mins. Put back on ice.
5. If you are transfecting into DH5 α , and the plasmid selectable marker is amp^r, then you can skip this step and proceed to step 6b. (The amp^r gene gets turned on right away, the others do not). Otherwise, if you are transforming into XL10 gold, or BL21 cells, which are cam^r, and/or have a plasmid with kan^r you're going to have to recover your cells. Recovering cells: add 1 ml of LB to your microcentrifuge tubes tape/put on a rack on the shaker in the 37°C. Vigorously shake at 37°C for 30-60 min.
6. a) If you had to recover your cells, harvest them 13.2 krmp, 0.5 min. Aspirate the LB leaving 100 μ l of LB in the tube. Gently resuspend the cells, plate on prewarmed 37°C selective LB agar plate (if applicable make sure you select for both plasmid and cells).
b) Otherwise, plate your entire 50 μ l + 10 μ l diagnostic digest of ligation rxn.
7. Incubate at 37°C O/N or on your bench over the weekend.

VI. PCR check colonies

1. Start 5 ml selective LB O/N cultures of your colonies⁷.
2. The next day use 2 μ l of this O/N in a PCR.
3. Put your O/Ns in the 4°C.
4. Check your PCRs. Miniprep two correct colonies with a Qiagen Miniprep Kit.
5. Send for sequencing. Put the other correct colonies back in the 4°C in case these two are incorrect.

VII. Sequencing

1. Look on Genewiz's website how to setup your sequencing rxns. Remember, if you are using a commercial primer, Genewiz usually has a universal primer they will add for you, i.e. T7 promoter, T7 terminal, SP6, 5GEX, 3GEX, etc. This setup is slightly different, than if you were going to add your own primer (PLEASE NOTE THAT "primer" is SINGULAR.... WHEN SEQUENCING ONLY ADD ONE PRIMER!!).

⁵ Some examples of rubidium competent cells I use are XL10 gold for plasmids I am transfecting into *P. falciparum*, and DH5 α for everything else.

⁶ This volume is specific for a 42°C heat shock transformation from a diagnostic digest of a ligation reaction. If you're using straight plasmid, for example, this is 20X what you will need!!

⁷ I use to do this PCR screen right off my plate, however, for some reason I would get "false" positives. Doing a PCR from an overnight ensures that you will not waste your time on a completely false positive (you may still get point mutations, and such...)

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2. When you get your sequencing results back, Clustal align them to verify that the sequence is what you want.

VIII. Other Troubleshooting Ideas

A. pGEM-T (TOPO-TA like cloning system)

When things are just not working, and I have no idea where I'm going wrong I use this system and it always works.

1. **Incubate** your cleaned up PCR product according to the kit's instructions (I use 1/2 of all the volumes they suggest), BUT make sure you've used the proper DNA polymerase to amplify your product to ensure you have the hanging A's.
 2. **Transform** into DH5 α cells. Spread plate on LBagar + Carb or Amp + Xgal plates (per instructions on kit).
 3. **Screen** the white colonies by making 5 ml LB + Carb or Amp O/N cultures, and doing PCRs.
 4. **Sequence** two colonies that have the correct size fragment.
 5. **Digest** correct plasmid in 20 μ l rxn. (Don't worry about heat inactivating, put in -20°C when not using). Run 1-2 μ l of your reaction on a gel besides an equal concentration of uncut vector to verify you have linearized plasmid.
 6. **Gel Purify** 10 μ l of your digest rxn. (I also gel purify my linearized vector so that I can keep the same proportions as I always use).
 7. **Ligate** like above.
 8. **Diagnostic Digest** if you feel like it.
 9. **Transform, Screen, Sequence** like above.
- B. Dephosphorylate your vector prior to ligating.**